Collection of Scientific Papers 2013

Research articles in medicine & pharmacy

Internal Medicine
Surgery
Medical Basic Sciences
Stomatology
Pharmacy

Rīga
RSU
2014

UDK 61(063)

Rīga Stradiņš University.
Collection of Scientific Papers: Research articles in medicine & pharmacy, 2013: Internal Medicine. Surgery. Medical Basic Sciences. Stomatology. Pharmacy. – Rīga: Rīga Stradiņš University, 2014. – 107 p.

IPD Nr. 13-255

ISBN 978-9984-793-43-6
ISSN 1691-497X

Contents

Internal Medicine

Surgery

Medical Basic Sciences

Diversity of CD1a Positive Cells in Case of 25-hydroxyvitamin D Deficiency in Patients with Metabolic Syndrome

Jana Janovska [1], *Julia Voicehovska* [2], *Regina Kleina* [3], *Janis Kisis* [4], *Raimonds Karls* [4], *Olga Zubova*

Rīga Stradiņš University, Latvia
[1] *Department of Doctoral studies,*
[2] *Department of Internal Diseases,*
[3] *Department of Pathology,*
[4] *Department of Infectology and Dermatology*

Abstract

Introduction. Vitamin D has immunomodulatory properties and it affects the immune system through a number of mechanisms including the influence on dendritic cells (DCs). Langerhans cells (LC) are dendritic cells in epidermis and they belong to the skin immune system. DCs are professional antigen-presenting cells playing a major role in the induction of immune responses by activating native T cells. In literature, there are no reports regarding the influence of vitamin D on DCs in patients with metabolic syndrome.

The aim of the study is to explore the potential immunomodulatory activity of vitamin D on LC in case of metabolic syndrome.

Material and methods. In this study, we have analysed a group of patients of both genders with metabolic syndrome aged 40–55 years. Patients' clinical examinations, measurement of blood pressure and waist circumference were conducted. Blood biochemical analyses (cholesterol, HDL, LDL, vitamin D level) were determined. Full-thickness circular 4 mm *Punch* biopsies were taken in 49 patients. Specimens were stained by haematoxylin and eosin, as well as immunohistochemistry using a transmembrane CD1a Langerhans cells marker was done.

Results. Average age in both genders is 43 years, mean waist circumference – 95 cm, total cholesterol – 5.5 mmol/l, LDL – 2 mmol/l, average 25-hydroxyvitamin D – 27.0 ng/ml. In the skin conditioned with MS and low vitamin D level, evident perivascular accumulation of Langerhans cells (LC) in epidermis is observed; in some cases, there was diffuse mild interstitial cluster of LC. LC activity, amount and filling of them with Birbeck granules change in cases with 25-hydroxyvitamin D deficiency.

Conclusion. In patients with low 25-hydroxyvitamin D level, average LC quantity in one field of vision is higher in comparison with those, who have normal amount of 25-hydroxyvitamin D. A deeper investigation is necessary on the role of LC and vitamin D in case of metabolic syndrome in order to reveal their interaction with lymphocytes, plasma cells and mast cells as a part of skin immune system.

Keywords: dendritic cells, metabolic syndrome, vitamin D.

Introduction

The skin is the largest organ in the human body. A network composed of delicate physical, chemical, and immunological barriers in the skin makes it a perfect organ to protect the integrity of the human body. Anatomically, skin is divided into epidermis, dermis, and subcutaneous tissue, from the superficial to the deep tissues. Dendritic cells (DCs) are found in epidermis, which tend to migrate and drain in deeper layers to lymphatic system. DCs represent a heterogeneous cell population residing in most peripheral tissues, particularly at sites of interphase with the environment, e.g. skin and mucosa. They represent 1–3% of the total cell numbers of these tissues [1].

In the periphery, immature DC (iDC) can capture and process antigens. Thereafter, they migrate towards T-cell-rich areas of the secondary lymphoid organs through the afferent lymphatic vessels.

Antigens are presented in such a way that antigen-specific native T cells become activated and start to proliferate. Moreover, when DC initiate a T-cell-mediated adaptive immune response, they also play an important role in polarization of T-cell reactivity [2].

In skin, these cells are dedicated antigen-presenting cells (APCs) and play a key role in sensing danger and initiating both innate and adaptive responses, as well as protect skin from invading pathogens.

The Langerhans cells have captured considerable interest since student Paul Langerhans first described them in 1865 as aureophilic cells present within the epidermis. At first, they were falsely described as melanocytic generation cells, due to their appearance. However, later, it was discovered that LC express MHC class I molecules [3], that indicates LC as important immunological antigen-presenting cells in the body [4]. LC represent approximately 1–3% of epidermal cells and form a constituent part of the skin immune system. LC are typically characterized by the expression of CD1a and a unique cytoplasmic organelle named the Birbeck granule (BG). BGs constitute a subdomain of the endosomal recycling compartment, perhaps being involved in antigen loading processing [5].

It has been postulated that bone-marrow-derived myeloid cutaneous lymphocyte-associated antigen (CLA)-expressing LC precursors travel through peripheral blood through the dermis into the epidermis. The final differentiation of LC precursors depends on the cytokine environment of the epidermis [5]. The cutaneous cytokines granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin-15 (IL-15), and TGF-β1 contribute to the establishment of immature LC in the epidermis. During inflammation, however, circulating precursors might have an important role in replenishing the local pool of LC.

LC has different function according to location; thus in periphery immature LC capture and process antigens. In afferent lymphatic vessels, LC presents antigens and acquire the capacity to present antigens to native T cells (named as maturation of LC). Moreover, there are T cell activation and proliferation (named T cell priming), as well as polarization of T cell reactivity towards type 1 and / or type 2 responses. In epidermis and dermis, LC participate in recognition of invading pathogens (viral, bacterial, etc.), toxins and harmful irradiations. [6] In case of over expression of ultraviolet radiation (UV) from sun, LC might play role in skin immunosuppression. Under UV radiation in epidermis develop direct (intrinsic) keratinocyte damage via apoptosis with clustering of death receptors in the cell surface (extrinsic) and generation of reactive oxygen species (ROS). When apoptotic keratinocytes are processed by adjacent immature Langerhans cells, the inappropriately activated LC could result in immunosuppression. Furthermore, UV can deplete LC in the epidermis and impair its migratory capacity [7].

Vitamin D_3 exerts pluripotent effects on adaptive immune functions such as T cell activation and maturation of dendritic cells. In addition, vitamin D_3 is suggested to increase innate immunity in skin and to enable efficient antimicrobial defense at epithelial surfaces [8].

The surface of our skin is constantly being challenged by a wide variety of microbial pathogens; still cutaneous infections are relatively rare. Within cutaneous innate immunity, the production of antimicrobial peptides (AMPs) is a primary system for protection against infection. Many AMPs can be found in the skin and these include molecules that were discovered for their antimicrobial properties, and other peptides and proteins first known for activity as chemokines, enzymes, enzyme inhibitors and neuropeptides. Cathelicidins were among the first families of AMPs discovered on the skin. Now they are known to have two distinct functions: they have direct antimicrobial activity and initiate a host cellular response resulting in cytokine release, inflammation and angiogenesis [9].

Synthesis of pre-vitamin D_3 from 7-dehydrocholesterol occurs in skin and involves UVB radiation that penetrates the epidermis. 7-dehydrocholesterol absorbs UV light most effectively at wavelengths between 270–290 nm and thus the production of vitamin D_3 will occur at those wavelengths. Calciol, which is the product of the transformation of 7-dehydrocholesterol, is an inactive, unhydroxylated form of vitamin D_3. To form the active hormone calciol must be hydroxylated twice to form calcidiol (25-hydroxyvitamin D_3, $25D_3$) and finally, active calcitriol (1,25-dihydroxyvitamin D_3, $1,25D_3$) (Figure 1). The two enzymes responsible for activating vitamin D_3 – vitamin D 25-hydroxylase (CYP27A1) and 25-hydroxyvitamin D_3. 1-α-hydroxylase (CYP27B1) were initially identified in liver and kidney [10, 11]. Keratinocytes express both enzymes and are capable of producing active $1,25D_3$ independent of renal and hepatic hydroxylation steps [12].

Vitamin D_3 exerts a pluripotent effects on adaptive immune function by [13, 14]:

1) adaptive T cell immune reaction;
2) maturation and proliferation of LC from immature dendritic cells;
3) increase of innate immunity in skin and regulation of antimicrobial defense at epithelial surfaces (cathelcidin expression);
4) regulation of keratinocyte differentiation and proliferation, as well as production of intact epidermal barrier;
5) changes in serum D vitamin level which may impact skin immune barrier functions and inflammatory reactions.

In skin, the presence of vitamin D_3 is essential for normal keratinocyte development, differentiation and function [15]. Any alteration in local vitamin D_3 concentrations and / or activation will likely affect normal cutaneous immune function, barrier function and inflammatory responses [16, 17].

The minimal concentration of vitamin D per day is well known – for adults who are not in risk group the amount is 1000 IU per day. In case of risk group (the elderly, dark skin, season, latitude, time of day, use of sunscreens, fat malabsorption, anticonvulsant use, chronic kidney disease, obesity) – at least 2000 IU is needed.

It has been reported that exposure of 6–10% of the body surface to 1 minimal erythemal dose, which is the minimum amount of UVB radiation that produce redness in 24 hours after exposure, is equivalent to ingesting about 600–1000 IU of vitamin D [18].

Obesity is one of the risk factors for low vitamin D status; we investigated patients with metabolic syndrome. According to the International Diabetes Federation definition [Alberti, et al., 2006] a person can be characterized as having the metabolic syndrome if he has central obesity (waist circumference for women ≥ 80 cm, men ≥ 94 cm) and additionally two of any of the following factors: raised triglycerides, reduced HDL cholesterol, raised blood pressure, raised plasma glucose. In skin, metabolic syndrome causes a chronic latent inflammation that impact all skin functions, such as barrier, immune and endocrine function. Due to obesity, there are inadequate balance between high-density lipoproteins and low-density lipoproteins, that may induce defence skin immunological responses to external factors. The lipid abnormalities might facilitate and maintain the inflammatory reaction in the skin. Mainly, activity of macrophages and increased activity of matrix metalloproteinase are involved. There are no reports regarding the influence of vitamin D on DCs in MS patients.

The aim

The aim of our research is to explore the potential immunomodulatory activity of vitamin D on LC in case of metabolic syndrome.

Material and methods

Generally, 49 40–55-year old patients of both genders have been analyzed (I-III Fitzpatrick's skin phototype) in the Clinic of Dermatology, Rīga, Latvia. The respective study employed the group of patients with clinically and biochemically proved diagnosis of MS based on IDF 2006 criteria [International Diabetes Federation, 2006].

In the course of the study, several parameters were evaluated in all patients:

- clinical examination entailed measurement of the arterial blood pressure and waist circumference;
- instrumental examination of the skin surface with ARAMO SG – Skin Analyser (Aramhuvis Co) with 3 different lenses (×1; ×10; ×60) for determining the moisture, sebum level, evenness of the surface, dyspigmentation, capillary characteristics and skin micro-relief;
- biochemical testing: total cholesterol level (TH), fasting plasma glucose, high density lipoproteins (HDL), low density lipoproteins (LDL) levels, 25-hydroxyvitamin D;
- full-thickness circular 4 mm *Punch* biopsies were obtained from the dorsal surface of the palm. Specimens were stained with haematoxylin-eosin and with Masson's trichrome. Immunohistochemical antibody staining was conducted with Dako Cytomation EnVision system (Denmark) for the following antigens: CD34, CD117. Subsequently, capillaries and CD117 positive cells and fibres were quantified per 1 mm^2. Axiostar Plus microscope with a ruler was used to measure adipocyte dimensions under ×400 magnification. CD1a positive Langerhans cells were evaluated in 3 fields of vision. The average quantity of CD1a positive Langerhans cells was calculated under ×400 magnification. All data were documented and analysed using Microsoft Excel 2012 software and MS Excel programmes.

Results

In the group of patients with MS, mean waist circumference for both genders was 95 cm. Average body mass index – 25.5 kg/m^2. Women : men ratio – 1.6 : 1. Mean 25-hydroxyvitamin D minimal and maximal values are shown in Figure 1, assumed normal vitamin D level in serum in adults varies from 30 to 100 IU.

Regarding the blood biochemistry: average cholesterol level is 6.1 mmol/l, LDL – 3.5 mmol/l, HDL – 0.5 mmol/L, and CRP – 2.1 mg/L, respectively.

By dermatoscopic findings, in case of MS facial skin dyspigmentation and pronounced telangiectasia were revealed, yet skin micro-relief has not been altered.

In patients with MS, CD34 positive endothelial cells composed up to 4.5 capillaries per 1 mm^2, in patients without MS, 3.1 capillaries per 1 mm^2, respectively. The epidermal basal membrane was thickened in MS cases. CD117 positive mast cells were evenly distributed in the dermis and between adipocytes (8 CD117 positive cells per 1 mm^2 on average).

Figure 1. 25-hydroxyvitamin D level in serum

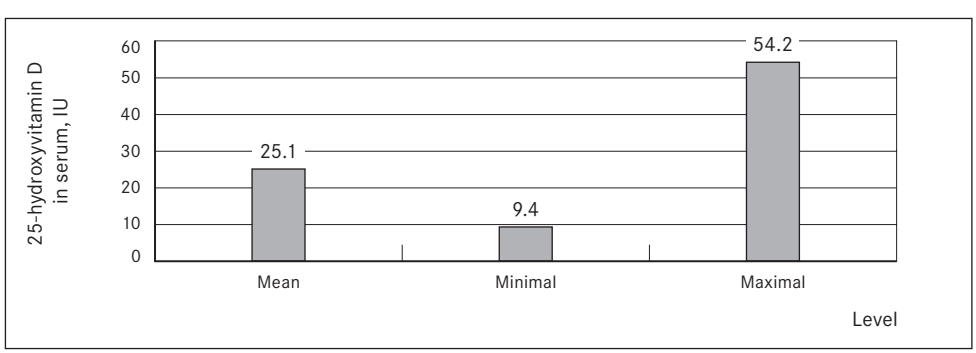


Table 1. Association of 25-hydroxyvitamin D level in serum and amount of LC in epidermis

25-hydroxyvitamin D in serum, IU	Average amount of LCs per field of vision
8.3	18.0
10.3	25.0
20.8	13.1
Up to 30	7.7

Immunoreactivity of bcl-2 protein had a patchy pattern in basal cells with a mean quantity of 39.1 per 100 basal cells in MS and 6.4 in persons without it. Average thickness of epidermis was 0.71 mm (patients with MS) and 0.46 mm (without MS). Thickness of *stratum spinosum* was 0.48 mm (patients with MS) and 0.25 mm (without MS). Thickness of *stratum corneum* was 0.18 mm (patients with MS) and 0.16 mm (without MS).

Enlargement of adipocytes up to 0.13 mm and fibrosis of deep vessels has been revealed.

Amount of DCs in epidermis varied from 32.6 to 13.5, the quantity per field of vision is illustrated in Table 1. In cases with low vitamin D level number of $CD1a^+$ cells was 16.2 and there are tendency in increase of LC in epidermis (Figure 2).

Significant accumulation of DCs around the cluster of inflammatory cells, acanthosis and hyperkeratosis were observed. Variable content of Birbeck granules in patients with MS was evaluated, as well as in some cases migration of CD $1a^+$ cells into papillary dermis (Figure 3). $CD1a^+$ cells were revealed around apoptotic lesion in epidermis, as well as a mild inflammatory reaction around two skin appendages (Figure 4).

Figure 2. Migration of Langerhans cells into dermis (×400, Dako)

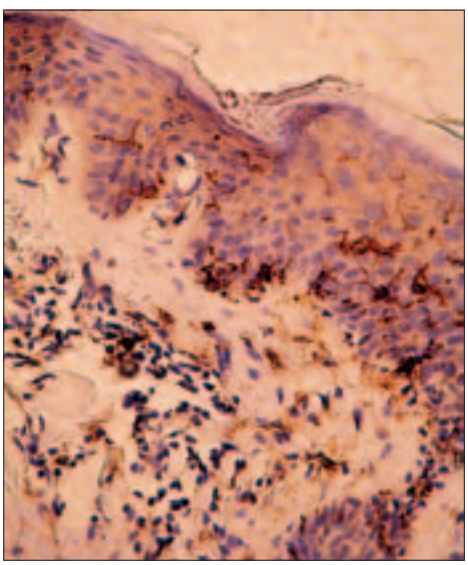
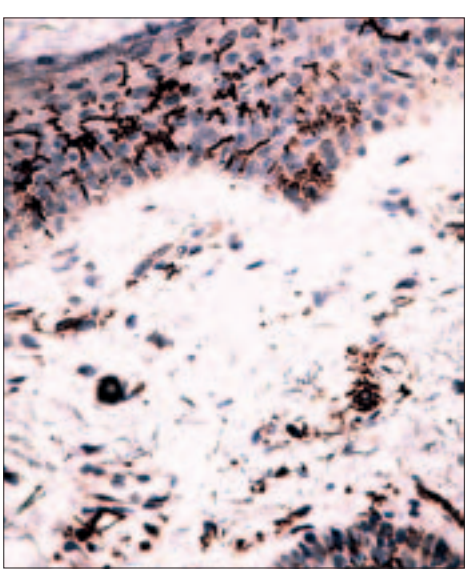


Figure 3. Adequate amount of LC amount (right) (×100, Dako); increased number of LC in case of vitamin D deficiency (left) (×400, Dako)



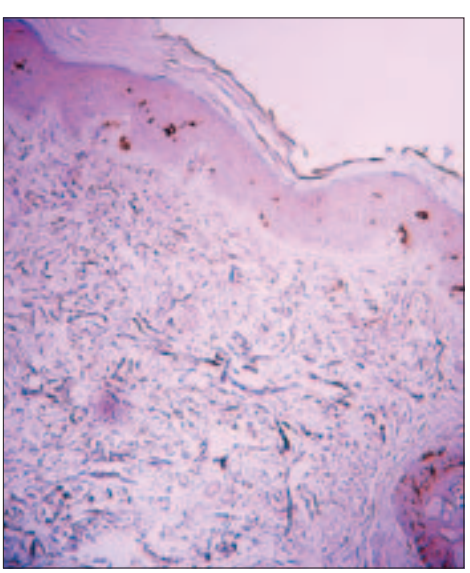
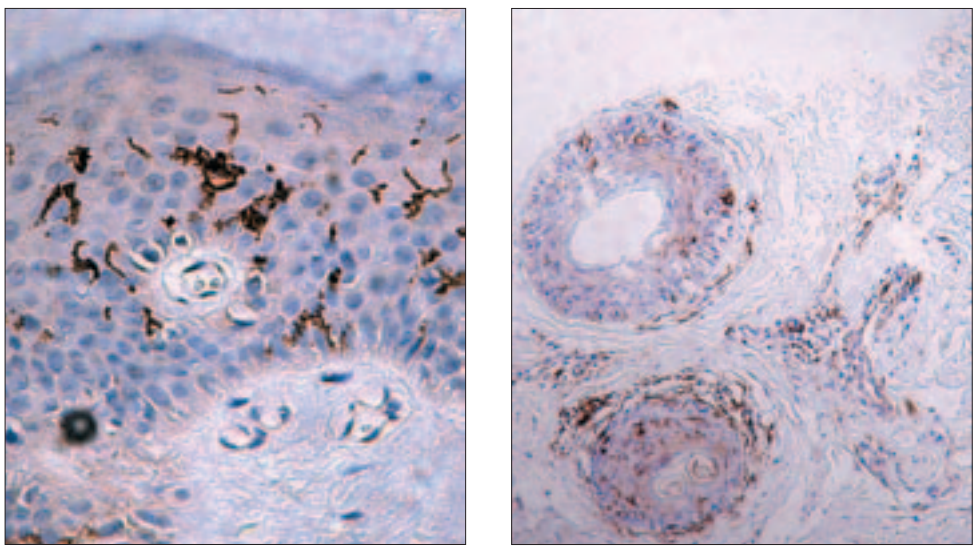


Figure 4. Concentration of CD1a+ cells around apoptotic keratinocytes (left) (×400, Dako), mild inflammatory reaction and CD1a+ cells around appendages (right) (×100, Dako)



Discussion

The immune system exists in a delicate equilibrium between inflammatory responses and tolerance. DCs are the primary professional antigen-presenting cells (APCs) initiating adaptive immune response. In this review, we summarize accumulating evidence about the role of regulatory DCs in situations where the balance between tolerance and immunogenicity has been altered leading to pathologic conditions such as diffuse chronic inflammation [19]. There are a lot of articles describing LC peculiarities in case of local skin lesion such as melanoma, basal cell carcinoma, etc. The role and characteristics of LC in basal cell carcinoma (BCC) is revealed. Thus, the lower density and fewer morphological changes of LC in the epidermis overlying BCC may give rise to alterations in the immune response to BCC [20]. I. Santos has described significant increase in the number of LC in the group with lower potential of local aggressiveness, as compared with the number of cells in the epidermis superposed to the basal cell carcinoma, thus limiting the aggressiveness of the neoplasm [21]. Patapova, et al. and Prignano, et al. underline the role of LC in skin chronic inflammatory diseases such as psoriasis and atopy, where LC participated in the reaction of allergic contact dermatitis and recognized them as antigen-presenting cells [22, 23].

As considered in case of MS, chronic latent inflammation and metabolic disturbances persist in the skin due to oxidative stress, that may affect skin immune system, as it was published before by J. Janovska, et al. [24]. Changes in CD3, CD20, CD8, accumulation of pro-apoptotic protein bcl-2, thickness of basal cell membrane of epidermis and mild inflammatory reaction around capillaries were revealed.

Our study showed that 25-hydroxyvitamin D deficiency in MS patients is associated with higher amount of dendritic cells and increase of their activity in epidermis. In patients with low 25-hydroxyvitamin D level in serum average LC quantity in 1 field of vision is higher (up to 25) compared with those, who have normal amount of 25-hydroxyvitamin D less than 10 CD1a positive cells. These peculiarities may be associated with skin immunomodulatory process and characterizes LC as antigen-presenting cells. Different amount of Birbeck's granules filling is revealed, of those patients, whose skin is conducted with metabolic syndrome. In case of MS expansion of adipocytes and dermal fibrosis may point to hypoxia in tissue, which could be directly associated with presence of oxidative stress under metabolic syndrome.

Conclusions

1. In patients with low vitamin D level in serum, average amount of Langerhans cells quantity in one field of vision is higher in comparison with those, who have sufficient rates of vitamin D.

2. In patients with metabolic syndrome and vitamin D deficiency, Langerhans cells activity is higher and filling of Birbeck's granules are more pronounced in comparison with those, who have sufficient vitamin D status.
3. It is necessary to investigate more regarding Langerhans cells and vitamin D effect in case of metabolic syndrome in order to reveal more interaction with lymphocytes, plasma cells and mast cells as a part of skin immune system.

References

1. Banchereau J., Steinman R. M. Dendritic cells and the control of immunity (Review) // Nature, 1998; 392: 245–252.
2. Kissenpfennig A., Malissen B. Langerhans cells revisiting the paradigm using genetically engineered mice (Review) // Trends Immunol, 2006; 27: 132–139.
3. Tony C., Ronald J. The normal Langerhans cell and the LCH cells // Br J Cancer, 1994; 70.
4. Austyn J. M. Lymphoid dendritic cells // Immunology, 1987; 6: 161–170.
5. Erna J., Breve J., et al. Antigen-bearing Langerhans cells in skin draining lymph nodes: phenotype and kinetics of migration // J of Invest Dermatol, 1994: 217–220.
6. Elbe A., Tschachler E., Steiner G., et al. Maturational steps of bone marrow-derived dendritic murine epidermal cells. Phenotypic and functional studies on Langerhans cells and Thy-1+ dendritic epidermal cells in the perinatal period // J Immunol, 1989; 143: 2431–2438.
7. Schaerli P., Willimann K., Ebert L. M., et al. Cutaneous CXCL14 targets blood precursors to epidermal niches for Langerhans cell differentiation // Immunity, 2005; 23: 331–342.
8. Toebak M. J., Gibbs S. Dendritic cells: biology of the skin // Contact Dermatitis, 2009; 60: 2–20.
9. Lee C. H., Wu S. B., Molecular mechanisms of UV-induced apoptosis and its effects on skin residential cells: the implication in UV-based phototherapy // Int J Mol Sci, 2013 Mar 20; 14 (3): 6414-35. doi: 10.3390/ijms14036414.
10. Yim S., Dhawan P., Ragunath C., et al. Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D(3) // J Cyst Fibros, 2007; 6: 403–410.
11. Schauber J., Dorschner R. A., Yamasaki K., et al. Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli // Immunology, 2006; 118: 509–519.
12. Prosser D. E., Jones G. Enzymes involved in the activation and inactivation of vitamin D // Trends Biochem Sci, 2004; 29: 664–673.
13. Dixon K. M., Tongkao-on W. Vitamin D and death by sunshine // Int J Mol Sci, 2013; 18; 14 (1): 1964–1977.
14. Van Etten E., Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts // J Steroid Biochem Mol Biol, 2005; 97: 93–101.
15. Schauber J., et al. Antimicrobial peptides and the skin immune defense system // J Aller Clin Imunol, 2009; 124 (3).
16. Yim S., Dhawan P., et al. Induction of cathelicidin in normal and CSF bronchial epithelial cells by D(3) // J Cyst Fibros, 2007; 6: 403–410.
17. Bikle D. D. Vitamin D regulated keratinocyte differentiation // J Cell Biochem, 2004; 92: 436–444.
18. Oda Y., Sihlbom C., Chalkley R. J., et al. Two distinct co-activators, DRIP/mediator and SRC/p160, are differentially involved in VDR transactivation during keratinocyte differentiation // J Steroid Biochem Mol Biol, 2004; 90: 273–276.
19. Bikle D., Chang S., Crumrine D., et al. Mice lacking 25OHD 1alpha-hydroxylase demonstrate decreased epidermal differentiation and barrier function // J Steroid Biochem Mol Biol, 2004; 5: 347–353.
20. Ran Zhang, Naughton D. P. Vitamin D in health and disease // Nutrition Journal, 2010; 9: 65.
21. Schmidt S., Andrea C., et al. Regulatory dendritic cells: there is more than just immune activation // J Frontiers in Immunology, 2012; 3: 274.
22. Mardones F., Zemelman V., Sazunic I., et al. CD1a+ Langerhans cells in the peritumoral epidermis of basal cell carcinoma // Actas Dermosifiliogr, 2009; 100: 700–705.
23. Santos I., Mello R. J., Santos I. B., et al. Quantitative study of Langerhans cells in basal cell carcinoma with higher or lower potential of local aggressiveness // An Bras Dermatol, 2010 Mar–Apr; 85 (2): 165–171.
24. Potapova O., Luzgina N., Shkurupiy V. Immunomorphological study of Langerhans cells in skin of patients with atopic dermatitis // Bull Exp Biol Med, 2008; 146 (6); 809–811.
25. Prignano F., Gerlini G., Fossombroni V., et al. Control of the differentiation state and function of human epidermal Langerhans cells by cytokines in vitro // J Eur Acad Dermatol Venerol, 2001 Sept; 15 (5): 433–440.
26. Janovska J., Kisis J., Voicehovska J., et al. Pilot study of early skin changes due to metabolic syndrome // Collection of scietific papers 2012. – Rīga: RSU, 2013. – Pp. 11–17.

Comparative Study of Oveall Survival of Hereditary (HNPCC) and Other Forms of Colorectal Cancer according to Family History

Inese Eglite, Miki Nakidzawa-Miklasevica, Gunta Purkalne, Elina Skuja, Zanda Daneberga, Janis Gardovskis, Edvins Miklasevics

Rīga Stradiņš University, Institute of Oncology, Latvia

Abstract

Cancer is a very common disease; many families have at least a few members who have had cancer. Sometimes, certain types of cancer affect some families. This can be caused by a number of factors but in some cases cancer is caused by mutation and determinated genetically.

The purpose of this study was to describe compatibility to Amsterdam criteria II (ACII) of various substitute classification systems by comparing overall survival of hereditary non-polyposis colorectal cancer (HNPCC) patients and colorectal (CRC) patients with suspicion to HNPCC syndrome in family; patients with cancer aggregation among more than 3 first-degree relatives in family; and other patient groups with hereditary cancer of localizations related to Lynch syndrome.

The case-record data of 1423 patients were analyzed, classified by clinical diagnosis, cancer history in family, age at which cancer was diagnosed, stages of cancer by TNM classification, criteria matching and mortality.

Patients with CRC diagnosis without known cancer cases of any localization among first and second-degree relatives in family have worst survival rate compared to patients in HNPCC group matching to Amsterdam criteria II. Comparing the survival between patients with sporadic and hereditary colorectal cancer, survival rate as prognostic factor among different patients groups with or without cancer diagnosis (CRC, breast or other types), anamnesis in family comparing with HNPCC syndrome the study reported improved prognosis for HNPCC patients compared to sporadic colorectal cancer patients.

Probably survival of HNPCC patients depends not only on family history, tumour localization, pathohistological findings, but also on biological and genetic features.

In this context, molecular genetic testing of MMR gene mutations would play a key prognostic role.

Keywords: CRC, HNPCC, Lynch syndrome, Amsterdam criteria II, MMR genes, MSI.

Introduction

Cancer is such a common disease that many families have at least a few members who have had cancer. Sometimes, certain types of cancer seem to run in some families. This can be caused by a number of factors, like smoking, unhealthy life style, obesity that tend to influence cancer risk. In some cases cancer is caused by mutation that is being passed over from generation to generation. Although this is often referred to as an inherited cancer, what is inherited is the mutation that can lead to cancer, not the cancer itself. Only about 5–10% of all cancers are hereditary – resulting directly from mutation inherited from a parent [2].

One cause of hereditary colon cancer is a disease called familial adenomatous polyposis (FAP). People with this disease start getting colon polyps in adolescence, and over time may have many of polyps in their colon. If left alone, at least one of these polyps will become cancer. The gene for this syndrome is called *APC*, and testing for mutations in this gene is available. If FAP is diagnosed early in life, surgery to remove the colon is often used to stop the cancer from developing. The most common inherited syndrome that increases a person's risk for colon cancer is hereditary non-polyposis colorectal cancer (HNPCC), or Lynch syndrome. People with this syndrome have a high risk of colorectal cancer. Most of these cancers occur before age 50. HNPCC also leads to a high risk of endometrial cancer in women. Other cancers linked with HNPCC include cancer of the ovary, stomach, small intestine, pancreas, kidney, brain, urethras and bile duct [1].

HNPCC is caused by mutations in one of DNA mismatch repair (MMR) genes – *MLH1*, *MSH2*, *MSH6*, *PMS1*, or *PMS2*. Mutations in these genes can be found by genetic testing. Another option for people with colorectal cancer is to have the tumour tissue tested for changes that can be caused when one of these genes is faulty. These changes are known as microsatellite instability (MSI). Having normal findings (no MSI) implies that HNPCC is not present and that the genes that cause it are normal [10.] Synchronous and metachronous cancers are common, and multiple colorectal cancers can be observed in 20–40% of people with HNPCC [12].

The ones who are known to carry an MMR gene mutation should start colonoscopy screening at a young age in order to diagnose cancers and polyps at an early stage. Women with HNPCC should be screened for endometrial cancer.

Originally, HNPCC is a clinical diagnosis, based on the Amsterdam criteria I (ACI). In 1999, the ACI were superseded by the Amsterdam criteria II (ACII), which included extracolonic HNPCC-associated tumours. After the discovery of germline MMR gene mutations as the cause of HNPCC, it soon became challenged that these criteria are too stringent, as a large proportion of families carrying germline MMR gene mutations did not fulfill either ACI or ACII criteria. Due to cultural and socio-economic reasons and the size of Latvian families, in clinical practice it is often hard to fulfill ACII criteria and get an appropriate anamnesis for patients diagnosed with CRC. Consequently, several groups formulated new sets of criteria to select patients for mutation analysis in the MMR genes. The best known are the Bethesda criteria, published in 1997 and revised in 2004 and HNPCCsusp. groups by Józef Kładny and Jan Lubiński [8].

The aim

The aim of this study is to describe usefulness and compatibility to ACII of various substitute classification systems by comparing overall survival of HNPCC patients and CRC patients classified as HNPCCsusp. (suspicion to HNPCC syndrome in family); patients with cancer aggregation among more than 3 first-degree relatives in family (group CFA); and other patient groups with hereditary cancer of localizations related to Lynch syndrome.

The results should enable to give recommendations to rational guidelines regarding whether or not to offer mutation analysis to a patient with cancer who is at risk of HNPCC and could be used as prognostic marker in clinical practice.

Material and methods

The case-record data of 1423 patients at mean age 67.1 years were obtained from Rīga Stradiņš University Institute of Oncology data base. Patients were classified by clinical diagnosis, cancer history in family, age at which cancer was diagnosed, stages of cancer by TNM classification, criteria matching. The mortality was analyzed using the Cox proportional hazards models. The diagnosed group, age, and stratified tumour stages were tested with multivariable linear regression models adjusted for covariates. Methodology and criteria by Józef Kładny and Jan Lubiński were used to divide patients into HNPCC and HNPCCsusp. groups [8].

The R software version 2.15.1 was used for the statistical analyses. All hypotheses were two sided and p-value less than 0.05 was considered as statistically significant.

Results

Patient groups. 114 (8.6%) patients were diagnosed stage I cancer, 501 (37.7%) – stage II, 424 (31.9%) patients – stage III, 289 (21.8%) patients – stage IV cancer by TNM classification [6]. For 113 patients cancer stage data were not available. We analyzed 356 (25%) patients with 2 cases of any cancer localization in family (2CA group); 144 (10.1%) patients with more than 3 first-degree relatives with any cancer diagnosis (CFA group); 85 (6%) patients with 2 cases of CRC within I0 blood relatives older than 50 years (FCC1 group – Family Colorectal Cancer); 30 (2.1%) patients with 2 cases of CRC within II0 blood relatives at any age (FCC2 group); 16 (1.1%) patients with hereditary breast cancer in family (HBC group) – at least 3 breast cancer cases in one family at any age, one of them a first-degree relative to other two; 47 (3.3%) patients matching to Amsterdam criteria II; 76 (5.3%) patients (HNPCCsusp. group – suspicion to HNPCC syndrome in family) – at least 2 first-degree relatives had HNPCC syndrome related cancer, at least one out of them diagnosed before age 50; 669 (47%) CRC patients as a control group without any detected hereditary cancer or known cancer anamnesis among first or second-degree relatives in family, defined as NEGATIVE group (Table 1).

Mean survival of all analyzed patients were 48.2 months.

Statistical analysis. Survival of different patients groups graphically shows Kalpan-Meijer survival curves. Vertical axis represents estimated probability of survival for a hypothetical cohort (survival rate, the percentage of subjects who have survived), horizontal axis represents living for a certain amount of time after treatment, measured in months.

It appears that patients with CRC diagnosis without known cancer cases of any localization among first and second-degree relatives in family (NEGATIVE group) have worst survival rate compared to patients in HNPCC group matching to Amsterdam criteria II. Cox multivariate analysis showed that the hazard ratio of risk of death from cancer was 0.4 (95% CI 0.2–0.8; p = 0.01) in HNPCC (Figure 1).

Also 2 CRC cases among first or second-degree blood relatives in anamnesis do show significantly (p = 0.0315 and p = 0.0367, respectively) worst survival prognosis than HNPCC group patients and death risk from cancer is higher in both FCC1 and FCC2 than in HNPCC group (Figures 2, 3). Results did not reach statistical significance that patients of groups FCC1 and FCC2CRC (at least 2 cases of CRC cases among first and second degree relatives (p = 0.261 and p = 0.784)) would have higher death risk from cancer than patients without known cancer anamnesis in family (NEGATIVE group) (Figures 2, 3).

Analysis also showed lower potential risk to die from cancer being diagnosed with HNPCC by Amsterdam criteria II than for patients (HNPCCsusp. group) with first-degree relatives who have had HNPCC syndrome related cancers (colonic, or extra-colonic) (p = 0.0021). However, sporadic CRC cannot be considered as a better prognostic factor for disease outcome compared to patients' group with anamnesis of at least 2 relatives having HNPCC syndrome related cancers and one of them diagnosed before age 50 (p = 0.784) (Figure 4).

Patients group matching to Amsterdam criteria II (HNPCC) did show better survival prognosis than group with any type of cancer diagnosis aggregation within family among the first-degree relatives (CFA group) (p = 0.049). Comparing CFA group with patients without known cancer anamnesis in family (NEGATIVE group) median survival difference is 5.2 months; however there is no statistical significance reached proving risk for death within 14–15 years (p = 0.060) (Figure 5).

Also 2 CRC cases among first or second-degree relatives in anamnesis (2CA group) showed significantly (p = 0.007) worst survival prognosis than HNPCC group patients, but death risk from cancer is not higher (p = 0.444) compared to patients without relatives having had any known cancer diagnosis (NEGATIVE group) (Figure 6.)

Although patients' group having had hereditary breast cancer cases in family (HBC group) showed worst survival prognosis compared to HNPCC group (p = 0.0106) and median survival difference is 12.4 months, there is no proven survival benefit of NEGATIVE group over HBC group (p = 0.639) (Figure 7).

Table 1. Groups of patients, diagnosis and clinical data analyzed, spread by cancer stages*

Group of patients	Clinical description	Number of patients, n	Age median, years (IQR)	Survival median, months (IQR)	Stage of cancer – number of patients in the group, n (%)
2CA	Two cases of any cancer localization in consanguineous patients	356	68 (60, 75)	46.2 (17.2, 69.7)	I – 30 (9.0%)
					II – 132 (39.6%)
					III – 103 (30.9%)
					IV – 68 (20.4%)
					23 – stage data unavailable
CFA	Cancer familial aggregation: ≥ 3 diagnosis of cancer among first-degree relatives	144	68.5 (59, 76)	50.5 (22.3, 74.5)	I – 15 (10.9%)
					II – 58 (42.3%)
					III – 40 (29.2%)
					IV – 24 (17.5%)
					7 – stage data unavailable
FCC1	Family colorectal cancer 1: 2 CRC cases among first-degree-relative patients with age-of-onset ≥ 50 years	85	68 (62, 75)	51.1 (24.9, 70.5)	I – 7 (8.9%)
					II – 32 (40.5%)
					III – 25 (31.6%)
					IV – 15 (19.0%)
					6 – stage data unavailable
FCC2	Family colorectal cancer 2: 2 CRC cases among second-degree-relative patients with any age-of-onset	30	62 (53.2, 71.5)	51.9 (16.7, 77.2)	I – 5 (17.9%)
					II – 9 (32.1%)
					III – 8 (28.6%)
					IV – 6 (21.4%)
					2 – stage data unavailable
HBC	Hereditary breast cancer: ≥ 3 breast cancer cases in family with any age-of-onset; one first-degree relative to other two diagnosed cases	16	62.5 (57.8, 72.5)	44.7 (25.4, 68.5)	I – 0 (0.0%)
					II – 4 (26.7%)
					III – 7 (46.7%)
					IV – 4 (26.7%)
					1 – stage data unavailable
HNPCC	Amsterdam's criteria II: 1) ≥ 3 relatives diagnosed with HNPCC or related cancer (colorectal, endometrial, small intestines', renal, urethral cancer), one first-degree relative to other two diagnosed cases; 2) diagnosed cancer in two generations; 3) ≥ 1 case of cancer before age of 50; 4) diagnosis of FAP should be excluded	47	66 (57.5, 73)	57.1 (20.9, 88.8)	I – 5 (11.6%)
					II – 16 (37.2%)
					III – 17 (39.5%
					IV – 5 (11.6%)
					4 – stage data unavailable
HNPCC susp.	HNPCC syndrome in family suspected: ≥ 2 cases of HNPCC syndrome related cancer in first-degree relatives with one case diagnosed before age of 50	76	60 (48.8, 69)	35.4 (16.4, 69.9)	I – 5 (7.4%)
					II – 25 (36.8%)
					III – 22 (32.4%)
					IV – 16 (23.5%)
					8 – stage data unavailable
Negative	Control group: diagnose of CRC without hereditary cancer or known cancer anamnesis in family	669	70 (63, 76)	45.3 (15.5, 68.4)	I – 47 (7.5%)
					II – 225 (36.0%)
					III – 202 (32.3%)
					IV – 151 (24.2%)
					44 – stage data unavailable

* Stage of cancer by TNM classification.

Figure 1. Survival of HNPCC and NEGATIVE patients groups

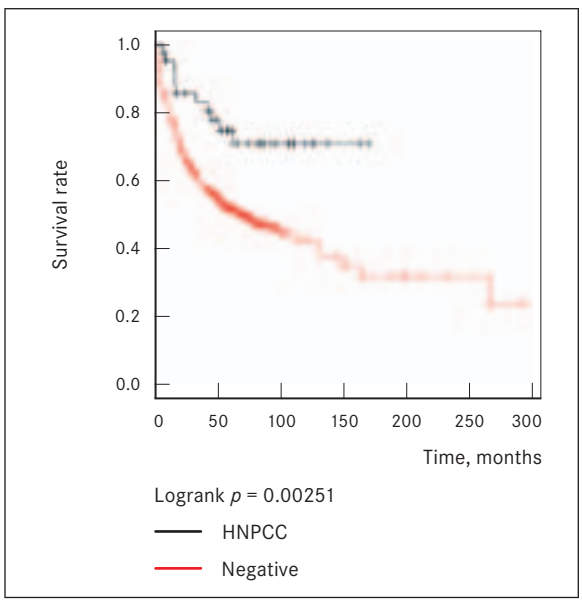


Figure 2. Survival of HNPCC, NEGATIVE and FCC1 groups

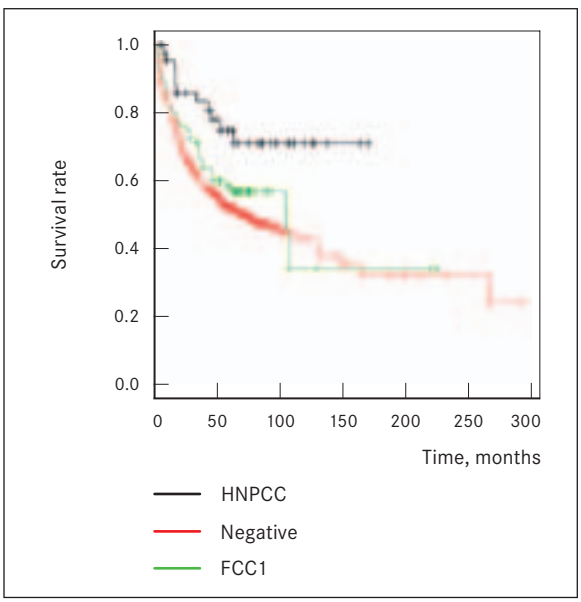


Figure 3. Survival of HNPCC, NEGATIVE and FCC2 groups

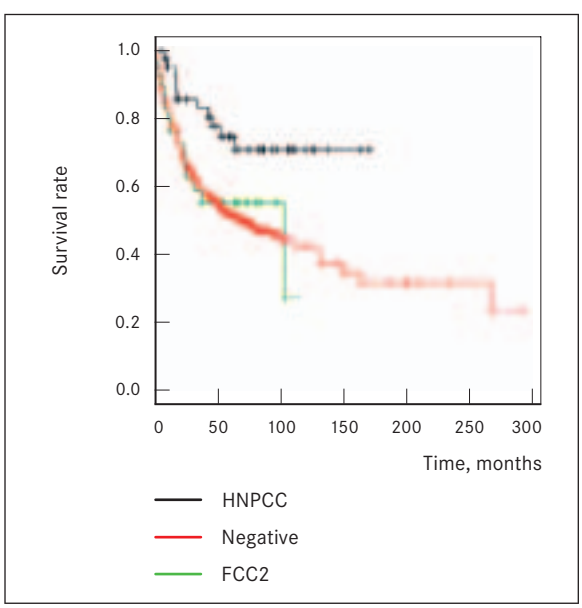


Figure 4. Survival of HNPCC, NEGATIVE and HNPCCsusp. groups

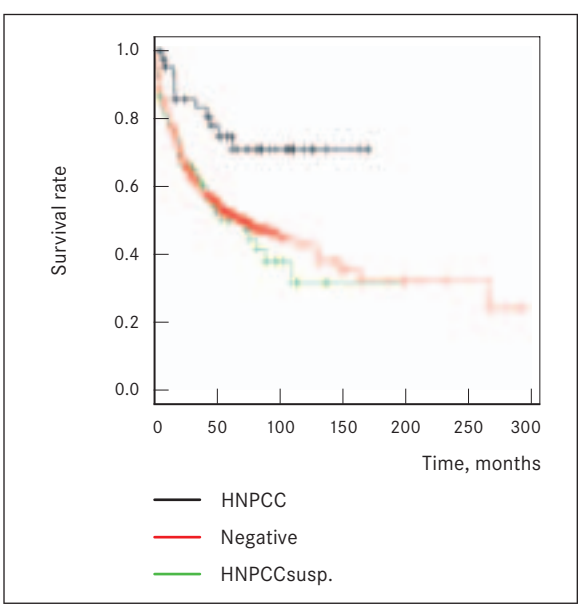


Figure 5. Survival of HNPCC, NEGATIVE and CFA groups

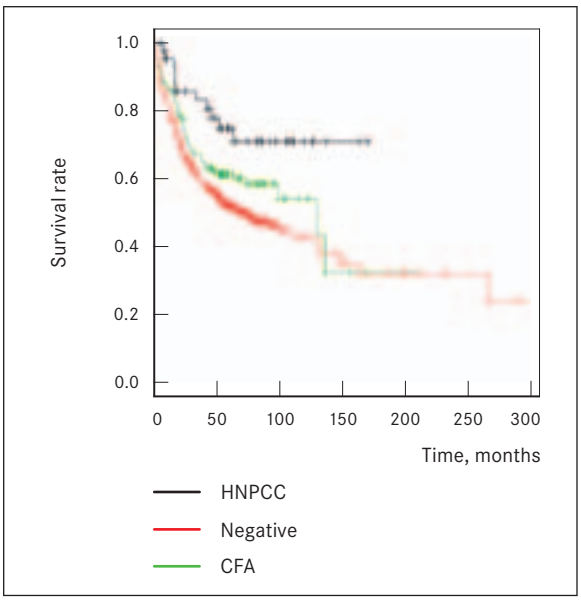


Figure 6. Survival of HNPCC, NEGATIVE and 2CA groups

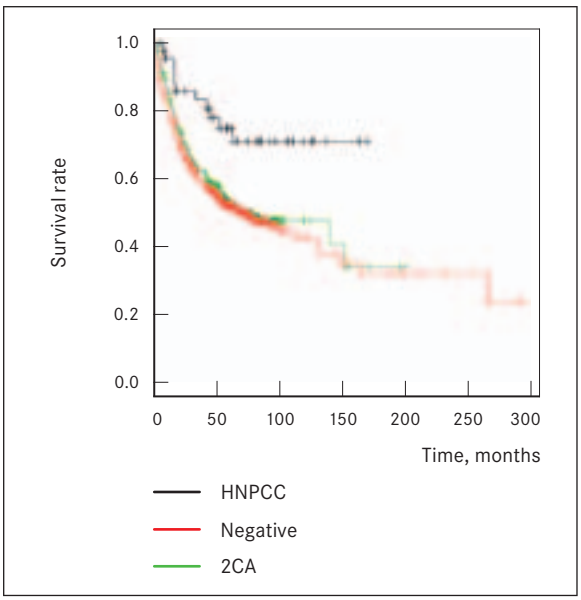
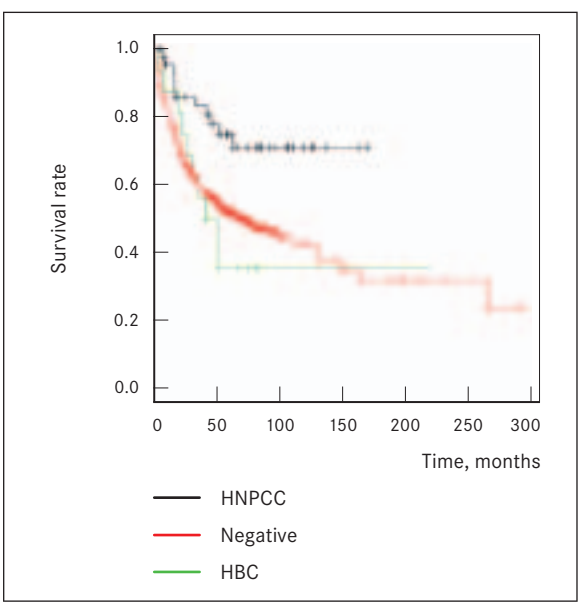


Figure 7. Survival of HNPCC, NEGATIVE and HBC groups



Discussion

Different survival rates of patients with colorectal cancer have been investigated in several studies.

The results are sometimes conflicting because of the different pathogenetic mechanism of tumour genesis between sporadic and familiar types of colorectal syndrome (HNPCC in particular). These differences are probably due to different clinical pathological characteristics and genetic alterations [4].

If we compare the survival between patients with sporadic and hereditary colorectal cancer, survival rate as prognostic factor among different patients groups with or without cancer diagnosis (CRC, breast or other types) anamnesis in family compared to HNPCC syndrome, then a couple of studies have reported improved prognosis for HNPCC patients compared to sporadic colorectal cancer patients, but some studies did not confirm it [15].

The localization of tumour is an important prognostic factor for survival [4], because some types of cancer are more aggressive than the other ones. Moreover, the same colorectal cancer based on anatomical localization (rectum, colon), distribution and histological characteristic of tumour can influence survival prognosis.

This different anatomical distribution between HNPCC and sporadic CRC, confirmed in literature [5] is one of the Amsterdam criteria for the diagnosis of HNPCC and determines a better prognosis, being less aggressive.

We have considered survival rate, stratified patients by groups based on cancer anamnesis in family among first and second degree relatives – whether they had CRC or any other cancer localization, were diagnosed at an early age (< 50 years) or by criteria suspected to HNPCC syndrome.

5-year survival in HNPCC group was significantly better than in sporadic CRC group, it means less death risk from cancer (95% CI 0.2–0.8; p = 0.01) in HNPCC.

Survival rate of HNPCC showed also benefit versus patients with any type of cancer aggregation within family (2CA and CFA groups), also previous history of 2 cases of CRC diagnosis among first and second-degree relatives could be considered as poorer prognostic factor for survival than HNPCC syndrome.

Interestingly, even patients group suspected to HNPCC syndrome (HNPCCsusp.) showed much worst survival prognosis than by Amsterdam criteria defined HNPCC group (p = 0.002).

Hereditary breast cancer patient group (HBC) with at least 3 breast cancer cases in family showed much worst survival prognosis than HNPCC group (p = 0.01).

Although patients in each group were spread statistically similarly taking into account cancer stage by TNM classification (Table 1), prevalence of localized tumours (stage II and stage III) in HNPCC group is higher than in HNPCCsusp. group where more advanced disease (stage IV for 16 patients out of 76 vs. 5 patients out of 47) is found more often. Current analysis did not compare different patient groups by different cancer stages; this could be valuable information for further research based on some studies [9, 5] indicating that a good prognosis can be based on a favourable stage at diagnosis.

When matched stage for stage, colon cancers in individuals with Lynch syndrome are associated with a better prognosis than sporadic colon cancers. Also, poorly differentiated histology of Lynch syndrome-related colon cancers is typically associated with a poor prognosis. Histologic characteristics of Lynch syndrome-related colon cancers include: poor differentiation, tumour-infiltrating lymphocytes, mucin, and signet ring or cribiform histology [14]. Histological findings were not taken into account at this study.

Further studies should deal with different therapies patients of different groups received – whether adjuvant chemotherapy was received or not, what was the regimen, the number of chemotherapy cycles.

Lynch syndrome is caused by mutations in genes involved with MMR pathway, and this pathway functions identify and remove single nucleotide mismatches or insertions and deletion loops. Mutations in four of the MMR genes can cause Lynch syndrome [14]. The functions of the mismatch repair genes can be disrupted by missense mutations, truncating mutations, splice site mutations, large deletions,

or genomic rearrangements. In addition, germline deletion within *EPCAM*, which is not an MMR, can disrupt the MMR pathway. Genetic modifiers of cancer risk in Lynch syndrome have been reported by many authors [14]. To establish the probability of Lynch syndrome and to identify which gene is most likely to have a causative germline mutation in a person with Lynch syndrome, tumour testing is recommended.

Although the Amsterdam criteria can be a significant predictor of a germline mutation in an MMR gene in families that fulfill the criteria, the Amsterdam criteria, nonetheless, fail to identify a large portion of persons with a germline MMR gene mutation. Therefore, family history and the Amsterdam criteria cannot be relied upon to identify all individuals with a germline mutation in one of the MMR genes. Sjursen et al. [2010] found the sensitivity of the Amsterdam criteria II to be 87%, 62%, 38%, and 48% for identifying persons with a *MLH1*, *MSH2*, *PMS2*, or *MSH6* germline mutation, respectively [14]. Hampel et al. also reported that in a population-based study of persons with colon cancer, only three of 23 persons with a germline mutation in an MMR gene met the Amsterdam criteria [6].

Therefore besides cancer anamnesis in family, assessment matching to Amsterdam criteria, life style evaluation and clinical assessment of molecular genetic testing is recommended.

There are reported advantages of Microsatellites Instabity (MSI) testing. MSI testing is an effective method for determining which tumours arise from MMR deficiency. Studies have demonstrated that the sensitivity of MSI testing for identifying tumours in individuals with a germline MMR gene mutation is 93% [16].

BRAF mutations the most commonly occur in 15% of colorectal cancers. *BRAF* mutations are thought to be rare in Lynch syndrome-related cancers and, thus, in general the presence of a *BRAF* mutation rules out the diagnosis of Lynch syndrome [14].

There are many Colorectal Cancer Screening testing strategy guidelines developed by professional associations in Europe and the USA, e.g., the National Comprehensive Cancer Network (NCCN) [11].

The screening of tumour tissue for MSI allow to identify individuals who may have Lynch syndrome.

Predictive testing for at-risk asymptomatic adult family members requires prior identification of the disease-causing mutation in the family. Penetrance of colon cancer associated with mutations in a MMR gene or *EPCAM* is less than 100%. Therefore, some individuals with a cancer-predisposing mutation in one of the MMR genes never develop colon cancer [14].

Clinicians and researchers working in the area of hereditary colon cancer have suggested returning to the Lynch syndrome instead of HNPCC in order to specify individuals and families with defective MMR and to distinguish them from other forms of familial colon cancer [3].

Colon cancer surveillance should be ensured by regular colonoscopy, removal of precancerous polyps reduces the incidence of colon cancer in individuals with Lynch syndrome. A 2009 study of a Finish cohort with high compliance with screening found no increase in mortality for individuals with Lynch syndrome over their mutation-negative relatives, indicating that annual colonoscopy could help with the prevention and detection of colon cancer [7]. Therefore, current recommendations are to have colonoscopy every one to two years beginning between ages 20 and 25 years or ten years before the earliest diagnosis in the family, whichever is earlier [7].

Some authors also report that cigarette smoking increases the risk of colorectal cancer in Lynch syndrome [13].

Early recognition of cancers associated with Lynch syndrome may allow for timely intervention and improved final outcome.

When an MMR gene mutation has been identified in a family with Lynch syndrome, molecular genetic testing for the mutation should be offered to all first-degree relatives.

Several factors can hinder the diagnosis of Lynch syndrome based on family history. Screening and removal of precancerous polyps and prophylactic surgery may prevent colon or endometrial cancer in some at-risk relatives; some who died young from other causes may never have developed cancer [14].

However genetic cancer risk assessment and testing is not useful in predicting whether symptoms will occur, and if they do, what the age of onset, severity and type of symptoms, or rate of disease progression will be. When testing at-risk individuals for Lynch syndrome, an affected family member should be tested first to confirm the molecular diagnosis in the family.

Conclusions

1. Our findings appear to confirm previous studies which detected that a better survival for colon cancer in HNPCC, compared to sporadic CRC, usually occur. Moreover, HNPCC patient has better prognostic survival prognosis than hereditary breast cancer or CRC or other localization cancer diagnosis in family (within first and second-degree relatives).
2. Probably survival of HNPCC patients depends not only on family history, tumour localization, pathohistological findings, but also on biological and genetic features.
3. In this context, molecular genetic testing of MMR gene mutations would play key prognostic role. MSI mutation pattern plays an important prognostic role since colon cancer with MSI has a better prognosis than tumours without MSI.
4. HNPCC group should be enlarged and divided by stages, clinical features, tumour histological type and dispensed therapy, and be examined to confirm this data. Only family cancer history and compatibility to ACII criteria with various substitute classification systems is an insufficient prognostic marker for survival in clinical practice.
5. Research should be widened to give recommendations to rational guidelines of molecular genetic analysis and result interpretation to a patient with cancer who is at risk of HNPCC based on clinical, pathological and genetic findings.

References

1. American Cancer Society Cancer Information Database. Colorectal Cancer // http://www.cancer.org/Cancer/Colonand-RectumCancer/DetailedGuide/indexon12/14/2011 (viewed 2013.05.11).
2. American Cancer Society Cancer Information Database. Heredity and Cancer // http://www.cancer.org/cancer/cancercauses/geneticsandcancer/heredity-and-cancer (viewed 2013.05.11).
3. Bellizzi A. M., Frankel W. L. Colorectal cancer due to deficiency in DNA mismatch repair function: a review // Adv Anat Pathol, 2009; 16: 405–417.
4. Cosimelli M., Stigliano V., Assisi D., et al. Survival of hereditary non-polyposis colorectal cancer patients compared with sporadic colorectal cancer patients // Journal of Experimental & Clinical Cancer Research, 2008; 27: 39.
5. Frattini M., Balestra D., Suardi S., et al. Different genetic features associated with colon and rectal carcinogenesis // Clin Cancer Res, 2004 Jun 15; 10 (12 Pt 1): 4015–4021.
6. Hampel H., Stephens J. A., Pukkala E., et al. Cancer risk in hereditary nonpolyposis colorectal cancer syndrome: later age of onset // Gastroenterology, 2005; 129: 415–421.
7. Järvinen H. J., Renkonen-Sinisalo L., Aktán-Collán K., et al. Ten years after mutation testing for Lynch syndrome: cancer incidence and outcome in mutation-positive and mutation negative family members // J Clin Oncol, 2009; 27: 4793–4797.
8. Kadny J., Lubiński J. Hereditary Lynch syndrome (HNPCC) // Cancer in Clinical Practice, 2008; 6 (2): 99–102.
9. Kohlmann W., Gruber M. S., Stephen B., MD, PhD. Lynch Syndrome synonyms: HNPCC, Hereditary Non-polyposis Colon Cancer. Includes: *EPCAM*-related Lynch Syndrome, *MLH1*-related Lynch Syndrome, *MSH2*-related Lynch Syndrome, *MSH6*-related Lynch Syndrome, *PMS2*-related Lynch Syndrome // http://www.ncbi.nlm.nih.gov/books/NBK1211/#hnpcc.Molecular_Genetics / Initial posting: February 5, 2004; last revision: September 20, 2012 (viewed 2013.04.24).
10. National Human Genome Research Institute. Chromosome abnormalities fact sheet // www.genome.gov/11508982 on December 14, 2011 (viewed 2013.04.20).
11. NCCN Clinical Practice Guidelines in Oncology: Colon Cancer // http://www.nccn.org/professionals/physician_gls/pdf/colon.pdf (viewed 2013.04.20).

12. Niessen R. C., Berends M. J., Wu Y., et al. Identification of mismatch repair gene mutations in young patients with colorectal cancer and in patients with multiple tumours associated with hereditary non-polyposis colorectal cancer // Gut, 2006; 55: 1781–1788; doi: 10.1136/gut.2005.090159.
13. Pande M., Lynch P. M., Hopper J. L., et al. Smoking and colorectal cancer in Lynch syndrome: results from the Colon Cancer Family Registry and the University of Texas M. D. Anderson Cancer Center // Clin Cancer Res, 2010; 16: 1331–1339.
14. Peltomaki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer // J Clin Oncol, 2003; 21: 1174–1179.
15. Percesepe A., Benatti P., Roncucci L., et al. Survival analysis in families affected by hereditary nonpolyposis colorectal cancer // Int J Cancer, 1997; 71: 373–376.
16. Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part 1: The utility of immunohistochemistry // J Mol Diagn, 2008; 10: 293–300.

Sedation with a Target-controlled Infusion during Colonoscopy

Inga Vevere [1], *Agnese Malinovska* [2], *Romans Dzalbs* [3], *Anete Mickevica-Lepenika* [3], *Biruta Mamaja* [1, 2]

[1] *Rīga Eastern Clinical University Hospital, Latvia*
[2] *Rīga Stradiņš University, Department of Anaesthesiology and Reanimatology, Latvia*
[3] *Rīga Stradiņš University, Faculty of Medicine, Latvia*

Abstract

The best methods for analgesia and sedation during gastrointestinal endoscopy are still debated, ranging from minimal (anxiolysis), moderate (conscious) or deep sedation to general anaesthesia. Accurate titration of sedative / analgesic medications is essential to improve patient comfort and safety and to avoid the risk of over-sedation. Therefore, target-controlled infusion (TCI) systems have been developed to provide constant blood concentration of intravenous anaesthetics.

The aim of the study was to assess our first experience with a propofol sedation using target-controlled infusion for colonoscopy with a focus on the effectiveness and safety of the method.

Material and methods. A prospective study was performed for 91 unpremedicated patients, 31 males (34%) and 60 females (66%) undergoing colonoscopy, ASA I – 33 (36%); ASA II – 37 (41%); ASA III – 21 (23%). All patients were divided into two groups depending on whether patient received only TCI propofol or TCI propofol plus 50 mcg of fentanyl for sedation. Fentanyl was used either before the procedure, if difficult colonoscopy was anticipated, or during the procedure, if higher than 6 mcg/mL propofol concentration was necessary. Pulse oximetry, non-invasive blood pressure and ECG monitoring was applied. Entropy electrodes were applied to 48 randomly cases to evaluate the depth of sedation.

Results. The age cohorts: 20–40 yrs – 17 (19%), 41–60 yrs – 28 (30%), > 60 yrs – 46 (51%). There were 39 (43%) inpatients and 52 (57%) outpatients. The mean plasma concentration of propofol used was 3,62 mcg/mL (range 1 – 6 mcg/mL) and the total propofol consumption was 70 – 521 mg. 41% of patients received 50 mcg of fentanyl.

The values of response entropy (RE) were 65 (± 5.42) and 64 (± 5.14); the values of state entropy (SE) were 70 (± 5.33) and 72 (± 5.05) for propofol group and for propofol plus fentanyl group, respectively.

Incidence of hypotension was 27%, mostly in patients with arterial hypertension. Five patients required mandibular advancement. No other sedation-related adverse events (arrhythmia, O_2 desaturation < 90% or short time assisted manual ventilation) were observed.

Conclusion. Data from this study suggest that the titration of propofol concentration with a target-controlled infusion device (with or without low dose of fentanyl) depending on the required clinical effect provides adequate and safe sedation during colonoscopy and maintains spontaneous ventilation and low incidence of adverse events.

Keywords: sedation, colonoscopy, target-controlled infusion (TCI).

Introduction

Although oesophagogastroduodenoscopy (EGD) and colonoscopy can be performed without sedation [1], both procedures are better tolerated in terms of patient satisfaction and willingness to repeat the examination when sedation is administered [2]. On the other hand, sedation delays patient recovery and discharge, increases the risks and costs associated with endoscopy [3]. Cardiopulmonary events related to sedation and analgesia (such as hypoxemia, hypoventilation, airway obstruction, aspiration, apnea, arrhythmias, hypotension and vasovagal episodes) are the most frequent causes of gastrointestinal endoscopy-related mortality [4].

The best methods for analgesia and sedation during gastrointestinal endoscopy are still debated, ranging from minimal (anxiolysis), moderate (conscious) or deep sedation to general anaesthesia [5, 6]. This categorisation is very subjective, with no objective cut-off line between the categories, and it does not consider sedative / hypnotic dosing strategies. There are overlapping zones between categories. In clinical practice, there is a gray area between deep sedation and general anaesthesia; many so-called "deep sedations" are virtually general anaesthesia [7]. Several sedation scales and scoring systems have been developed to describe the level of consciousness by a patient's response to verbal, light tactile, or painful stimuli.

Table 1. Sedation scales

Ramsay sedation scale	ASA Continuum of depth of sedation (responsiveness)	Observer's assessment of alertness / sedation (OAA/S)
6 – no response	General anesthesia	0 – no response to pain
5 – sluggish to light glabellar tap / noise	Deep sedation / analgesia	1 – no response to mild prodding / shaking
4 – response to light glabellar tap / noise 3 – responds to commands only	Moderate sedation / analgesia	2 – responds to mild prodding / shaking 3 – responds to loud noise or repeated name 4 – lethargic response to name called
2 – cooperative, oriented, calm	Minimal sedation to awake	5 – responds to name, alert
1 – anxious, agitated, restless	Not defined	6 – anxious, agitated, restless

The degree of sedation should be titrated to achieve patient comfort and a successful procedure. Optimal sedation allows the patient the greatest degree of comfort while preserving the greatest degree of safety [8]. Patients may require different levels of sedation for the same procedure and may attain varying levels of sedation during a single procedure [9]. For example, a patient undergoing colonoscopy may experience greater pain and require more analgesia / sedation at points in the procedure when the colon wall is being stretched. It is suggested that the best strategy should be tailored to the individual patient, based on the clinical risk evaluation and the type of procedure to be done. Most endoscopic procedures have been accomplished with moderate or deep sedation [10, 11, 12].

The properties of an ideal sedative agent for endoscopy would include rapid onset of action, analgesic and anxiolytic effects and ease of titration to desired level of sedation, rapid recovery and an excellent safety profile. The short-acting hypnotic agent propofol is increasingly being used due to its rapid onset of action, short recovery profile, anti-emetic proprieties and good amnesic effects [13]. One of the drug's disadvantages, though, is its very narrow therapeutic window. Due to significant variations among patients, it is uncommon for a dose that typically produces moderate sedation to lead to a level of deep sedation or even general anaesthesia in some patients [14].

The ASA guidelines recommend accurate titration of sedative / analgesic medications to improve patient comfort and safety and avoid the risk of over-sedation. Therefore, computer-controlled intravenous drug delivery systems, or target-controlled infusion (TCI) systems have been developed to achieve a user-defined drug concentration in a body compartment or tissue of interest. Using special algorithms (based on real-time pharmacokinetic models, derived from populational studies) TCI pumps calculate their infusion

rates according to patient factors such as age, weight, gender and height [15]. Among different systems available for administration of propofol, the TCI pump is undoubtedly one of the most sophisticated, and several studies have shown that target-controlled drug infusion ensures excellent and safe sedation during endoscopic procedures [5, 16].

In 2010, European Society of Gastrointestinal Endoscopy (ESGE), European Society of Gastroenterology and Endoscopy Nurses and Associates (ESGENA), and the European Society of Anaesthesiology (ESA) published a guideline on non-anaesthesiologists administration of propofol for gastrointestinal endoscopy, providing non-anaesthesiologists with a comprehensive framework on how to implement and practice non-anaesthesiologist-administered propofol (NAAP) [17, 18]. According to this guideline, specific NAAP training courses are necessary for endoscopists and nursing staff to ensure patient comfort and safety, including basic life support (BLS) and advanced cardiac life support (ACLS). The Board of Directors of the ESA initially endorsed the report, but later the endorsement of this guideline was retracted, and the ESA continues to maintain that only anaesthesia specialists should administer propofol [19, 20).

The aim

The aim of the study was to describe and to assess our first experience with a propofol sedation using target-controlled infusion for colonoscopy outside operating room focusing on the effectiveness and safety of the method.

Material and methods

A prospective study was performed for 91 unpremedicated patients, 31 males (34%) and 60 females (66%) undergoing colonoscopy, ASA I – 33 (36%); ASA II – 37 (41%), ASA III – 21 (23%).

The Alaris® PK Syringe Pump was used to administer TCI propofol.

The mean procedure duration was 15.45 (± 6.02) minutes. Duration of procedure was calculated as the time from the start of the procedure (insertion of the endoscope) to the end of the procedure (time of withdrawal of the endoscope).

A starting propofol concentration of one mcg/mL was chosen (using the Marsh model) and it was increased in incremental doses (0.5–1.0 mcg/mL) to achieve loss of response to verbal stimulation with spontaneus breathing. During maintenance of sedation, the propofol target concentration was increased or decreased according to the patient response (movements, moaning) and the degree of difficulty to perform the colonoscopy. In the absence of signs of inadequate sedation, the propofol target concentration was titrated downwards to avoid maintaining propofol concentration higher than clinically necessary. As soon as terminal ileum was reached, the propofol concentration was gradually decreased and finally stopped 1–2 minutes before the end of the procedure.

Additionally, 50 mcg of fentanyl was used either before the procedure, if difficult colonoscopy was anticipated, or during the procedure, if higher than 6 mcg/mL propofol concentration was necessary.

All patients were divided into two groups depending on whether fentanyl was used or not.

Supplemental oxygen was delivered via a face mask (6 litres per minute). The patients were in left lateral position. Pulse oximetry (SpO_2), non-invasive blood pressure and ECG monitoring was applied.

Entropy electrodes were applied to 48 randomly cases. The values of response entropy (RE) and state entropy (SE) were recorded continuously throughout the endoscopic sedation to evaluate the depth of sedation.

Safety of the method was evaluated based on incidence rates of adverse events and changes in vital signs.

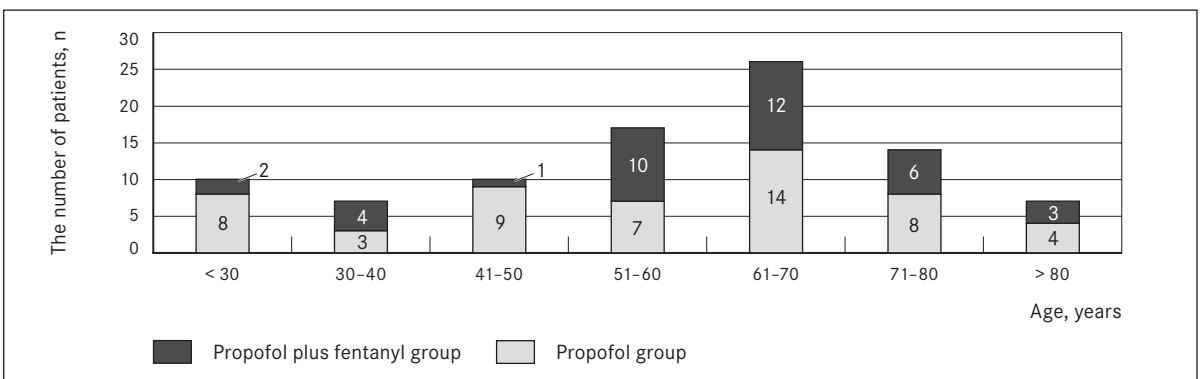
Data processing and statistical analysis was made by STATISTICA 7.0 software package using descriptive analysis and one-way ANOVA. Categorical variables are presented as percentages. Continuous values are expressed as mean ± SD. Categorical values were compared using chi-square (χ^2) test or Fisher exact tests, as appropriate. P value of < 0.05 was considered significant.

Results

The age cohorts: 20–40 yrs – 17 (19%), 41–60 yrs – 28 (30%), > 60 yrs – 46 (51%). There were 39 (43%) inpatients (ASA I–II – 24, ASA III – 15) and 52 (57%) outpatients (ASA I–II – 46, ASA III – 6). The mean plasma concentration of propofol used was 3.62 mcg/mL (range 1–6 mcg/mL) and the total propofol consumption was 70–521 mg. 41% of patients received 50 mcg of fentanyl (Figure 1, Figure 2, Table 2).

Incidence of hypotension (fall in systolic blood pressure > 25% from the baseline value) was 27% (25 cases), mostly (14 cases) in patients with arterial hypertension, this was transient and resolved spontaneously without any additional medication (Table 3). Five patients (6%) required mandibular advancement (similar number of cases in both groups). No other sedation-related adverse events (arrhythmia, O_2 desaturation < 90% or short time assisted manual ventilation) were observed.

Figure 1. Distribution of cases by age in propofol group and propofol plus fentanyl group



Groups	Age, years						
	< 30	30–40	41–50	51–60	61–70	71–80	> 80
Propofol plus fentanyl group	2	4	1	10	12	6	3
Propofol group	8	3	9	7	14	8	4

Figure 2. Total distribution of cases by weight

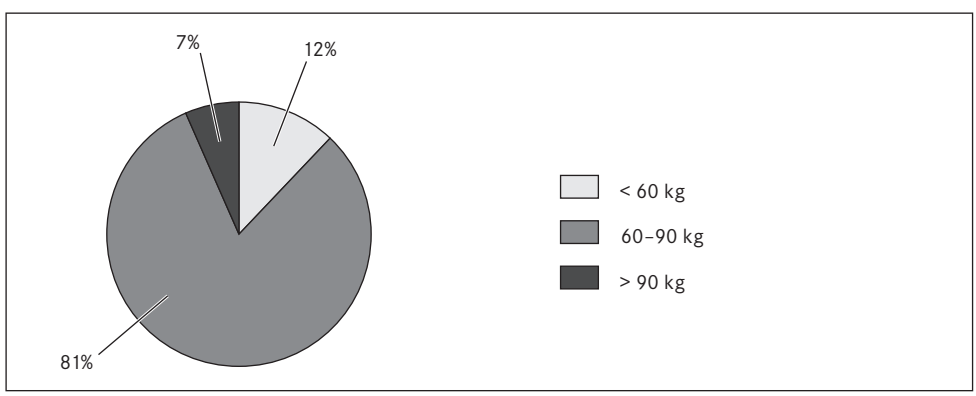


Table 2. Distribution of cases by weight in propofol group and propofol plus fentanyl group

Patient weight category, kg	Total number, n	Propofol used, n	Propofol plus fentanyl used, n
< 60	11	6	5
60–90	74	43	31
> 90	6	4	2

Table 3. Baseline clinical characteristics

Characteristics	Total (n = 91)	Propofol used (n = 53)	Propofol plus fentanyl used (n = 38)
Propofol plasma concentration, mean ± SD	3.62 ± 1.08	3.93 ± 0.97	3.19 ± 1.09
Propofol consumption (mg), mean ± SD	243.42 ± 80.73	255.77 ± 74.41	226.18 ± 86.88
Incidence of hypotension, n (%)	25 (28%)	14 (26%)	11 (29%)

Table 4. Entropy parameters

Parameters	Sedation	
	Propofol used	Propofol plus fentanyl used
Mean SE (± 95% CI)	65 (± 5.42)	64 (± 5.14)
Mean RE (± 95% CI)	70 (± 5.33)	72 (± 5.05)

Ten minutes after the procedure, all patients were able to dress up and to walk. Inpatients were transported to the endoscopy unit and then back to the ward by wheelchair. Outpatients were taken to the recovery room until they are fully awake.

The level of satisfaction with the sedation was high for a patient, an anaesthetist, and endoscopist. Outpatients were discharged in about 30 minutes.

Discussion

As shown by the results of our study, colonoscopy requires deep sedation (Table 4), sometimes very close to general anaesthesia (when the target range for entropy values is 40–60) [21]. Fentanyl use did not affect the entropy parameters.

The degree of sedation may be proportional to the difficulty of performing colonoscopy. There is a number of factors that affect the the endoscopy – loops or angulation in the colon, diverticular disease, altered anatomy and adhesions from prior surgery, as well as technical skill of the endoscopist and the quality of the bowel preparation [22, 23]. The desired level of sedation can be effectively and safely provided using propofol [24, 25, 26].

There are different techniques of propofol administration. Some anaesthetists are skilled in the delivery of intravenous anaesthetic agents by intermittent bolus injection. However, the plasma concentration of drug and the anaesthetic effect fluctuate widely, and the technique is acceptable only for procedures of short duration. A bolus injection followed by a continuous infusion results in achievement of an excessive concentration (with an increased incidence of side effects), and this is followed by a prolonged dip below the intended plasma concentration. In order to achieve a reasonable constant plasma concentration in this case, it is necessary to use a multistep infusion regimen, which requires complex calculations [27].

TCI system allows an anaesthetist to set (and change) a desired concentration of anaesthetic agent, based on the clinical observation of the patient. Multi-compartmental pharmacokinetic models are used by TCI systems to calculate the infusion rates required to achieve the target concentration. When the anaesthetist increases the target concentration, the system administers a rapid infusion (bolus) to quickly fill the central compartment (which is calculated from an equation using height, weight, age and gender as variables). When the new target concentration is achieved, it stops the rapid infusion, and commences an infusion at a lower rate that is gradually decreasing. When the anaesthetist decreases the target concentration, the system stops the infusion, and waits until it estimates that the blood concentration has reached the target. Then it will re-start the infusion at a lower rate to maintain the target concentration [15].

The main complications associated with propofol use include respiratory depression, hypotension and bradycardia [28], therefore patient's level of consciousness and pulmonary ventilation, oxygenation and hemodynamics should be monitored continuously during moderate or deep sedation. As there can be variable individual patient response to sedative or analgesic agent, the recommended procedure is to deliver an initial bolus and then titrate the drug by incremental dosing until the desired level of sedation is achieved. Adequate time must elapse between doses to assess drug effect before subsequent dose administration.

Hypotension is a known dose-dependent complication of propofol that appears to be caused by both vasodilatation and myocardial depression. Literature reports a 25–40% reduction of systolic blood pressure using propofol induction dose of 2 to 2.5 mg/kg [29, 30, 31, 32]. Transient decreases in blood pressure are more prominent during bolus administration. Thus, most patients are recommended slow initial infusions [33]. In our study, hypotension occured in 27% of cases. This is a relatively low number, which means that TCI device allows to minimise the propofol-related blood pressure drop. Hypotension occured less frequently in propofol group than in propofol plus low dose fentanyl group (26% vs. 29%).

Heart rate did not change significantly, and it was not analysed.

An induction dose of propofol results in a 25–30% incidence of apnea [29]. We did not observe any apnea episodes, but we did observe hypoventilation in five cases (6%), which was managed by head extension and / or chin lift. There was also no reduction in SpO_2 on pulse oximetry below 90%. We explain this with the administration of supplemental oxygen during sedation. Since studies have shown limited utility of the SpO_2 measurement during oxygen supplementation, we would prefer to use capnography for better respiratory function assessment in further studies [34, 35].

Both propofol alone and propofol in combination with opiates or benzodiazepines are frequently used during colonoscopy. Hsieh et al. noticed that for sedated colonoscopy, propofol in combination with meperidine is better than propofol alone for improving patients' tolerance and recovery [36]. Our study showed no significant difference between propofol group compared with propofol plus low dose fentanyl group in terms of sedation depth (Table 3), complication rate (hypoventilation in 5 cases (6%), no other sedation-related adverse events in both groups) or recovery time (10 minutes). The propofol target concentration and the propofol consumption were lower when fentanyl was added.

In a study of 17 999 endoscopic procedures performed over 8 years, the authors concluded that deep sedation during endoscopic procedures is safe [37]. They noted that adverse events occurred in a small proportion of patients (4.5%) and six complications, i.e., hypotension, desaturation, bradycardia, hypertension, arrhythmia, and aspiration, occurred in more than 0.1% of patients.

In summary, although deep sedation was necessary to perform colonoscopy and relatively high doses of propofol were used (with or without low dose of fentanyl), the complication rate was low.

Conclusion

Data from this study suggest that the titration of propofol concentration with a target-controlled infusion device (with or without low dose of fentanyl) depending on the required clinical effect provides adequate and safe sedation during colonoscopy and maintains spontaneous ventilation and low incidence of adverse events. This is particularly important for patients who are at risk for hemodynamic detoriation.

References

1. Terruzzi V., Paggi S., Amato A., Radaelli F. Unsedated colonoscopy: a never-ending story // World J Gastrointest Endosc, 2012 April 16; 4 (4): 137–141.
2. Baudet J. S., Aguirre-Jaime A. The sedation increases the acceptance of repeat colonoscopies // Eur J Gastroenterol Hepato, 2012 Jul; 24 (7): 775–80.
3. Aisenberg J., Brill J. V., Ladabaum U., et al. Sedation for gastrointestinal endoscopy: new practices, new economics // Am J Gastroenterol, 2005; 100: 996–1000.

4. Sharma V. K., et al. A national study of cardiopulmonary unplanned events after GI endoscopy // Gastrointestinal Endoscopy, 2007July; 66: 27–34.
5. Fanti L., Testoni P. A. Sedation and analgesia in gastrointestinal endoscopy: What's new? // World J Gastroenterol, 2010 May 28; 16 (20): 2451–2457.
6. Trummel J. Sedation for gastrointestinal endoscopy: the changing landscape // Curr Opin Anaesthesiol, 2007; 20: 359–364.
7. Liu H., Fox C., Kalarickal F., et al. Moderate and deep sedation in clinical practice / R. D. Urman, A. D. Kaye (eds). – Cambridge: Cambridge University Press, 2012. – Pp. 135–149.
8. Vargo J. J., Delegge M. H., Feld A. D., et al. Multisociety sedation curriculum for gastrointestinal endoscopy // Am J Gastroenterol, 2012 May 22.
9. Greenwald D. Sedation: propofol versus conscious sedation. Who should administer it? // http://www.gastrohep.com/freespeech/freespeech.asp?id=101
10. VanNatta M. E., Rex D. K. Propofol alone titrated to deep sedation versus propofol in combination with opioids and/or benzodiazepines and titrated to moderate sedation for colonoscopy // Am J Gastroenterol, 2006; 101: 2209–2217.
11. Gasparović S., Rustemović N., Opacić M., et al. Clinical analysis of propofol deep sedation for 1,104 patients undergoing gastrointestinal endoscopic procedures: a three-year prospective study // World J Gastroenterol, 2006; 12: 327–330.
12. Hata K., Andoh A., Hayafuji K., et al. Usefulness of bispectral monitoring of conscious sedation during endoscopic mucosal dissection // World J Gastroenterol, 2009 Feb 7; 15 (5): 595–598.
13. Singh H., Poluha W., Cheung M., et al. Propofol for sedation during colonoscopy / Cochrane Database Syst Rev. 2008 Oct 8; (4): CD006268 // doi: 10.1002/14651858.CD006268.pub2.
14. Wainscott J. K., Fragneto R. Y. Anesthesia for gastrointestinal endoscopic procedures // Anaesthesia outside of the operating room. Urman R. D., Gross W. L., Philip B. K. (eds). – New York: Oxford University Press, 2011.
15. Absalom A. R., Struys M. M. R. F. An overview of TCI & TIVA. – 2nd ed. – Gent: Academia Press, 2007.
16. Dal H., Izdes S., Kesimci E., Kanbak O. Comparison of intermittant bolus versus target-controlled infusion of propofol sedation for colonoscopy: 2AP2-5 // Eur J Anaesthesiol, 2010 Jun; 27 (47): 38.
17. Vargo J. J., Cohen L. B., Rex D. K., Kwo P. Y. Position statement: nonanesthesiologist administration of propofol for GI endoscopy // Am J Gastroenterol, 2009; 104 (12), 2886–2892.
18. Dumonceau J. M., Riphaus A., Aparicio J. R., et al. European Society of Gastrointestinal Endoscopy, European Society of Gastroenterology and Endoscopy Nurses and Associates, and the European Society of Anaesthesiology // Eur J Anaesthesiol, 2010 Dec; 27 (12): 1016–1030.
19. Perel A. Non-anaesthesiologists should not be allowed to administer propofol for procedural sedation: a Consensus Statement of 21 European National Societies of Anaesthesia // Eur J Anaesthesiol, 2011 Aug; 28 (8): 580–584.
20. Pelosi P., Board of the European Society of Anaesthesiology. Retraction of endorsement: European Society of Gastrointestinal Endoscopy, European Society of Gastroenterology and Endoscopy Nurses and Associates and the European Society of Anaesthesiology Guideline–non-anaesthesiologist administration of propofol for gastrointestinal endosco // Eur J Anaesthesiol, 2012 Apr; 29 (4): 208.
21. Coté G. A., Hovis R. M., Ansstas M. A. Affiliations Department of Anesthesiology, Washington University, St. Louis, Missouri, et al. Incidence of sedation-related complications with propofol use during advanced endoscopic procedures // Clinical Gastroenterology and Hepatology, 2010 Feb; 8: 137–142.
22. Oh S. Y., Sohn C. I., Sung I. K., et al. Factors affecting the technical difficulty of colonoscopy // Hepatogastroenterology, 2007 Jul–Aug; 54 (77): 1403–1406.
23. Witte T. N., Enns R. The difficult colonoscopy // Can J Gastroenterol, August 2007; 21 (8): 487–490.
24. Daorong Wang, Chaowu Chen, Jie Chen, et al. The use of propofol as a sedative agent in gastrointestinal endoscopy: a meta-analysis // PLoS One, 2013; 8 (1): e53311.
25. Vevere I., Putka K., Stepanovs J., Mamaja B. Sedation outside the operating room during colonoscopy with a target controlled infusion // Acta Medica Lituanica, 2012; 19 (3): 354.
26. Krievane I., Mamaja B. Sedation with a target controlled infusion during colonoscopy // RSU Zinātniskās konferences tēzes, 2013. – Rīga: RSU, 2013. – P. 470.
27. Aitkenhead A. R. Textbook of anaesthesia / Aitkenhead A. R., Rowbotham D. J., Smith G. (eds) – 4th ed. – London: Churchill Livingstone, 2001. – Pp. 169–183.
28. Qadeer M. A., Vargo J. J., Khandwala F., et al. Propofol versus traditional sedative agents for gastrointestinal endoscopy: a meta-analysis // Clin Gastroenterol Hepatol, 2005 Nov; 3 (11): 1049–1056.
29. Reves J. G., Glass P., Lubarsky D. A., et al. Miller's anaesthesia / Miller R. D. (ed.). – 7th ed. – London: Churchill Livingstone, 2010, Vol. 1. – Pp. 719–769.

30. Claeys M. A., Gepts E., Camu F. Haemodynamic changes during anaesthesia induced and maintained with propofol // Br J Anaesth, 1988; 60: 3–9.
31. Hertzog J. H., Campbell J. K., Dalton H. J., Hauser G. J. Propofol anaesthesia for invasive procedures in ambulatory and hospitalized children: experience in the Pediatric Intensive Care Unit // Pediatrics, 1999; 103: e30.
32. Bilotta F., Fiorani L., La Rosa I., et al. Cardiovascular effects of intravenous propofol administered at two infusion rates: a transthoracic echocardiographic study // Anaesthesia, 2001 March; 56 (3): 266–271.
33. Triantafillidis J. K., Merikas E., Nikolakis D., Papalois A. E. Sedation in gastrointestinal endoscopy: current issues // World J Gastroenterol, 2013 January 28; 19 (4): 463–481.
34. Arakawa H., Kaise M., Sumiyama K., et al. Does pulse oximetry accurately monitor a patient's ventilation during sedated endoscopy under oxygen supplementation? // Singapore Med J, 2013 Apr; 54 (4): 212–215.
35. Lightdale J. R., Goldmann D. A., Feldman H. A., et al. Microstream capnography improves patient monitoring during moderate sedation: a randomized, controlled trial // Pediatrics, 2006 Jun; 117 (6): 1170–1178.
36. Hsieh Y. H., Chou A. L., Lai Y. Y., et al. Propofol alone versus propofol in combination with meperidine for sedation during colonoscopy // J Clin Gastroenterol, 2009; 43: 753–757.
37. Agostoni M., Fanti L., Gemma M., et al. Adverse events during monitored anaesthesia care for GI endoscopy: an 8-year experience // Gastrointest Endosc, 2011; 74: 266–275.

Changing *emm* Types Epidemiology and Macrolide Resistance of Group A Streptococcus Isolated from Children in Latvia

Dace Zavadska [1, 2], ***Dace Berzina*** [3], ***Nina Pugacova*** [2], ***Ivonna Selga*** [2], ***Dace Gardovska*** [1, 2], ***Edvins Miklasevics*** [1, 3]

[1] *Rīga Stradiņš University, Department of Paediatrics, Latvia*
[2] *Children's Clinical University Hospital, Latvia*
[3] *Pauls Stradiņš Clinical University Hospital, Latvia*

Abstract

The aim of the respective study was to identify and compare *emm* types and determine macrolide resistance genes in group A streptococci (GAS) cultures isolated from children with pharyngitis before and after an intervention to reduce macrolide consumption.

96 GAS strains from 2002–2006 and 47 strains from 2009 obtained from children aged 2–12 years with pharyngitis were compared. The *emm* type and subtype assignments were made according to CDC guidelines. The presence of the *ermA*, *ermB* and *mefA* genes was established using amplification of streptococcal DNA with specific primers.

In total, 22 different *emm* types were identified in 2002–2006 and 17 different emm types in 2009. The most prevalent *emm* type in 2002–2006 was *89.0* (34/96; 35%), in 2009 – *emm 3.1* (8/47; 17%). A new GAS *emm 1.51* subtype was found.

High level of resistance to macrolides was revealed: in 2002–2006 78 % (n = 75), in 2009 47% (n = 22). *emm 89.0* type was prevailingly associated with macrolide resistance (n = 34) in 2002–2006, whereas *emm 3.1* type (n = 5) in 2009.

Significant change in the distribution of *emm* types has occurred during 5 + 1 year period within an 8-year period in Latvia and there still exists high antimicrobial resistance of GAS to macrolides in this country.

Keywords: S. pyogenes, emm types, resistance.

Introduction

Group A streptococci (GAS), also called *S. pyogenes*, are among the most common pathogenic bacteria that infect children and adolescents; they are associated with a wide variety of infections and disease states [1]. Accurate identification and typing of group A haemolytic streptococci is an essential part of epidemiological and pathogenesis studies of streptococcal diseases. A serotyping system based on antigenic variation of a surface exposed M protein and developed by Rebecca Lancefield has been used since 1928 [2]. Adequate knowledge of the dynamics of *emm* types in a region may shed light on the pathogenesis of GAS infections and is crucial for selecting appropriate antimicrobial agent [3–4].

At the beginning of the 21st century GAS is unique in the sense that as of to date there has not been found such GAS clinical isolate which would produce *in vitro* resistance to penicillin, perhaps, the cheapest and most affordable antimicrobial agent [1]. Nevertheless, determination of antimicrobial sensitivity is important in providing rational antimicrobial therapy when penicillin cannot be an optional preparation. Testing of isolates is required for not only the needs to choose appropriate therapy, but also to monitor and possibly control the spread of antimicrobial-drugs-resistant microorganisms in public places and hospitals. Acute pharyngitis is one of the most frequent causes of irrational antibacterial therapy because doctors willingly appoint antimicrobial drugs for children with exudative pharyngitis, and abandon them in cases of catarrhal inflammation. This points to the strategy of inappropriate diagnostics and treatment of patients with pharyngitis symptoms, and in the result of which, during last ten years more and more studies show convincing data on the rise of GAS antibacterial resistance to macrolides [5–9]. Earlier, in Latvia, we have found an extremely high GAS antibacterial resistance to macrolides [10]. Within the course of the study, such results urged us to develop nationwide recommendations regarding antimicrobial use in GAS infections. Later results of the previous study were compared to the second time period after the recommendations have been introduced. Both previous and current results are expected to be of value to the institutions responsible for nationwide recommendations regarding antimicrobial use in terms of growing multidrug-resistance of microorganisms.

Material and methods

Group A streptococcal isolates. Ninety-six group A streptococci strains obtained from throat swabs of inpatients and outpatients aged from 24 months to 12 years having acute pharyngitis symptoms from 2002 to 2006 and 47 GAS strains obtained in identical circumstances in 2009 were compared.

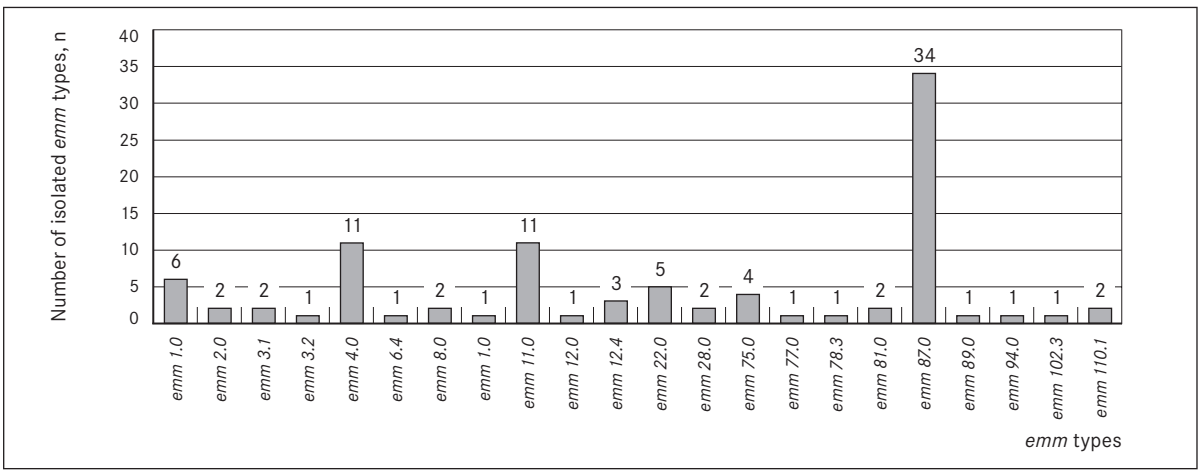
Detection of antimicrobial resistance. The presence of the *ermA*, *ermB* and *mefA* genes was established by the amplification of streptococcal DNA with specific primers. PCR mixture and conditions were made according to CDC guidelines, and *emm* type and subtype assignments were determined as described in CDC website (http://www.cdc.gov/ncidod/biotech/strep/doc.htm).

Results

GAS *emm* type distribution in 2002–2006. In total, 22 different *emm* types were identified among 96 non-invasive isolates during the 2002–2006 period (Figure 1). The most frequent *emm* types were *89.0* (34/96; 35%), followed by *4.0* and *12.0* (11/96; 11%), *1.0* (6/96; 6%) and *28.0* (5/96; 5%). The rest of isolated *emm* types are shown in Figure 1.

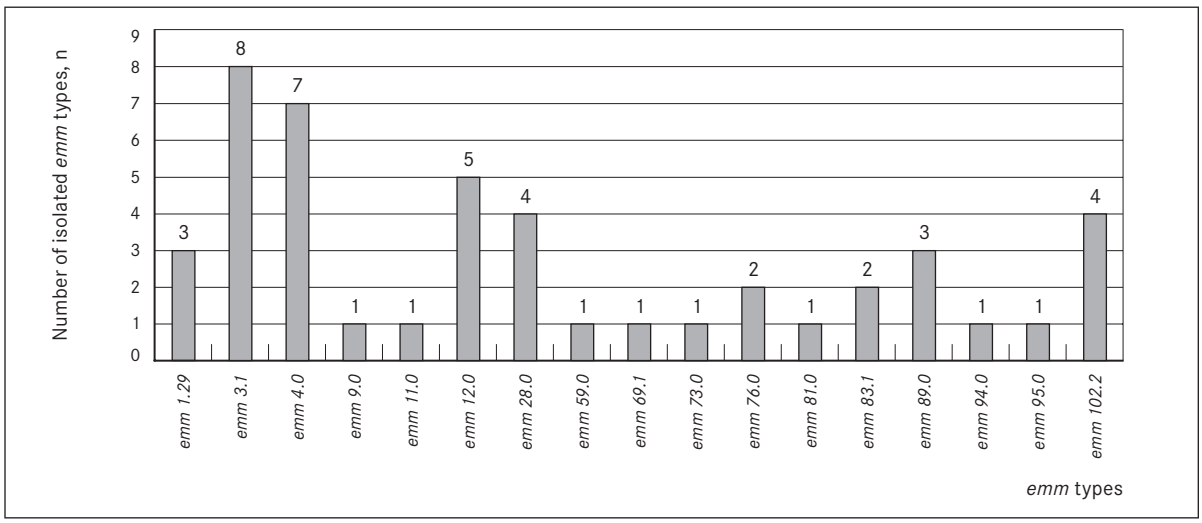
Two different isolates were attributed to a novel *emm* subtype – *1.51*.

Figure 1. GAS *emm* type distribution from 2002 to 2006 (n = 22)



GAS *emm* type distribution in 2009. There were 17 different *emm* types isolated in 2009 (Figure 2). The most prevalent *emm* type was *3.1* (8/47; 17%), then followed *4.0* (7/47; 15%) and *12.0* (5/47; 10%). The rest of isolated *emm* types in 2009 are in small frequency and shown in Figure 2.

Figure 2. GAS *emm* type distribution in 2009 (n = 17)



Antimicrobial resistance during 2002–2006. Totally, 96 GAS isolates from throat swabs of both inpatients and outpatients with sore throats were used to detect antimicrobial resistance. Antimicrobial susceptibility tests revealed that all the strains tested were sensitive to vancomycin, linezolid, penicillin and ceftriaxone. At the same time, high levels of resistance to macrolides were evident; 78% (n = 75) of the isolates were resistant to clindamycin and erythromycin.

Molecular studies in 2002–2006 demonstrated that the majority of strains harboured *ermA* (n = 27; 28%), *ermB* (n = 23; 23%) or both genes (n = 24; 25%). *mefA* was detected only in one strain (1%) (Table 1).

Table 1. Frequency of macrolide resistance genes in 2002–2006 and in 2009

Resistance genes	2002–2006 (from total n = 96)		2009 (from total n = 47)	
	n	%	n	%
ermA	27	28	7	15
ermB	23	23	6	13
ermA + ermB	24	25	5	11
mefA	1	1	3	6
ermA + mefA	0	–	1	2
ermB + mefA	0	–	0	–

Emm 89.0 type was found prevailingly associated with antimicrobial resistance to erytromycin and clindamycin, all *emm 4.0* (n = 11) showed resistance to both MLS_B (macrolide-lincosamide-streptogramin B) antibiotics.

Antimicrobial resistance in 2009. 47 GAS strains obtained in identical circumstances, as described above, were analyzed for constitutive macrolide resistance in 2009. As previous antimicrobial susceptibility tests revealed, all the strains tested were again sensitive to vancomycin, linezolid, penicillin and ceftriaxone. There was a notable decline, though still high, of resistance to macrolides – 47% (n = 22) vs. 78% (n = 75) in comparison to 2002–2006. Type *emm 3.1* (n = 5) was most frequently associated with resistance to macrolides within this time period.

There was a slight change compared to 2002–2006 in the distribution of resistance genes. Still the most prevalent resistance gene among isolates was *ermA* (n = 13; 28%), then followed the *ermB* (n = 11; 23%). Both resistance genes (n = 5; 11%) in the isolates were less frequent than in 2002–2006. Comparatively, *mefA* was found more frequently than in previous period (n = 1; 1% in 2002–2006 vs. n = 4; 8.5% in 2009).

Discussion

The distribution of streptococcal *emm* types in cases of non-invasive and invasive diseases tends to vary over time and within different geographic regions [2–19], and a similar situation occurred during our study [10]. The results of our research on certain GAS pharyngeal isolates, e.g. non-invasive, *emm* type epidemiology, indicate that data acquired in Latvia partly coincides with the previously found in European countries [5–16]. For example, *CDC (Centers for Disease Control and Prevention)* in 2009 published 25 most common *emm* types contributing to pharyngeal disease in the Established Market Economy countries, from which 14 are found in our study within 2002–2006: *emm 1*, *emm 2*, *emm 3*, *emm 4*, *emm 6*, *emm 11*, *emm 12*, *emm 22*, *emm 28*, *emm 75*, *emm 77*, *emm 81*, *emm 87* and *emm 89* and 7 in 2009: *emm 4*, *emm 9*, *emm 11*, *emm 12*, *emm 28*, *emm 7*, and *emm 89*, respectively [4]. Most common *emm* types in CDC data were close to most often found types within the same year in Latvia.

Emm 89.0 type, most frequently found during 2002–2006, in studies more often has been found in association with invasive streptococcal diseases [6–9], which is different from the previous [10] and recent study – it had been isolated from acute pharyngitis, e.g. non-invasive, cases. Taking into account that before 2002 no studies on streptococcal *emm* type epidemiology had been done in Latvia, any speculations on *emm 89.0* frequency in this country would not be fair. It should be noted that a significant change in the distribution of the *emm* types in Latvia during this 5 + 1 year period within an 8-year period has been observed.

During *emm* typing, two different isolates were attributed to a novel *emm* subtype – *1.51*. The data of the different isolates were sent to *CDC*, and the confirmation on the new subtype was received.

Although GAS is susceptible to penicillin and many other antimicrobial agents, GAS infections present considerable clinical and public health problems [1, 3, 10, 22–24]. They are responsible for up to 30% of cases of pharyngitis in children [20–22]. An important issue arises on the necessity and effectiveness of antimicrobial therapy. It is, therefore, crucial to determine the antimicrobial susceptibility of a clinical isolate for optimal treatment of infected patients using antimicrobial therapy [3, 5–13, 18–27].

The antibiotic of choice for treating streptococcal pharyngitis is penicillin as it is effective, safe and costs relatively low. Patients who are allergic to penicillin or other β-lactam antibiotics should be treated with erythromycin or other macrolides [20–22]. Despite the extensive use of β-lactams for more than half a century, there have been no reports on resistance to them by GAS. However, increased macrolide usage following the introduction of the second-generation macrolides has been directly associated with the relatively high increase in resistance to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics, and has been reported in many countries [10, 23, 24]. Normally, two mechanisms are involved in *Streptococcus pyogenes* resistance to macrolides: firstly, modification of the target by the *erm*-encoded gene products; secondly, efflux provided by *mefA* products [27]. Similarly, to the previously published data, there have been suggestions that the high rate of macrolide resistance was caused by *emm 89.0* type, harbouring *erm* (B) gene [10, 18, 19].

Having found the alarming results of an extremely high (78%) antimicrobial resistance of GAS to macrolides in outpatient children, which can be explained by frequent macrolide use in outpatient treatment, after the first stydy period [10], Finland's example [23, 24] was found to be equal to the situation described in our study, where in 1992, Seppälä, et al. [23] reported a high rate of erythromycin resistance (44%). Since 1988, there had been a rapid and substantial increase in resistance to erythromycin in group A streptococci in different areas of Finland linked to an increased rate of erythromycin consumption in outpatients. Consequently, policies regarding outpatient antibiotic therapy were changed, and nationwide recommendations were issued that called for reductions in the use of macrolide antibiotics for respiratory and skin infections in outpatients [24]. Macrolides were the most widely prescribed class of antibiotics in Latvia by general practitioners for treatment of upper respiratory tract infections during the study

of 2002–2006 [10]. Within the first period of the study, results on extremely high macrolide resistance in Latvia urged the need to develop nationwide recommendations regarding antimicrobial use in GAS infections. In the second time period after the recommendations have been introduced, there has been a notable decline of resistance to macrolides, though still high – 47% (n = 22) vs. 78% (n = 75) in 2002–2006. Besides, we have to take into account that the declining macrolide resistance might be associated also with significant change in the distribution of *emm* types, int. al. with the change of resistant genotypes.

Conclusion

Significant change in the distribution of *emm* types has occurred during 5 + 1 year period within an 8-year period in Latvia, and there still exists a high antimicrobial resistance of GAS to macrolides in this country.

Taking into account GAS resistance data in the world and the data of the research done in Latvia it is important to remember that still today, penicillin as an optional preparation should be prescribed in treatment of streptococcal acute pharyngitis.

Adequate penicillin therapy prevents from developing of rheumatic fever in a later stage, even if the treatment is started 9 days after the onset of acute streptococcal pharyngitis [20, 22]; it shortens also the clinical course, reduces the risk of transmission, as well as decreases the risk to develop purulent complications. For these reasons giving antimicrobial therapy in cases of acute pharyngitis is not urgent, and doctors may make decisions on how to start the treatment after awaiting and receiving all laboratory results that they think necessary to have.

Acknowledgements

This work was supported by:

1. *ESF National Programme "Support for Implementation of Doctoral Programmes and Postdoctoral Research in Medical Sciences", Contract No. 2004/0005/VPD1/ESF/PIAA/ 04/NP/3.2.3.1./ 0001/0004/0066.*
2. *ESF Project "Support for PhD Programme Studies and Earning of Scientific Degree at RSU", Agreement No. 2009/0147/1DP/1.1.2.1.2./09/IPIA/VIAA/009.*
3. *WHO Multicentric (Egypt, Latvia, Brazil, Croatia) Study The Grasp Study, 2001–2004.*
4. *Latvian Council of Science (LCS) Grant (Project Nr.04.1211), 2004–2008.*
5. *Ministry of Education and Science, Development of Scientific Activities in 2007. Branch of Science: Medicine Research direction and subdirection: Study of factors causing mortality and disability in children, children's infectious diseases, paediatric surgery, children's rheumatology, immunogenetics, and embryology. Project: Study of factors causing children's mortality and invalidity related with development of new undertakings of diagnostics, treatment and prevention in order to improve children's health in Latvia.*
6. *State Research Programmes – SRP 7 "Reduction of children's mortality by improving early diagnostics, treatment results and prophylaxis of life-threatening diseases in Latvia, employing methods of modern molecular biology, cytometry and immunogenetics", within the project in 2008–2009.*

References

1. Long S., Pickering L., Prober C. Principles and practice of paediatric infectious diseases. – 3rd ed. – Churchill Livingstone, 2008. – Pp. 700–711.
2. Lancefield R. C. Current knowledge of type specific M antigens of group A streptococci // J Immunol, 1962; 89: 307–313.

3. Jaggi P., Beall B., Rippe J., et al. Macrolide resistance and *emm* type distribution of invasive pediatric group A streptococcal isolates // Pediatr Infect Dis J, 2007; 26: 253–255.
4. Steer A., Law I., Matatolu L., et al. Global *emm* type distribution of group A streptococci: systematic review and implications for vaccine development // The Lancet Infectious Diseases, 2009; 9 (10): 611–616.
5. Jasir A., Tanna A., Efstratiou A., et al. Unusual occurrence of M type 77, antibiotic-resistant group A streptococci in Southern Sweden // J Clin Microbiol, 2001; 39: 586–590.
6. Rivera A., Rebollo M., Miro E., et al. Superantigen gene profile, *emm* type and antibiotic resistance genes among group A streptococcal isolates from Barcelona, Spain // J Med Microbiol, 2006; 55: 1115–1123.
7. Zampaloni C., Cappelletti P., Prenna E., et al. *emm* gene distribution among erythromycin-resistant and susceptible Italian isolates of *Streptococcus pyogenes* // J Clin Microbiol, 2003; 41 (3): 1307–1310.
8. Creti R., Imperi M., Baldassarri M., et al. *emm* types, virulence factors, and antibiotic resistance of invasive *Streptococcus pyogenes* isolates from Italy: What has changed in 11 years? // J Clin Microbiol, 2007; 45 (7): 2249–2256.
9. Creti R., Gherardi G., Imperi M., et al. Association of group A streptococcal *emm* types with virulence traits and macrolide-resistance genes is independent of the source of isolation // J Med Microbiol, 2005; 54: 913–917.
10. Zavadska D., Berzina D., Drukalska L., et al. Macrolide resistance in group A beta haemolytic Streptococcus isolated from outpatient children in Latvia // APMIS, 2010; 118 (5): 366–370.
11. Buter C. C., Mouton J. W., Klaassen C. H., et al. Prevalence and molecular mechanism of macrolide resistance in beta-haemolytic streptococci in the Netherlands // Int J Antimicrob Agents, 2010; 35 (6): 590–592.
12. Koh E., Kim S. Decline in erythromycin resistance in group A streptococci from acute pharyngitis due to changes in the *emm* genotypes rather than restriction of antibiotic use // Korean J Lab Med, 2010; 30 (5): 485–490.
13. Pavlovic L., Grego E., Sipetic-Grujicic S. Prevalence of macrolide resistance in *Streptococcus pyogenes* collected in Serbia // Jpn J Infect Dis, 2010; 63 (4): 275–276.
14. Mijac V., Ranin L., Markovic M., et al. Distribution of *emm* types among group A streptococcal isolates from Serbia // Clin Microbiol Infect, 2010; 16: 295–298.
15. Tewodros W., Kronvall G. M. Protein gene (*emm* type) analysis of group A beta-hemolytic streptococci from Ethiopia reveals unique patterns // J Clin Microbiol, 2005; 43: 4369–4376.
16. Langeland N., Mylvaganam H., Kittang B. R. Distribution of *emm* types and subtypes among non-invasive group A, C and G streptococcal isolates in western Norway // APMIS, 2008; 116: 457–464.
17. Vivek S., Rajesh K., Nirmal K. G., et al. Comparative analysis of *emm* type pattern of Group A Streptoccocus throat and skin isolates from India and their association with closely related SIC, a streptococcal virulence factor // BMC Microbiology, 2008; 8: 150.
18. Yu-Fang S., Shin-Min W., Ya-Lan L., et al. Changing epidemiology of *Streptococcus pyogenes emm* types and associated invasive and non-invasive infections in Southern Taiwan // J. Clin Microbiol, 2009; 47 (8): 2658–2661.
19. Wajima T., Somay Y. M., Katsuhiko S., et al. Distribution of *emm* type and antibiotic susceptibility of group A streptococci causing invasive and non-invasive disease // Journal of Medical Microbiology, 2008; 57: 1383–1388.
20. Bisno A. L., Gerber M. A., Gwaltney J. M. Jr., et al. Practice guidelines for the diagnosis and management of group A streptococcal pharyngitis // Clin Infect Dis, 2002; 35: 113–125.
21. Basco W. T. Jr. Viewpoint: acute pharyngitis in children: properly managed? // Paediatrics, 2006; 117: 1871–1878.
22. Pechere J. C., Kaplan E. L. (eds). Streptococcal pharyngitis. Issues Infect Dis. – Basel: Karger, 2004.
23. Seppälä H., Nissinen A., Järvinen H., et al. Resistance to erythromycin in group A streptococci // N Engl J Med, 1992; 326: 292–297.
24. Seppälä H., Klaukka T., Voupio-Varkila J., et al. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland // N Engl J Med, 1997; 337: 441–446.
25. Bandak S. I., Turnak M. R., Allen B. S., et al. Oral antimicrobial susceptibilities of *Streptococcus pyogenes* recently isolated in five countries // Int J Clin Pract, 2000; 54: 585–588.
26. Arvand M., Hoeck M., Hahn H., et al. Antimicrobial resistance in *Streptococcus pyogenes* isolates in Berlin // J Antimicrob Chemother, 2000; 46: 621–623.
27. Fluit A. C., Visser M. R., Schmitz F. J. Molecular detection of a antimicrobial resistance // Clin Microbiol Rev, 2001; 14 (4): 836–871.

Morphological Picture of Psoriatic Skin

Elga Sidhom [1], ***Mara Pilmane*** [1], ***Janis Kisis*** [2]

Rīga Stradiņš University, Latvia
[1] *Institute of Anatomy and Anthropology,*
[2] *Department of Infectology and Dermatology*

Abstract

Psoriasis is a chronic, inflammatory, complex and multifactorial lifelong condition of the skin with Munro microabscesses and spongiform pustules of Kogoj as morphological diagnostic criteria and red, scaly, indurated plaques clinically. Psoriasis patients have altered innate immune system and changes of antimicrobial peptide expression can play an important role in pathogenesis of psoriasis. The aim of the study was to evaluate the expression of human beta defensin 2 (HBD2), tumour necrosis factor-alpha (TNF-alpha), calcitonin gene related peptide (CGRP) and protein gene product 9.5 (PGP 9.5) and subsequent morphological events – inflammation, local immune response and neuropeptides-containing innervation in skin biopsies of psoriatic lesions.

We evaluated 40 *Psoriasis vulgaris* patients' skin samples obtained using routine punch method. All tissue specimens were stained with hematoxylin and eosin and by immunohistochemistry for HBD2, TNF-alpha, CGRP and PGP 9.5. The intensity of staining we graded semiquantitatively.

We observed proliferation of basal layer, reduction of granular layer and parakeratosis, marked infiltration of inflammatory cells in subepithelial layer of the skin, presence of epitheloid cells, macrophages and Munro microabscesses. Arteriole sclerosis and sweat gland cell vacuolization were detected. Numerous to abundant epitheliocytes, macrophages and fibroblasts expressed HBD2, with particular increase in sites of well defined inflammation. TNF-alpha and CGRP positive cells (lymphocytes and macrophages) were found in almost all skin samples – particularly in subepithelium, walls of blood vessels and eccrine sweat glands. A moderate number of neuropeptide-containing nerve fibres (PGP 9.5) was noticed as fine bundles in subepithelial area with prominent innervation of epithelium and sweat glands.

Psoriatic skin shows compensatory prominent local antimicrobial protein expression and neuropeptides-containing innervation on the basis of moderate inflammation and thus may play a role in the pathogenesis of psoriasis.

Keywords: antimicrobial peptides; cytokines; neuropeptides; psoriasis.

Introduction

Psoriasis is a chronic, inflammatory, complex and multifactorial lifelong condition of the skin, often involving nail and joint damage. Large amount of heterogenous genetic and immunologic factors take part in pathogenesis. Morphologically, parakeratosis thinning or complete absence of granular layer, acanthosis and focal spongiosis can be observed due to abnormal epidermal proliferation, dilated blood vessels and perivascular infiltrate of lymphocytes in papillary dermis. The only truly diagnostic criteria are Munro microabscesses and spongiform pustules of Kogoj. Psoriatic skin lesions clinically are described as red, scaly, indurated plaques [5].

Relationship between ultraviolet B (UVB) radiation exposure and psoriasis is well known. There is variability of psoriasis prevalence across various geographic locations and therefore climate, race and environmental antigen exposure, but no relevant differences have been found between genders [4]. Skin works as a primary defense line against pathogens and one of the most important mechanisms is the innate immune system and production of antimicrobial peptides. Large source of antimicrobial peptides are keratinocytes in normal skin and leukocytes once the skin is inflamed. Most described antimicrobial peptides in skin are human β defensins (HBD) and cathelicidins [1, 6]. For a long time psoriasis has been considered T lymphocyte-mediated disease with active involvement of inflammatory cytokines. Cytokine role in the pathogenesis of psoriasis has been greatly exercised in creating new therapies with agents that target cytokines directly. Psoriasis patients often suffer from flare-ups after stressful situations; commonly they report depression. A bilateral link between skin and brain has been found and there is a sequence of neuropeptides found in skin. Psoriasis has altered innate immune system and changes of antimicrobial peptide, inflammatory cytokine and neuropeptide expression can play an important role in pathogenesis of psoriasis.

The aim

The aim of the study was to evaluate the expression of human beta defensin 2 (HBD2), tumour necrosis factor-alpha (TNF-alpha), calcitonin gene related peptide (CGRP) and protein gene product 9.5 (PGP 9.5) and subsequent morphological events – inflammation, local immune response and neuropeptides-containing innervation in skin biopsies of psoriatic lesions.

Material and methods

Patients. We created patient selection criteria to exclude possible affecting factors such as fierce tan, received local and systemic medication, concomitant diseases. The selected patients were between the age of 18 and 70, suffering from psoriasis at least 6 weeks, having visible characteristic psoriatic eruptions in typical localization sites. We selected 40 psoriasis patients with clinically and histologically confirmed diagnosis of *Psoriasis vulgaris*. Skin biopsies were obtained in local lidocaine anesthesia using routine 3 mm punch biopsy method. All biopsied skin was of new untreated psoriasis lesions.

The study was approved by the Ethical Committee of Rīga Stradiņš University, permit issued on September 10, 2009.

Methods. Hematoxylin and eosin. Skin biopsy tissue was fixed in Stefanini's solution, dehydrated, and embedded in paraffin. Further, four micrometer thick sections were prepared and stained routinely with hematoxylin and eosin.

Immunohistochemical method (IMH): HBD 2 (cat No AF 2758, obtained from goat, 1 : 100 dilution, R & D Systems, Germany), TNF-alpha (code ab 6671, obtained from rabbit, 1 : 100 dilution, Abcam, Cambridge, UK), CGRP (B 49–100, obtained from rabbit, 1 : 10 dilution, DakoCytomation, Denmark) and PGP 9.5 (code Z5116, obtained from rabbit, 1 : 600 dilution, DakoCytomation, Denmark) were used by biotin – streptavidin IMH [Hsu, et al., 1981].

The findings of the study were evaluated using Leica DC 300F camera and image processing and analysis software Image Pro Plus.

The intensity of immunostaining was graded semiquantitatively [Pilmane, 1995]:

- few positive structures in the visual field were labelled with +,
- moderate number of positive structures in the visual field was labeled with ++,
- numerous positive structures in the visual field were labeled with +++,
- and abundance of positive structures in the visual field was marked with ++++.

Results

Changes in epidermis were characterized by proliferation of basal layer, reduction of granular layer and parakeratosis, as well as general pronounced proliferation and thickening. Marked infiltration of inflammatory cells was observed in subepithelial layer of the skin, especially surrounding blood vessels (Figures 1–2). Inflammatory cells were found also in hair follicle and around sweat glands. Presence of epitheloid cells and macrophages, as well as Munro microabscesses were observed. Arteriole sclerosis and sweat gland cell vacuolization were detected. All results have been summarized in Table 1.

Numerous to abundant epitheliocytes, macrophages and fibroblasts expressed HBD2, with particular increase in sites of well-defined inflammation (Figure 3).

TNF-alpha positive cells (lymphocytes and macrophages) were found in almost all skin samples – particularly in subepithelium, walls of blood vessels and eccrine sweat glands and their number varied from few (+) to numerous (+++) positive structures in the visual field (Figure 4).

CGRP was established in most specimens – from few (+) to abundance (++++) of positive structures in subepithelium, eccrine sweat glands and walls of blood vessels. More pronounced expression was observed in skin from psoriasis patients with long course of the disease (Figure 5).

Moderate number of neuropeptide-containing nerve fibres (PGP 9.5) was noticed around blood vessels, beneath epithelium, in neuroendocrine cells of epithelium, in smooth muscle bundles and surrounding eccrine sweat glands in psoriasis patients. PGP 9.5-containing nerve fibers were characterized by fine bundles in subepithelial area. Notable innervation of epithelium (Figure 6) and sweat glands was present.

To obtain statistical data, non-parametric statistics was used and Spearman's rank correlation coefficient was calculated. We found moderate positive correlation between CGRP and PGP 9.5 (Spearman's rank correlation coefficient was 0.5286) and moderate positive correlation between TNF-alpha and PGP 9.5 (Spearman's rank correlation coefficient 0.4447). Between defensin and TNF-alpha (Spearman's rank correlation coefficient 0.3886), defensin and PGP 9.5 (Spearman's rank correlation coefficient 0.1258), TNF-alpha and CGRP (Spearman's rank correlation coefficient 0.2270) correlation was weak, while between defensin and CGRP statistically relevant correlation could not be detected.

Figure 1. Pronounced proliferation and thickening of epidermis (Hematoxylin and eosin, × 100)

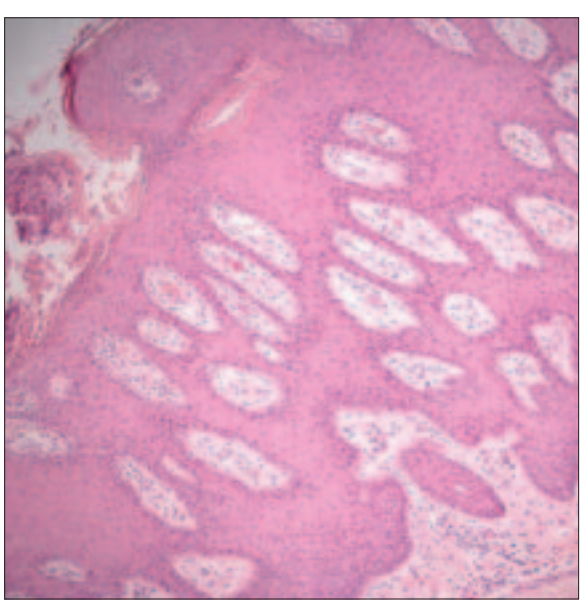


Figure 2. Arteriole sclerosis in papillary dermis surrounded by marked inflammation (Hematoxylin and eosin, × 250)

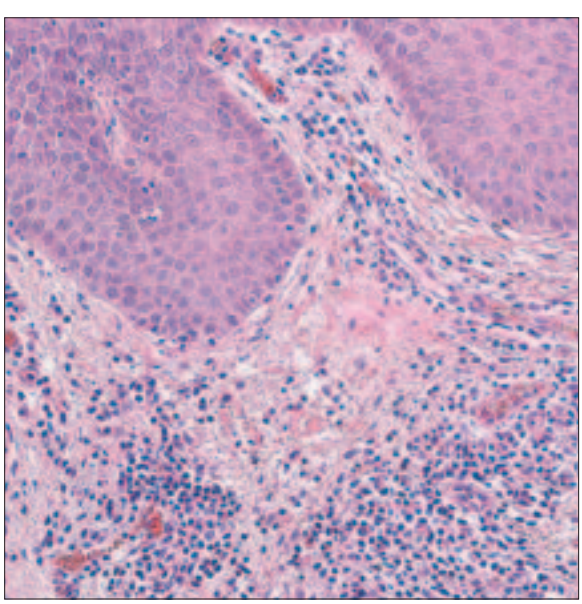


Table 1. Relative appearance of HBD2, TNF-alpha, CGRP and PGP 9.5 positive structures in psoriasis lesions

Patient	Age, years	Gender	Duration of disease	Human beta defensin 2	TNF-alpha	CGRP	PGP 9.5
No. 1	65	M	6 months	++	+	++	++
No. 2	47	M	6 months	++ / +++	+++	+++	+ / ++
No. 3	40	F	1 year	+++	++ / +++	+++	+++
No. 4	44	M	15 years	+++	+++	++	+ / ++
No. 5	34	F	5 years	+++	+++	++	+++
No. 6	25	M	3 months	+	++	+ / ++	+++
No. 7	43	M	6 months	++	+	+ / ++	++
No. 8	68	M	44 years	++++	+ / ++	++++	+++ / ++++
No. 9	37	M	2 years	++	++	+ / ++	+ / ++
No. 10	65	M	1 year	+	+++	++	+ / ++
No. 11	18	M	7 months	– / +	+ / ++	+	++
No. 12	70	M	5 years	– / +	++	++	++
No. 13	19	F	6 months	– / +	+++	+	++ / +++
No. 14	30	M	6 months	+	++	++	+ / ++
No. 15	19	M	6 years	+	++	+	+++ / ++++
No. 16	37	M	2 years	++++	+	+	– / +
No. 17	36	M	20 years	++++	+	+	+
No. 18	36	F	1 years	++++	– / +	–	– / +
No. 19	64	F	40 years	++++	++	+	– / +
No. 20	50	M	20 years	++++	+++	+	+ / ++
No. 21	43	M	18 years	++++	– / +	– / +	++
No. 22	48	F	10 years	++++	+ / ++	+	+ / ++
No. 23	69	M	3 years	+++ / ++++	++++	+	++
No. 24	38	M	7 months	+++ / ++++	++++	– / +	+++
No. 25	43	M	40 years	++++	++++	++	++
No. 26	18	M	16 years	+ / ++	++ / +++	+ / ++	+
No. 27	36	M	4 years	++++	++++	++ / +++	+++
No. 28	51	M	28 years	++++	++++	++	++/+++
No. 29	40	M	1 year	++++	++ / +++	+	+ / ++
No. 30	19	M	10 years	++++	+ / ++	– / +	++
No. 31	28	M	14 years	++	+ / ++	++ / +++	++
No. 32	45	F	20 years	+ / ++	–	+	++
No. 33	53	M	5 years	+	–	+	++
No. 34	27	F	6 months	++	+	++	+++
No. 35	45	F	1 year	+ / ++	–	+	+
No. 36	67	M	1.5 years	+	–	++ / +++	+
No. 37	30	M	1 year	+++	– / +	+++	++ / +++
No. 38	66	M	40 years	– / +	–	++	++
No. 39	29	M	2 weeks	++	– / +	+	+
No. 40	45	M	1 month	+++ / ++++	++	++++	++++
Mean	–	–	–	+++	++	+ / ++	++

Figure 3. Abundance of defensin-containing cells in psoriatic skin lesion (Human beta defensin 2 IMH, × 200)

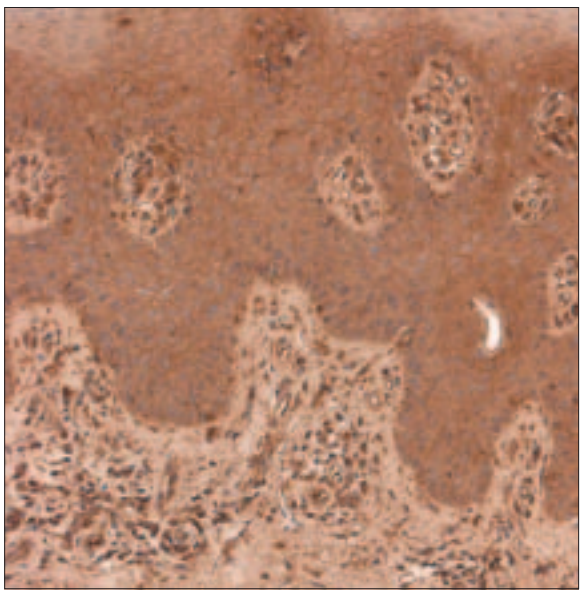


Figure 4. Numerous TNF-alpha positive cells in epidermis and subepidermal inflammatory infiltrates (TNF-alpha IMH, × 400)

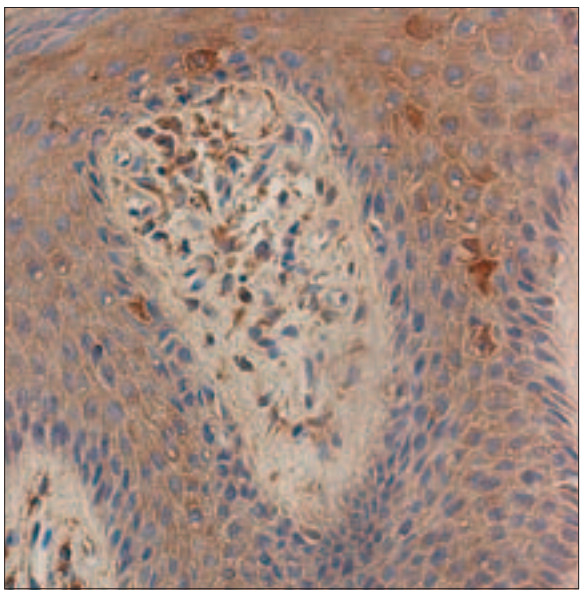


Figure 5. Pronounced expression of CGRP in subepithelium in skin from psoriasis patient with long course of the disease (CGRP IMH, × 400)

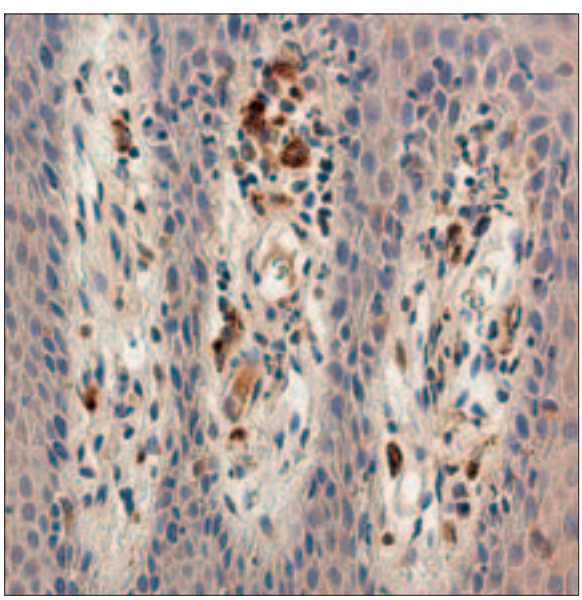
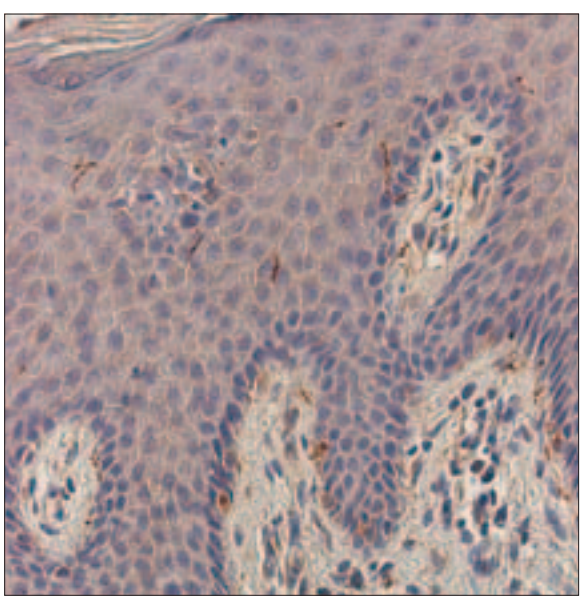


Figure 6. PGP 9.5 positive nerve fibers showing notable innervation of epidermis (PGP 9.5 IMH, × 400)



Discussion

This study displays the complex nature of psoriasis inflammation and morphological changes in skin.

Antimicrobial peptides are found in all living organisms including insects and plants and have been evaluated in many studies regarding various skin conditions. Currently, more than 20 different antimicrobial peptides have been found in skin. Antimicrobial peptides in psoriatic lesions are highly expressed and associations between psoriasis and such peptides as β-defensins, cathelicidin, S100 proteins,

RNase 7, elafin, lysozyme have been studied. For the purpose of this research, it was chosen to evaluate human beta defensin 2 in the selected tissue samples. This was expressed in all patients' skin with clearly pronounced distribution in inflammatory areas thus coinciding with current general understanding of psoriasis and innate immune system. It is believed that HBD2 could be used as a marker of psoriasis activity – recent study suggests that increased systemic levels of serum HBD2 in psoriasis patients with active disease are derived from high cutaneous production, and possibly quantitative amount of HBD2 could be used as a marker to show the disease activity in psoriasis [8–10, 13].

The importance of TNF-alpha in pathogenesis of psoriasis is crucial and is believed to induce expression of HBD2 in keratinocytes. Currently, manufactured TNF-alpha inhibitors have been successfully used in therapy of many inflammatory conditions including psoriasis. Accordingly, TNF-alpha was included in the respective study. Moderate distribution was observed in tissue samples with predominance in subepithelium, walls of blood vessels and eccrine sweat glands. Psoriasis belongs to group with predominantly Th1 cytokine profile. TNF-alpha in psoriasis lesions is associated with hyperplasia with proliferation of interpapillary cones (clinically developing psoriatic plaques) and capillary dilation, lymphocytic inflammatory infiltrate (erythema clinically) and that corresponds with our findings [2, 12].

The expression of CGRP was evaluated and PGP 9.5. CGRP was established in most specimens and moderate number of PGP 9.5-containing nerve fibres was noticed in epithelium and upper dermis, around blood vessels, in smooth muscle bundles and surrounding eccrine sweat glands. Psoriatic skin is highly innervated and activation of the autonomic nervous system and increase in the presence of various neuropeptides in the skin possibly correlates with psoriatic lesions. It is believed that not only dysregulated innate immune system but also dysregulated peripheral nervous system plays a role in psoriasis pathogenesis and locally secreted neuropeptides contribute to persistence of psoriatic lesions. Increased nerve fiber density and increased expression of neuropeptides such as catecholamines, substance P, CGRP, nerve growth factor has been found. Elevated expression of CGRP in papillary dermis has been found in psoriasis patients with high psychological stress levels [3]. The overexpression of CGRP in psoriatic lesions has been observed in Langerhans cells [7]. Another study that shows the importance of neuropeptides is of psoriasiform mouse skin model where denervation was followed by improvement of psoriatic skin lesions [11].

Conclusions

Psoriatic skin shows compensatory prominent local antimicrobial protein expression and neuropeptides-containing innervation on the basis of moderate inflammation and thus, may play a role in the pathogenesis of psoriasis.

References

1. Borkowski A. W., Gallo R. L. The coordinated response of the physical and antimicrobial peptide barriers of the skin // J Invest Dermatol, 2011; 131 (2): 285–287.
2. Brotas A. M., Lago E. H. J., Carneiro S. C. D., et al. Tumour necrosis factor-alpha and the cytokine network in psoriasis // An Bras Dermatol, 2012; 87 (5): 673–683.
3. Chapman B. P., Moynihan J. The brain-skin connection: role of psychosocial factors and neuropeptides in psoriasis // Expert Rev Clin Immunol, 2009; 5 (6): 623–627.
4. Enamandram M., Kimball A. B. Psoriasis epidemiology: the interplay of genes and the environment // J Invest Dermatol, 2013; 133: 287–289.
5. Freedberg I. M., Eisen A. Z., Wolff K., et al. Fitzpatrick's dermatology in general medicine. – 6th ed. – McGraw-Hill, 2003. – Chap. 43.
6. Hsu S., Raine L., Fanger H. The use of antiavidin antibody and avidin-biotin complex in immunoperoxidase technics // Am J Clin Pathol, 1981; 75 (6): 816–821.

7. Gallo R. L., Nakatsuji T. Microbial symbiosis with the innate immune defense system of the skin // J Invest Dermatol, 2011; 131 (10): 1974–1980.
8. He Y., Ding G., Wang X., et al. Calcitonin gene-related peptide in Langerhans cells in psoriatic plaque lesions // Chin Med J, 2000; 113 (8): 747–751.
9. Jansen P. A. M., Rodijk-Olthuis D., Hollox E. J., et al. β-defensin-2 protein is a serum biomarker for disease activity in psoriasis and reaches biologically relevant concentrations in lesional skin // Plos One, 2009; 4 (3): e4725.
10. Kim S. K., Park S., Lee E. S. Toll-like receptors and antimicrobial peptides expressions of psoriasis: correlation with serum vitamin D level // J Korean Med Sci, 2010; 25: 1506–1512.
11. Morizane S., Gallo R. L. Antimicrobial peptides in the pathogenesis of psoriasis // J Dermatol, 2012; 39 (3): 225–230.
12. Ostrowski S. M., Belkadi A., Loyd C. M., et al. Cutaneous denervation of psoriasiform mouse skin improves acanthosis and inflammation in a sensory neuropeptide dependent manner // J Invest Dermatol, 2011; 131 (7): 1530–1538.
13. Pilmane M., Luts A., Sundler F. Changes in neuroendocrine elements in bronchial mucosa in chronic lung disease in adults // Thorax, 1995; 50: 551–554.
14. Singh T. P., Schon M. P., Wallbrecht K., et al. Involvement of IL-9 in Th17-associated inflammation and angiogenesis of psoriasis // Plos One, 2013; 8 (1): e51752.
15. Weinberg A., Jin G., Sieg S., McCormick T. S. The Yin and Yang of human beta-defensins in health and disease // Front Immunol, 2012; 3: 294.

Comparison of Point Prevalence of Depression in General Population of Latvia in 2011 and 2012

Jelena Vrublevska [1], ***Marcis Trapencieris*** [2],
Sigita Snikere [2], ***Elmars Rancans*** [1]

[1] *Rīga Stradiņš University, Department of Psychiatry and Narcology, Latvia*
[2] *University of Latvia, Philosophy and Sociology Institute, Latvia*

Abstract

Depression is an extremely burdensome illness and the major cause of disability. It is associated with an increased risk of the lower functional abilities, and high degree of impairment as defined by poor self-perceived health [Murray, 1996; Paykel, 2005]. Despite the importance of this disorder, the rates and determinants of depression in many non-Western European countries are not well understood; in only two surveys their prevalence in Latvia up to now has been assessed. The aim of the study was to compare estimates of the point prevalence of depression in general population of Latvia.

A comparison was made between point prevalences of depression, which were estimated by the Patient Health Questionnaire-9 (PHQ-9) on the basis of defined algorithm and generally established cut-off score and the Mini International Neuropsychiatric Interview (MINI) in 2011 and 2012, accordingly.

In the study, the participants were interviewed by using the PHQ-9 with a cut-off point for depressive episode ≥ 10, the point prevalence of depression was 6.7%. Studies of related literature recommended the algorithm for depression, the estimated prevalence of depression was 3.6%. A study by the MINI shows the point prevalence of depression 4.9%.

Significant depression point prevalence rate differences were observed by using PHQ-9 and MINI. Further validation studies are necessary to estimate the certain cut-off score for the population of Latvia.

Keywords: depression, point prevalence, general population.

Introduction

Depression is the third burdensome disorder in Europe, and it affects 60.3 million of the European population with a 2 times higher risk of having depression in women than in men [Wittchen, 2011]. Moreover, depression is an extremely burdensome illness not only for the patient, but for their relatives and society as well [Murray, 1996]. Depression is the major cause of disability: it is associated with increased risk of lower functional abilities, and high degree of impairment as defined by poor self-perceived health [Paykel, 2005]. The WHO highlights the occurrence of depression in younger age groups (20–25 years) [Kessler, 2005]. However, some studies showed that prevalence of depression increases with age and the highest rate is observed in the senior age group [Kolchakova, 2003]. Western European countries show higher prevalence rates in women, middle-aged people, vulnerable groups [Paykel, 2005]. In Europe, 12 months prevalence of depression estimates range widely from 1.0% to 10.1% across cultures, methods of definition and case identification [Wittchen, 2011]. Despite the importance of this disorder, the rates and

determinants of depression in many non-Western European countries are not well-understood. There have been only two population-based prevalence studies carried out in Latvia up to now. Therefore, an exact estimate of the prevalence of depression in general population is of great interest.

However, measures of depression are very much dependent on the selected assessment instruments. Screening instruments may be used to detect depressive disorders, and the interest to researchers and clinicians is to know which of the available screening instruments can be recommended, the validity of the results and recognition [Atkins, 2001]. Users of the screening instruments need to know optimal cut-off points for detecting depressive disorders [Löwe, Spitzer, 2004]. There are reports on different valid cut-off scores within one screening tool [Löwe, Gräfe, 2004], thus different rates of depression may be expected.

The aim

The aim of the study was to compare estimates of the point prevalence of depression in general population of Latvia by using two different standardized instruments for screening and assessment of depression.

Material and methods

A comparison was made between point prevalences of depression in general population in Latvia assessed in two population-based studies in 2011 and 2012. The study conducted in 2011 was a part of general population survey on substance use in Latvia. The study design was face-to-face cross-sectional multi-stage stratified randomization, which included the population aged 15–64 years with total net sample size of 4493 persons [Snikere, 2012]. To measure the depression, the participants were interviewed using the Patient Health Questionnaire-9 (PHQ-9) on the basis of defined algorithm for major depressive syndrome [Spitzer, 1999] and with a cut-off score for depression ≥ 10. In its initial validation study, a score of 10 had sensitivity of 88% and specificity of 88% for the detection of depression, thus the score of 10 is recommended as a cut-off score for diagnosing this condition [Kroenke, 2001]. The PHQ-9 is a 9-item depression module for diagnosing depression on the basis of Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) which provides the criteria for major depression and can also produce symptom severity ratings. The depression model of the PHQ-9 has become increasingly popular in research and practice over the past decade [Manea, 2012].

The second study was done in 2012, which was a part of the Finbalt Health Monitoring survey in 2012. The Finbalt Health Monitoring is a collaborative project for monitoring health related behaviour, practices and lifestyles in Finland and the Baltic states. The study sample was identified using a combination of the stratified random sampling and the quota method. The sample was stratified by geographic population density and nationality. In Latvia, 1 549 011 people are aged between 15 and 64 [Central Statistical Bureau of Latvia, 2010]; a sample size of 3010 was considered as population representative sample. Face-to-face interviews at the living place of the respondents were used in Latvian or Russian, as chosen by the respondent. The survey was conducted from April to June 2012. The 3004 interviews were conducted in 390 locations. Additional age group quotas were provided as necessary to keep sample stratified by sex, age, place of residence (type and region) and ethnicity. Before processing, the data were weighted by following five factors – gender, age, type of territory, region and ethnicity.

The purpose of this weighting was to normalize the data with the population age structure. The fieldwork was carried out by 68 specially trained and instructed interviewers managed by two coordinators in Rīga and five coordinators outside the capital. Quality control was conducted in all stages of the study through interviewer training, clear interview procedure and data analysis. The interviews were controlled by repeating 15% of the interviews with separate telephone calls to the respondents and comparing the results. To determine the depression, the participants were interviewed using the depression module of the Mini International Neuropsychiatric Interview (MINI) on the literature-recommended algorithm basis. The MINI is a structured diagnostic interview, developed to assess the diagnoses of psychiatric patients according to DSM-IV and ICD-10 criteria in a shorter time than other diagnostic interviews. The good psychometric characteristics of the MINI make it a good choice for

the research purposes [Van Vliet, 2007]. MINI is rapidly administered diagnosing interview that can be incorporated into routine clinical settings and it is acceptable to patients and should improve diagnostic accuracy [Pinninti, 2003].

Both assessment tools were translated in Latvian and Russian, back-translated in English and checked for accuracy. Translations of PHQ-9 were also discussed in focus groups. Both of the instruments showed good internal consistency. To analyze the data, descriptive statistics and uncorrected Pearson chi-square (χ^2) were applied.

Results

The point prevalence of depression assessed by the PHQ-9 with cut-off score ≥ 10 was 6.7% (95% CI 5.6–7.8%). Depression was more common for women than men, 7.8% (95% CI 6.2–9.4%) and 5.6% (95% CI 4.2–6.9%) (p = 0.018). Using the algorithm of the PHQ-9, the point prevalence for major depressive syndrome was 3.6% (95% CI 2.9–4.4%), where the prevalence of women and men formed 3.2% (95% CI 2.2–4.2%) and 4.1% (95% CI 3.0–5.1%). Nevertheless, these data has lost their statistical significance. The study assessed by the MINI reveals the point prevalence of depression 4.9% (95% CI 4.1–5.7%); for women and men 5.7% (95% CI 5.1–6.5%) and 4.0% (95% CI 3.2–4.8%) (p = 0.03). The results are shown in the Figure 1.

According to the PHQ-9 assessment using cut-off score and algorithm, depression was more common in the oldest age group, 55–64 years. The point prevalence of depression determined by the PHQ-9 ≥ 10 in the age 55–64 years was 9.9%, while in the youngest age group 15–24 years 3.8% (p = 0.003). In contrary to these findings, in the study with the MINI, depression was most common in younger age group 45–54 years and was 7.1% for both genders but in the oldest age group (55–64 years) the point prevalence was 3.8%. Furthermore, these data are statistically significant. The distribution of prevalence rates among age groups are shown in the Figure 2.

Figure 1. Point prevalence of depression (%) in the general population for both genders assessed by PHQ-9 and MINI

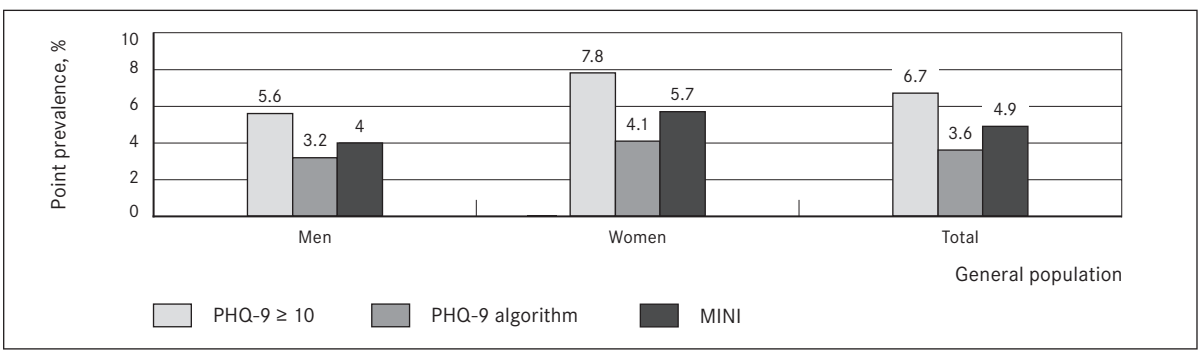
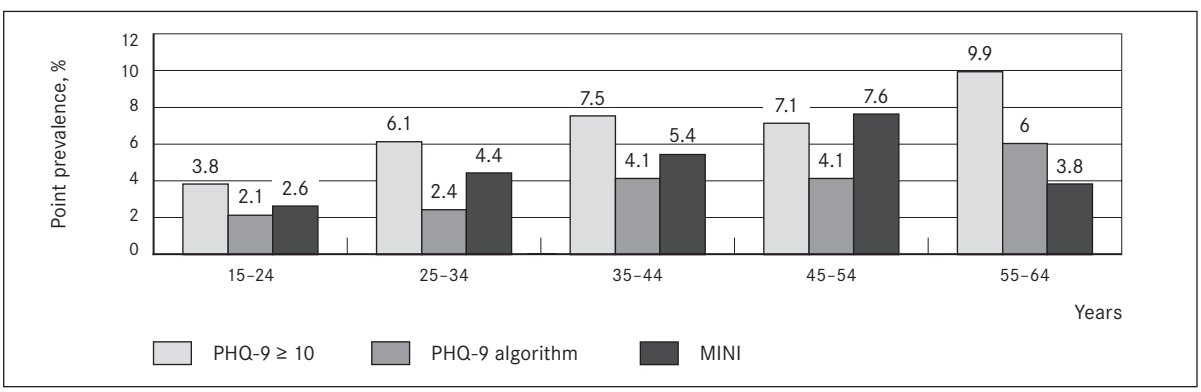


Figure 2. Point prevalence (%) of depression in age groups assessed by PHQ-9 and MINI



Discussion

In 2011 and 2012, the estimated point prevalence of depression by using internationally accepted diagnosing tools were 3.6%, 6.7% and 4.9%. Compared with the results from the previous Soviet bloc countries, depression rates are remarkably lower than the rates reported for Poland, the Czech Republic and Russia, where depression estimates in urban population were up to 40% [Bobak, 2006]. Such significant differences can be explained by methodological assessment. In the studies conducted in Latvia, structured interviews were applied, while Bobak et al. [Bobak, 2006] relied on the self-report of depressive symptoms using the Center of Epidemiologic Studies Depression Scale which includes mainly minor depression and psychological distress, rather than major or severe depression [Beekman, 1995]. Our results are closer to surveys conducted in Danish and Estonian populations [Kleinberg, 2006; Olsen, 2004].

We found differences in prevalence rates of depression in 2011 and 2012. These findings can be explained by using different assessment tools and the fact that the assessment was done in two separate studies. However, the range of prevalence rates of depression is less expressed compared to Europe.

We observed a rather significant difference in estimates of depression, using PHQ-9 in literature recommended cut-off score and diagnosing algorithm. In one of the recent reviews of PHQ-9, Kroenke and colleagues argued against inflexible adherence to a single cut-off score [Kroenke, 2010]. In recent meta-analyses of optimal cut-off score for diagnosing depression was found to have acceptable diagnostic properties to detect major depressive syndrome in outpatient clinics for cut-off scores between 8 and 11 with no statistical significance [Manea, 2012]. At the same time, there are studies which emphasize low specificity to cut-off score 10 [Löwe, Gräfe, 2004]. Moreover, optimal cut-off scores may vary across cultures and regions [Fountoulakis, 2003].

Differences in depression rates can also be explained by different assessment methodologies of screening tools. The assessment by PHQ-9 algorithm and MINI is more specified to ICD-10 and DSM-IV diagnosing criteria for depressive disorder, which includes general and additional symptoms. Patients have to fulfil at least one of the general symptoms to be considered for further assessment of depression. While PHQ-9 with cut-off score ≥ 10 does not require inclusion of the general criteria, thereby might identify other conditions with symptomatology of depression. Therefore, to provide optimal cut-off points and selection of the most appropriate instruments for general population of Latvia. Additional studies need to be carried out where both instruments could be applied simultaneously.

There is no consensus about the association between age and depression. There are studies that emphasize the protective role of age towards depression. Studies conducted in Latvia revealed substantially higher rates of depression in the age group above 40 years, which is consistent with the findings showing prevalence of depression increasing with age [Kolchakova, 2003]. Such phenomena can be explained by the association between depression and growing somatic diseases in later life. Moreover, in Eastern European countries lower incomes, social problems and failures in health care system are common [Paykel, 2005].

Conclusions

Significant depression point prevalence rate differences using PHQ-9 and MINI were observed. Further validation studies are necessary to estimate the certain cut-off score for general population of Latvia.

References

1. Atkins D. Screening for depression: recommendations and rationale // Internet Journal of Mental Health, 2002; 1 (2): 1.
2. Beekman A. T., Deeg D. J., Van Tilburg T., et al. Major and minor depression in later life: a study of prevalence and risk factors // Journal of Affective Disorders, 1995; 36 (1–2): 65–75.
3. Bobak M., Pikhart H., Pajak A., et al. Depressive symptoms in urban population samples in Russia, Poland and the Czech Republic // The British Journal of Psychiatry: The Journal of Mental Science, 2006; 188: 359–365.

4. Central Statistical Bureau of Latvia // http://www.csb.gov.lv/en/dati/statistics-database-30501.html. 2013
5. Fountoulakis K. N., Iacovides A., Kleanthous S., et al. The Greek translation of the symptoms rating scale for depression and anxiety: preliminary results of the validation study // BMC Psychiatry, 2003; 3: 21.
6. Kessler R. C., Berglund P., Demler O., et al. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication // Archives of General Psychiatry, 2005; 62 (6): 593–602.
7. Kleinberg A., Aluoja A., Vasar V. Point prevalence of major depression in Estonia. Results from the 2006 Estonian Health Survey // European Psychiatry, 2010; 25 (8): 485–490.
8. Kolchakova P. Y., Akabaliev V. H. A study of the effect of age on depressivity in Bulgarian urban population // Folia Medica 2003; 45 (4): 11–15.
9. Kroenke K., Spitzer R. L., Williams J. B. The PHQ-9: validity of a brief depression severity measure // Journal of General Internal Medicine, 2001; 16 (9): 606–613.
10. Kroenke K., Spitzer R. L., Williams J. B. W., Löwe B. The Patient Health Questionnaire Somatic, Anxiety, and Depressive Symptom Scales: a systematic review // General Hospital Psychiatry, 2010; 32 (4): 345–359.
11. Löwe B., Gräfe K., Zipfel S., et al. Diagnosing ICD-10 depressive episodes: superior criterion validity of the Patient Health Questionnaire // Psychotherapy & Psychosomatics, 2004; 7 (6): 386–390.
12. Löwe B., Spitzer R. L., Grafe K., et al. Comparative validity of three screening questionnaires for DSM-IV depressive disorders and physicians diagnoses // Journal of Affective Disorders, 2004; 78 (2): 131.
13. Manea L., Gilbody S., McMillan D. Optimal cut-off score for diagnosing depression with the Patient Health Questionnaire (PHQ-9): a meta-analysis // CMAJ: Canadian Medical Association, 2012; 184 (3): 191–196.
14. Murray C. J. L., Lopez A. D. The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020. – Harvard University Press, 1996.
15. Olsen L. R., Mortensen E. L., Bech P. Prevalence of major depression and stress indicators in the Danish general population // Acta Psychiatrica Scandinavica, 2004; 109 (2): 96–103.
16. Paykel E. S., Brugha T., Fryers T. Size and burden of depressive disorders in Europe // European Neuropsychopharmacology, 2005; 15 (4): 411–423.
17. Pinninti N. R., Madison H., Musser E., Rissmiller D. MINI International Neuropsychiatric Schedule: clinical utility and patient acceptance // European Psychiatry, 2003; 18 (7): 361–364.
18. Snikere S., Trapencieris M., Koroleva I., et al. Atkarību izraisošo vielu lietošanas izplatība iedzīvotāju vidū. [Substance use among the population in 2011. Analytic report]. – Slimību profilakses un kontroles centrs, 2012. – 13.–14. lpp.
19. Spitzer R. L., Kroenke K., Williams J. B. Validation and utility of a self-report version of PRIME-MD: the PHQ primary care study. Primary Care Evaluation of Mental Disorders. Patient Health Questionnaire // JAMA: The Journal of the American Medical Association, 1999; 282 (18): 1737–1744.
20. Van Vliet I. M., De Beurs E. The MINI-International Neuropsychiatric Interview. A brief structured diagnostic psychiatric interview for DSM-IV en ICD-10 psychiatric disorders // Tijdschrift Voor Psychiatrie, 2007; 49 (6): 393–397.
21. Wittchen H. U., Jacobi F., Rehm J., et al. The size and burden of mental disorders and other disorders of the brain in Europe 2010 // European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology, 2011; 21 (9): 655–679.

Domestic Drinking Water Systems as Source of *Legionella pneumophila* Infections in Latvia

Olga Valcina [1], ***Daina Pule*** [1], ***Svetlana Makarova*** [1],
Aivars Berzins [1], ***Irina Lucenko*** [2], ***Angelika Krumina*** [3]

[1] *Institute of Food Safety, Animal Health and Environment "BIOR", Latvia*
[2] *Centre for Disease Prevention and Control, Latvia*
[3] *Rīga Stradiņš University, Faculty of Medicine, Department of Infectology and Dermatology, Latvia*

Abstract

Legionella are heterotrophic bacteria commonly living in natural waters. Our aim was to study the occurrence of *Legionella* contamination in potable water supply systems in apartment and public buildings. Moreover, the correlation between *Legionella* spp. positive cases and water source, sampling point and temperature of water, received at the point of consumption was studied. A total of 250 water samples were collected from apartment and public buildings. Samples were taken in 21 cities and districts of Latvia. Only one *Legionella* species – *Legionella pneumophila* was isolated during this study. *L. pneumophila* was found in 72 out of 250 (29%) water samples. From all *Legionella* spp. positive samples, 19% represented *L. pneumophila* serogroup 1 and 78% *L. pneumophila* serogroups 2–14 (15). In 3% of *Legionella* spp. positive samples both serogroups 1 and 2–14 (15) where isolated. Statistically significant differences were observed in the distribution of *L. pneumophila* Serogroup 1 in samples from various water sources in different districts. *L. pneumophila* was found in 32% of the samples from private apartments and in 15% of the samples from public buildings. Data analysis confirmed the observation that the temperature of hot water significantly affects the frequency of *L. pneumophila* positive cases ($p < 0.05$).

Keywords: Legionella pneumophila, drinking water, Latvia.

Introduction

Legionella pneumophila, a gram-negative bacillus ubiquitous in aquatic environments, is responsible for Legionnaires' disease. Primary ecological niche of *Legionella* appears to be a parasite of protozoa, but humans may become secondarily infected after inhaling or aspirating organisms [Chiaraviglio, 2008]. Significant proportion of sporadic cases of Legionnaires' disease may be residentially acquired and associated with domestic potable water and disruptions in residential plumbing systems [Atlas, 1999; Stout, 1992]. Human infection with *Legionella* spp. is known to result from the inhalation of aerosols (ø 5 mm) filled with numerous infectious bacteria [Fields, 2002; Bollin, 1985]. More than 90% of legionellosis cases are caused by *Legionella pneumophila*. Most cases of legionellosis can be traced to man-made aquatic environments where the water temperature is higher than ambient temperature [Diederen, 2008]. Very low concentrations of legionellae in natural habitats can increase markedly in engineered hot water systems where water temperatures are below 55 °C [Mathys, 2008]. They have been

found in hot water taps and tanks, creeks, ponds, water-cooling towers and evaporative condensers and whirlpool spas [Bartram, 2007]. After entering the water supply system, *Legionella* can be isolated at all stages of the system – from preparation to delivery to the consumer.

In 2011, the increase of legionellosis case notifications was observed in Latvia indicating on possible legionellosis outbreak [Rozentale, 2011]. Extensive studies of occurrence of *Legionella* spp. have not been realized in Latvia before.

The aim

The aim of the study was to investigate the occurrence of *Legionella* contamination in potable water supply systems in apartment and public buildings. Moreover, the correlation between *Legionella* spp. positive cases and water source, sampling point and temperature of water was studied.

Material and methods

A total of 250 water samples were collected from apartment and public buildings from January 2011 through December 2012. The samples were taken in 21 cities and districts of Latvia, randomly representing Rīga, and regions of Vidzeme, Latgale, Kurzeme and Zemgale. Water samples were collected in sterile bottles (1 litre each). Water temperature was measured in each sampling point.

Isolation and identification of *Legionella pneumophila* was carried out by using the ISO 11731 standard [Anonymous, 1998]. One litre of water sample was filtrated and concentrated using membrane filtration with 0.45 µm pore-size polyamide filter (Millipore, USA). The filter membranes were cut into pieces and resuspended in 5 ml sterile distilled water and shaken for two minutes (Vortex Genie), and then kept in room temperature for ten minutes. Heat treatment and acid treatment were used to reduce the growth of other bacteria. A total of three 0.1 ml untreated, heat treated and acid treated aliquots of the sample were spread on Buffered Charcoal Yeast extract medium (GVPC, Oxoid, UK). The plates were incubated at 36 °C in a humidified environment for 10 days, and examined every day beginning on day 3. At least three characteristic colonies from each GVPC plate were selected for subculture onto plates BCYE (Buffered Charcoal Extract agar medium with L-cysteine, OXOID, UK) and BCYE-Cys (Buffered Charcoal Extract agar medium without L-cysteine, OXOID, UK) and incubated for at least 48 hours at the temperature of 36 °C. Colonies grown on BCYE were subsequently identified by latex agglutination test (*Legionella* Rapid Latex Test Kit, BIOLIFE Italiana S.r.l., Italy). *Legionella* Rapid Latex Test Kit allows for separation of the identification of *L. pneumophila* Serogroup 1 and Serogroups 2–15 (until December 2011, there were included 2–14 serogroups) and the identification of 10 non-pneumophila *Legionella* species. Colonies from all the plates were counted, confirmed and estimated, the number of *Legionella* was expressed as CFU/litre *Legionella* species and serogroup.

Microbiological analysis was carried out in Laboratory of Medical Microbiology (Institute of Food Safety, Animal Health and Environment "BIOR") which is the only laboratory in Latvia to investigate environmental samples for presence of *Legionella* spp. from 2008.

The data were analyzed with MS Excel based Data Analysis tool. The analysis of variance (one-way and two-way ANOVA) and post hoc tests were performed to determine possible significant differences for the occurrence of *Legionella pneumophila* depending on various factors. Correlation analysis was performed to assess the significance of correlation between temperature of water and occurrence and the level of *Legionella pneumophila* colonization.

Results

L. pneumophila was found in 72 out of 250 (29%) water samples. No significant difference in occurrence of *Legionella pneumophila* was observed between different districts of Latvia. However, 6% of *Legionella* positive samples were found in Vidzeme. The occurrence of *Legionella* ranged from 17% in Latgale and Zemgale to 29% and 32% in Kurzeme and Rīga, respectively.

L. pneumophila was found in 41% of hot water samples and in 13% of cold water samples. The occurrence of *L. pneumophila* was considerably higher in samples from private apartments (Table 1 and Table 2).

The temperature of potable water had an impact on frequency of *L. pneumophila* positive samples found in water systems, especially hot water samples (Figure 1). The obtained data presents that 85% of consumers received hot water at a temperature below 50 °C.

Two-way ANOVA was performed to determine possible differences for occurrence of *L. pneumophila* depending on the temperature of water and water source. Results ($F = 12.20$; $p = 0.020$) showed that the temperature of hot water had a significant effect on the occurrence of *Legionella* ($F > F_{0.05;2;4}$; $p < 0.05$). Tukey's HSD post hoc test indicated statistically significant differences in the occurrence of *Legionella* at temperature between 42 °C to 51 °C. No statistically significant differences were observed for the occurrence of *L. pneumophila* in cold water samples ($p > 0.05$).

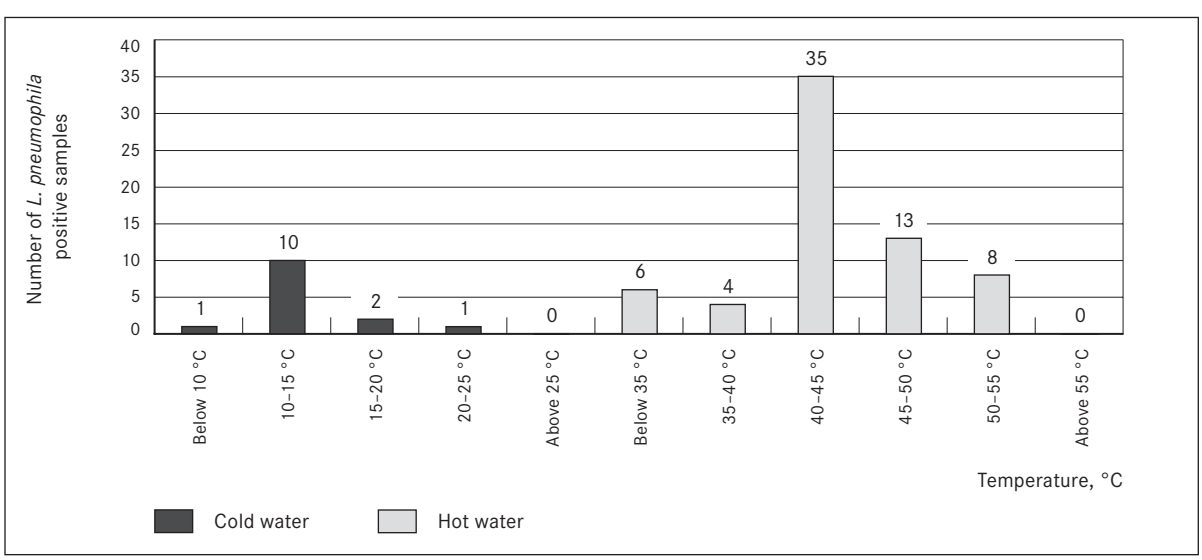
Table 1. Occurrence and colonization of *L. pneumophila* in hot water, characteristics of samples

Buildings	Positive samples, %	Temperature, °C						Colonization, CFU/L	
		All samples		Positive samples		Negative samples		average	min–max
		average	min–max	average	min–max	average	min–max		
Private apartments	45	45.4±0.5	25.0–68.0	43.9	28.0–52.0	47.1	25.0–68.0	2.0×10^3	1.0×10^2 – 8.0×10^3
Public buildings	27	48.3±1.5	24.3–60.0	45.9	42.0–48.0	49.2	24.3– 60.0	2.6×10^3	3.5×10^2 – 6.0×10^3
–	–	2.9	0.7–8.0	2.0	14.0–4.0	2.1	0.7–8.0	6.0×10^2	2.5×10^2 – 2.0×10^3

Table 2. Occurrence and colonization of *L. pneumophila* in cold water, characteristics of samples

Buildings	Positive samples, %	Temperature, °C						Colonization, CFU/L	
		All samples		Positive samples		Negative samples		average	min–max
		average	min–max	average	min–max	average	min–max		
Private apartments	15	15.8 ± 0.7	5.0–25.0	14.1	8.3–25.0	16.2	5.0–24.0	6.8×10^2	5.0×10^1 – 2.5×10^3
Public buildings	4	17.1 ± 1.0	10.0–25.6	12.0	12.0–13.8	17.5	10.0–25.6	2.3×10^2	2.0×10^2 – 2.5×10^2
–	–	1.3	5.0–0.6	2.1	3.7–11.2	1.3	5.0–1.6	4.5×10^2	1.5×10^2 – 2.3×10^3

Figure 1. Distribution of *L. pneumophila* in drinking water samples



No significant influence of water source on *L. pneumophila* positive cases ($F < F_{0.05;2;4}$; $p > 0.05$) was observed.

Correlation analysis confirmed statistically significant ($t > t_{inv}$) moderate negative correlation ($r = -0.60$) between the temperature of hot water and the occurrence of *L. pneumophila*. Weak negative correlation ($r = -0.27$) between the temperature and colonization of *L. pneumophila* was observed.

From all *Legionella* spp. positive samples, 19% represented *L. pneumophila* Serogroup 1 and 78% *L. pneumophila* Serogroups 2–14 (15). In 3% of *Legionella* spp. positive samples both serogroups 1 and 2–14 (15) were isolated.

Statistically significant differences were observed in the distribution of *L. pneumophila* Serogroup 1 in samples from various water sources in different districts of Latvia. Results of one-way ANOVA ($F = 13.14$; $p = 0.022$) showed that the occurrence of *L. pneumophila* Serogroup 1 is significantly higher in surface water ($F > F_{0.05;1;4}$; $p < 0.05$).

Discussion

L. pneumophila was found in 29% of water samples. The occurrence of *L. pneumophila* was considerably higher in hot water – 41%, which is significantly higher than in other European countries, where the occurrence of *Legionella* in water distribution systems varied from 22.6% in Italy [Borella, 2004], 26% in Germany [Zietz, 2001] to 30% in Finland [Zacheus, 1994]. The main reasons for these differences could be poor technical condition of apartment buildings and the unfavourable economic situation, which compels the population to spare water and energy [Rozentale, 2011]. Optimum temperature range for proliferation of legionellae is 32–35 °C but they are able to proliferate up to 45 °C [Levesque, 2004; Wadovsky, 1985]. The obtained data indicated that contamination with *L. pneumophila* was observed much more frequently in water at temperatures below 45 °C. Meanwhile, no contamination was detected in samples at temperature 55 °C or higher. At temperatures higher than 55 °C there is a break point (Figure 1), and this finding agrees with the observations from other studies which report that the range 55–60°C is a critical temperature region above which the proliferation of legionellae in the water supply systems is inhibited [Darelid, 2002; Wadowsky, 1982].

The studied data showed that temperature of the hot water has a significant influence on *Legionella* contamination in the water system. Average water temperature from contaminated systems was 3.2 °C lower than from systems where *Legionella* spp. was not detected. Similar results were presented in other studies [Alary, 1991].

Biofilms can maintain a stable population of legionellae, and provide an opportunity for survival and spread for them [States, 1987]. Unsterilized potable water contains enough nutrients for growth of legionellae [Yee, 1982]. Presence of other microorganisms is essentially important for the growth of legionellae because they provide necessary substances [Stout, 1985]. Data of other studies showed that in hot water supply systems with large surface area, up to 72% of bacteria may be located in the biofilms and only 26% of microorganisms are free-living bacteria [Bagh, 2004].

From all *Legionella* spp. positive samples, 19% represented *L. pneumophila* Serogroup 1 and 78% *L. pneumophila* Serogroups 2–14 (15). In 3% of *Legionella* spp. positive samples both serogroups 1 and 2–14 (15) were isolated. These data are consistent with the results of other studies. In Poland *L. pneumophila* 2–14 Serogroup was isolated from 73% and Serogroup 1 from 19.8% of *Legionella* spp. positive samples [Stojek, 2011], in Italy 75.6% and 22.6%, respectively [Borella, 2004].

Statistically significant differences were observed in the distribution of *L. pneumophila* Serogroup 1 in samples from various water sources in different districts of Latvia. 94% of *L. pneumophila* Serogroup 1 were isolated from samples taken in Rīga. Most cases of Serogroup 1 were observed in territories, which received treated surface water. Only 6% of *L. pneumophila* Serogroup 1 were isolated from samples taken in territories, which are provided mostly with underground water. *L. pneumophila* was isolated from 27% of samples taken in territories, which receive underground water. It indicates that underground water, which is delivered to consumer without any additional processing, may became a source of *Legionella*. Presence of legionellae in untreated underground water is confirmed also in Portugal [Costa, 2005] and in Canada [Riffard, 2001].

No significant difference in occurrence of *L. pneumophila* was observed between different districts of Latvia, except district of Vidzeme (6% of *Legionella* positive samples). This may be due to the relatively small number of samples (n = 18) taken in this district. In other cases, the amount of *Legionella* positive samples varied from 17% in Latgale and Zemgale up to 32% in Rīga. Higher occurrence of *L. pneumophila* in Rīga can be explained by a higher density of population and by different origin of potable water.

The occurrence of *L. pneumophila* in water samples correlated with overall incidence of legionellosis during years 2011 and 2012. Thus, the highest incidence of legionellosis (5.3/100 000) was observed in Rīga, where 32% of water samples were contaminated with *L. pneumophila*, whereas the lowest number of *L. pneumophila* positive water samples were found in Vidzeme, Latgale and Kurzeme with legionellosis incidence 0.25, 0.5 and 1.7 per 100 000 residents, accordingly.

Underdiagnosed Legionnaires' disease cases are a major bias in computing its incidence. Accurate diagnosis requires *Legionella* laboratory testing since the clinical manifestations are non-specific [Sabria, 2002]. Traditional clinical praxis does not favour routine laboratory testing for Legionnaires' disease unless the patient is admitted to the intensive care unit. World Health Organization recommends the use of *Legionella* laboratory tests in certain specific situations: for patients with enigmatic pneumonia, for patients who do not respond to ß-lactam treatment, and in the presence of an epidemic [Bartram, 2007]. The clinical manifestations considered characteristic of Legionnaires' disease in the early 1980s included high fever, diarrhea, confusion, hyponatremia, and high mortality. Thus, *Legionella* testing is often confined to patients with severe pneumonia and less likely to be ordered for patients who are not severely ill. However, clinical manifestations are not useful in predicting the likelihood of Legionnaires' disease [Mulazimoglu, 2001], so enigmatic pneumonia will remain enigmatic unless *Legionella* testing is applied. This has implications for the management of community acquired pneumonia, given the fact that Legionnaires' disease and pneumococcal pneumonia have the highest mortality rates [Yu, 2008].

Lack of data obtained in long-term research in Latvia suggests that the real level of contamination of water supply systems may be even higher. The high frequency of *Legionella* contamination in apartment buildings showed that regular preventive actions and controls are an important part of prevention against legionellosis. The high contamination level of *Legionella* of water supply systems and underreporting of legionellosis indicates that *Legionella* in Latvia is still overlooked.

Conclusions

1. Domestic drinking water supply systems should be considered as a significant source of *L. pneumophila* infection in Latvia.
2. Overall, 29% of the water samples were contaminated with *L. pneumophila*.
3. The occurrence of *L. pneumophila* was considerably higher in hot water samples (41%) than in cold water samples (13%).
4. The temperature of hot water was an important risk factor for survival and spread of *L. pneumophila*. Only in 15% of samples, water temperature was higher than 50 °C, which limits the growth of *Legionella* spp.

Acknowledgements

Laboratory of Medical Microbiology and Customer Service Experts (Institute of Food Safety, Animal Health and Environment "BIOR") are acknowledged for their tehnical support.

References

1. Alary M., Joly J. R. Risk factors for contamination of domestic hot water systems by legionellae // Applied and Environmental Microbiology, 1991; 57 (8): 2360–2367.
2. Anonymous. ISO 11731 Water quality – Detection and enumeration of legionella. – Geneva: International Standard Organization, 1998.

3. Atlas R. M. Legionella: from environmental habitats to disease pathology, detection and control // Environmental Microbiology, 1999; 1 (4): 283–293.
4. Bagh L. K., Albrechtsen H. J., Arvin E., Ovesen K. Distribution of bacteria in a domestic hot water system in a Danish apartment building // Water Research, 2004; 38: 225–235.
5. Bartram J., Chartier Y., Lee J. V., et al. Legionella and prevention of legionellosis. – Geneva: World Health Organization, 2007. – 252 p.
6. Bollin G. E., Plouffe J. F., Para M. F., Hackman B. Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets // Applied and Environmental Microbiology, 1985; 50 (5): 1128–1131.
7. Borella P., Montagna M. T., Romano-Spica V., et al. Legionella infection risk from domestic hot water // Emerging Infectious Diseases, 2004; 10 (3): 457–464.
8. Chiaraviglio L., Brown D., Kirby J. E. Infection of cultured human endothelial cells by *Legionella pneumophila* // PLoS One, 2008; 3 (4): 1–5.
9. Costa J., Tiago I., Costa M. S., Verissimo A. Presence and persistence of *Legionella* spp. in groundwater // Applied and Environmental Microbiology, 2005; 71 (2): 663–671.
10. Darelid J., Lofgren S., Malmvall B. E. Control of nosocomial Legionnaires' disease by keeping the circulating hot water temperature above 55 °C: experience from a 10-year surveillance programme in a district general hospital // Journal of Hospital Infection, 2002; 50: 213–219.
11. Diederen, B. *Legionela* spp. and Legionnaires' disease // Journal of Infection, 2008; 56, 1–12.
12. Fields B. S., Benson R. F., Besser R. Legionella and Legionnaires disease: 25 years of investigation // Clinical Microbiology Reviews, 2002; 15 (3): 506–526.
13. Levesque B., Lavoie M., Joly J. Residential water heater temperature 49 or 60 degrees Celsius? // Canadian Journal of Infectious Disease, 2004; 15 (1): 11–12.
14. Mathys W., Stanke J., Harmuth M., Junge-Mathys E. Occurence of Legionella in hot water systems of single family residences in suburbs of two German cities with special reference to solar and district heating // Int J Hyg Environ Health, 2008; 211: 179–185.
15. Mulazimoglu L., Yu V. L. Can Legionnaires disease be diagnosed by clinical criteria? // Chest, 2001; 4 (120): 1049–1053.
16. Riffard S., Douglass S., Brooks T., et al. Occurrence of Legionella in groundwater: an ecological study // Water Science and Technology, 2001; 43 (12): 99–102.
17. Rozentale B., Bormane B., Perevoscikovs J., et al. Increase of cases of legionelosis in Latvia, 2011 // Euro Surveillance, 2011; 16 (45): 1–3.
18. Sabria M., Yu V. L. Hospital-acquired legionellosis: solutions for a preventable infection // The Lancet Infectious Diseases, 2002; 2: 368–373.
19. States S. J., Conley L. F., Kuchta J. M., et al. Survival and multiplication of *Legionella pneumophila* in municipal drinking water systems // Applied and Environmental Microbiology, 1987; 53 (5): 979–986.
20. Stojek N. M., Dutkiewicz J. Co-existence of Legionella and other Gram-negative bacteria in potable water from various rural and urban spurces // Annals of Agricultural and Environmental Medicine, 2011; 18 (2): 330–334.
21. Stout J. E., Yu V. L., Best M. Ecology of *Legionella pneumophila* within water distribution systems // Applied and Environmental Microbiology, 1985; 49 (1): 221–228.
22. Stout J. E., Yu V. L., Muraca P., et al. Potable water as a cause of sporadic cases of community-aquired Legionnaires disease // The New England Journal of Medicine, 1992; 326 (3): 151–155.
23. Wadovsky R. M., Wolford R., McNamara A. M., Yee R. B. Effect of temperature, pH and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water // Applied and Environmental Microbiology, 1985; 49 (5): 1197–1205.
24. Wadowsky R. M., Yee R., Mezmar L., et al. Hot water systems as sources of *Legionella pneumophila* in hospital and nonhospital plumbing fixtures // Applied and Environmental Microbiology, 1982; 43 (5): 1104–1110.
25. Yee R. B., Wadowsky R. M. Multiplication of *Legionella pneumophila* in unsterilized tap water // Applied and Environmental Microbiology, 1982; 43 (6): 1330–1334.
26. Yu V. L., Stout J. E. Community-acquired Legionnaires disease: implications for underdiagnosis and laboratory testing // Clinical Infectious Diseases, 2008; 46: 1365–1367.
27. Zacheus O. M., Martikainen P. J. Occurrence of legionellae in hot water distribution systems of Finland apartment buildings // Canadian Journal of Microbiology, 1994; 40: 993–999.
28. Zietz B., Wiese J., Brengelmann F., Dunkelberg H. Presence of *Legionellaceae* in warm water supplies and typing of strains by polimerase chain reaction // Epidemiol Infect, 2001; 126: 147–152.

Frequency and Activity Phase of HHV-6 and HHV-7 Persistent Infection in Renal Transplant Recipients and Patients with Gastrointestinal Cancer

Alina Sultanova [1], *Maksims Cistjakovs* [1], *Svetlana Capenko* [1], *Simona Donina* [2], *Ieva Ziedina* [3], *Modra Murovska* [1]

[1] *Rīga Stradiņš University, A. Kirchenstein Institute of Microbiology and Virology, Latvia*
[2] *Rīga Eastern Clinical University Hospital, Latvian Oncology Centre, Latvia*
[3] *Pauls Stradiņš Clinical University Hospital, Department of Transplantation, Latvia*

Abstract

Human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) are ubiquitous beta-herpesviruses with immunmodulating abilities. Immunosuppression caused by medication after solid organ and hematopoietic stem cell transplantation or complications related to the underlying disease, like in oncology patients, may lead to these viruses reactivation causing worse outcomes.

Two groups were enrolled in this study: Group I – patients after renal transplantation (n = 47) and Group II – patients with gastrointestinal cancer (GIC) (n = 65).

Immunological parameters were determined by Becton-Dickinson (USA) laser flow cytofluorimeter using corresponding monoclonal antibodies to lymphocyte subpopulations: $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD95^+$ and $CD25^+$.

Nested polymerase chain reaction (nPCR) was used for the latent infection and active infection of persistent HHV-6 and HHV-7 infection.

Persistent beta-herpesvirus infection was detected in 42 out of 47 (89%) renal transplant recipients and in 44 out of 65 (68%) GIC patients (p = 0.01). A higher distribution of active beta-herpesvirus infection was found in renal transplant recipients compared with GIC patients (20/42, 48% and 14/44, 32%, respectively).

The most prevalent was active HHV-7 infection, with higher distribution in renal transplant recipients compared with GIC patients (11/16, 41% and 7/28, 25%, respectively).

Comparative analysis of cellular immune parameters in Groups I and II was performed dependently of beta-herpevirus infection. Renal transplant recipients with active viral infection had a tendency to decrease all cellular immunological parameters (leukocytes, monocytes, lymphocytes and $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$, $CD95^+$ and immunoregulatory index) compared with the subgroups with latent and without HHV-6 and HHV-7 infection.

Decrease of $CD4^+$ could be caused by HHV-7 reactivation in renal transplant recipients as well as in GIC patients.

Keywords: beta-herpesviruses, reactivation, immunosuppression.

Introduction

Human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) are ubiquitous T lymphotropic beta-herpesviruses with immunomodulating abilities [Castera, 2001]. Both are widely distributed in general population and the primary infection usually occurs in the early years of life and remains latent in the host for the lifelong period [Campadelli-Fiume, 1999]. These viruses can be reactivated in immunosuppressed conditions and can lead to severe complications in patients with solid organ transplantation [Lusso, 2006]. Also all neoplastic diseases including lymphoma/leukaemia as well as solid organ cancers are associated with immunosuppression reactivating HHV-6 and HHV-7 in the case of persistent infection [Quadrelli, 2011].

Active HHV-6 infection occurs in nearly 50% of all bone marrow and in 20–30% of solid-organ transplant recipients, 2–3 weeks following the procedure. It has been suggested that the viral activation results in clinical symptoms, such as fever, skin rash, pneumonia, bone marrow suppression, encephalitis, and transplant rejection [Dulery, 2012].

In contrast to studies of HHV-6 infection in organ-transplant recipients, the number of HHV-7 infection studies in these patients is limited. According to the latest published data, HHV-7 may act as a cofactor for cytomegalovirus disease in organ-transplant recipients [Yoshikawa, 2003].

HHV-6 and HHV-7 share a high degree of genomic homology and have some similar biological properties. Thus, these herpesviruses might share a similar oncogenic potential [Chan, 2001]. HHV-6 has immunomodulating properties and is a powerful inducer of cytokines. One important mechanism of HHV-6 pathogenesis is the engagement of the primary viral receptor, CD46, a complement-regulatory cell surface molecule that provides a key link between innate and adaptive immune responses [Santoro, 1999]. It has been recently indicated that exposure to HHV-6 results in a dramatic inhibition of IL-12 p70 production by differentiated human macrophages in the absence of a productive viral infection, a phenomenon that is likely to be mediated by CD46 engagement [Smith, 2003]. Other mechanisms of immune dysregulation by HHV-6 include defective antigen presentation by dendritic cells and aberrant cytokine production by peripheral blood mononuclear cells [Kakimoto, 2002]. Virus-induced changes in cytokines secretion can lead to changes in tumor microenvironment and deviation of anti-tumor immune response. HHV-6 may also contribute to cancer circuitously through immune suppression. HHV-6 can directly infect $CD4^+$ T cells and induce apoptosis, as an effective $CD4^+$ T cells response is believed to prevent tolerance induction by tumor antigen [Krueger, 1990; Schonnebeck, 1991; Kennedy, 2006].

Despite HHV-7 and HHV-6 similarities, important differences between these viruses exist, including the fact that HHV-7 binds to the cellular CD4 molecule and uses this protein as a necessary component of its receptor, while HHV-6 binds to a different receptor. Furthermore, the pathogenesis and sequelae of HHV-7 infection remains very poorly understood [Dewhurst, 1997].

Immunosuppression caused by medication after solid organ and hematopoietic stem cell transplantation or complications related to the underlying disease, like in oncology patients, may lead to these viruses' reactivation causing worse outcomes.

The aim

The aim of this study was to evaluate HHV-6 and HHV-7 infection reactivation in two patient groups with immunosuppression of different origin.

Material and methods

Two groups of patients were enrolled in this study: Group I – patients after renal transplantation (n = 47) and Group II – patients with gastrointestinal cancer (n = 65).

In the first group, renal transplant recipients 3 months after transplantation were enrolled, 27 male (57.5%) and 20 female (42.5%) aged 28–78 years. In the second group, patients with histologically

confirmed various stages of gastrointestinal cancer (GIC) were included, 22 male (33.8%) and 43 female (66.2%) aged 39–85 years. The cohort was established with the approval of the Ethics Committee of Rīga Stradiņš University and all participants gave their informed consent prior to the examination.

Immunological parameters were determined by Becton-Dickinson (USA) laser flow cytofluorimeter using corresponding monoclonal antibodies to lymphocyte subpopulations: $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD95^+$ and $CD25^+$.

Nested polymerase chain reaction (nPCR) was used for the detection of persistent infection (viral genomic sequences in whole blood DNA) and active phase (viral genomic sequences in cell free plasma DNA) of persistent HHV-6 and HHV-7 infection. Total DNA was isolated from 0.5 ml of whole blood by phenol-chloroform extraction. For DNA purification from 200 μl of cell free blood plasma, QIAamp Blood Kit (QIAGEN, Germany) was used. The plasma samples were treated with Deoxyribonuclease I before DNA purification. To assure the quality of the whole blood DNA as well as to exclude contamination of plasma DNA by cellular DNA, globin PCR was performed. PCR amplification for the viruses was carried out in the presence of 1 μg of whole blood DNA and 10 μl of plasma DNA (corresponding to 100 μl of plasma). The detection of HHV-6 and HHV-7 DNA was performed according to Secchiero, et al. (1995) and Berneman, et al. (1992), respectively. Positive (viruses genomic DNA, ABI, USA) and negative (DNA without virus-specific sequences) as well as water controls were included in each experiment.

HHV-6 variants were identified using restriction endonuclease analysis. The restriction enzyme *Hin*dIII (Fermentas, Lithuania), which cuts the 163-bp HHV-6B amplimer into two fragments of 66 and 97 bp, but does not cut the HHV-6A amplimer, was used for this purpose.

Statistical difference in the prevalence of latent and active HHV-6 and HHV-7 infection was assessed by Fisher's exact test. Student's t-test was used to compare significance in changes of blood cell counts.

Results

Comparative analysis of cellular immune parameters in both patient groups showed no significant differences in number of leukocytes in 1 mm^3 of peripheral blood independently of beta-herpesvirus infection. However, mean absolute number of lymphocytes and lymphocytes' subpopulations in Group I were significantly lower than in Group II. Comparative analyses of the lymphocyte subsets showed significant decrease of immunological parameters ($CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$ and $CD95^+$) in Group I – approximately a two-time decrease in comparison with Group II. Lowering of the immunoregulatory index was also revealed (Table 1).

Persistent beta-herpesvirus infection was detected in 42 out of 47 (89%) renal transplant recipients (Group I) and in 44 out of 65 (68%) patients with GIC (Group II). In renal transplant recipients, the frequency of beta-herpesvirus infection was significantly higher ($p = 0.01$) than in GIC patients (Figure 1).

There were no significant differences in the presence of latent single HHV-6 and HHV-7 infection in both patient groups. Latent single HHV-6 infection was found in 1/2 of Group I patients and 2/3 of Group II patients; latent single HHV-7 infection – in 16/27 (59%) renal transplant recipients and in 21/28 (75%) patients with GIC (Figure 2, Table 2). A higher level of double latent infection (HHV-6 + HHV-7) was found in Group I patients (5/13, 38%) compared with Group II patients (7/13, 54%). A higher frequency of active beta-herpesvirus infection in renal transplant recipients (20/42, 48%) was observed compared with GIC patients (14/44, 32%). The most prevalent was active HHV-7 infection, the higher incidence was found in renal transplant recipients (11/27, 41%) compared with GIC patients (7/28, 25%); however, statistical analysis showed no significance (Figure 2, Table 2).

In all HHV-6 positive DNA samples, isolated from renal transplant recipients, and GIC patients white blood cells and plasma HHV-6B variant was identified.

Table 1. Immunological parameters defined in renal recipients (Group I) and in GIC patients (Group II)

Parameters	Group I (n = 47)		Group II (n = 65)		p
	Mean* absolute count ± SD	Count, %	Mean* absolute count ± SD	Count, %	
Leu	7490 ± 2500	–	6970 ± 2310	–	0.25
Ly	1230 ± 650	17.04	1770 ± 780	25.39	< 0.01
$CD3^+$	940 ± 590	72.02	1240 ± 610	75.46	< 0.01
$CD4^+$	370 ± 300	34.72	690 ± 340	39.15	< 0.01
$CD8^+$	350 ± 260	34.32	520 ± 330	30.35	< 0.01
$CD38^+$	360 ± 280	29.74	510 ± 280	28.74	< 0.01
$CD16^+$	110 ± 70	14.62	350 ± 270	19.55	< 0.01
$CD19^+$	90 ± 60	8.60	150 ± 100	8.29	< 0.01
$CD95^+$	560 ± 400	44.43	860 ± 420	49.92	< 0.01
$CD25^+$	40 ± 40	3.53	140 ± 130	8.14	< 0.01
$CD4^+$ / $CD8^+$	1.20 ± 0.70	–	1.43 ± 0.75	–	0.10

* Absolute number of cells in 1 mm^3 of peripheral blood.

Figure 1. Distribution of persistent HHV-6 and HHV-7 infection in renal transplant recipients and GIC patients

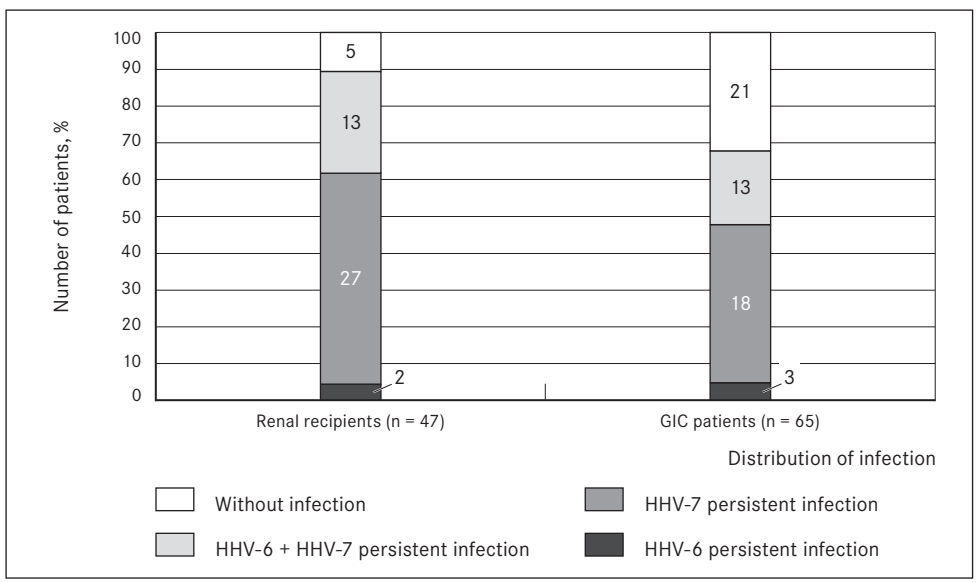


Table 2. Distribution of beta-herpesvirus infection in renal transplant recipients (Group I) and in GIC patients (Group II)

Patients groups	HHV-6 persistent (latent / active) infection	HHV-7 persistent (latent / active) infection	HHV-6 + HHV-7 persistent (latent / active) infection	Without HHV-6 or HHV-7 infection
Patients group I (n = 47)	2/47 (4.3%) (1/1) (50%) / (50%)	27/47 (57%) (16/11) (59%) / (41%)	13/47 (27.6%) (5/8) (39%) / (62%)	5 (11%)
Patients group II (n = 65)	3/65 (4.6%) 2/1 (67%) / (33%)	28/65 (43%) 21/7 (75%) / (25%)	13/65 (20%) 7/6 (54%) / (46%)	21 (32%)

Figure 2. Distribution of latent and active HHV-6 and HHV-7 infection in renal transplant recipients and GIC patients

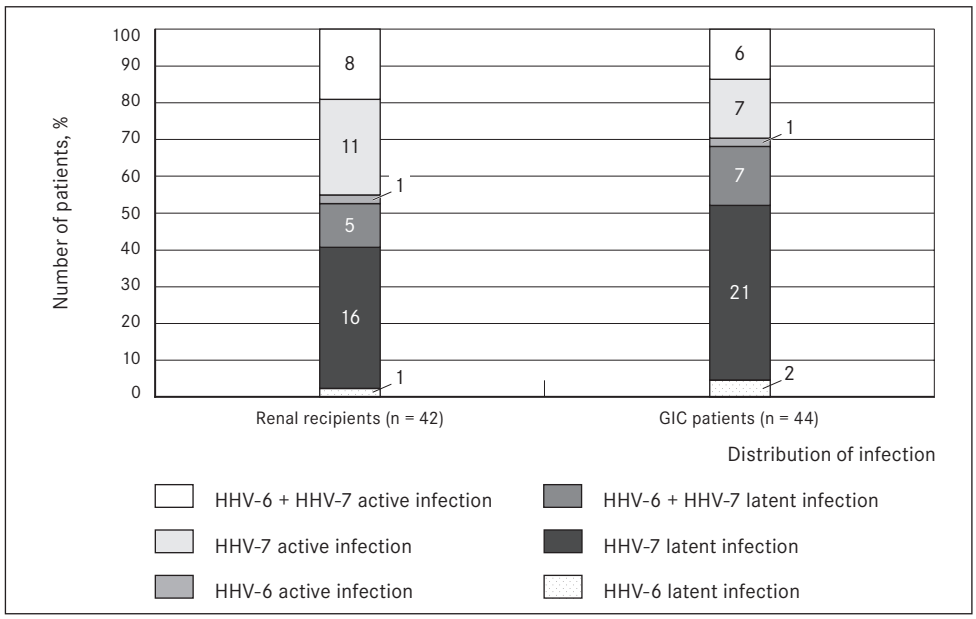


Table 3. Immunological parameters in Groups I and II dependently of beta-herpes viruses infection

Parametrs	Without HHV-6 and/or HHV-7			Latent HHV-6 and/or HHV-7			Active HHV-6 and/or HHV-7		
	Group I (n = 5)	Group II (n = 21)	p	Group I (n = 22)	Group II (n = 30)	p	Group I (n = 20)	Group II (n = 14)	p
Leu	7620 ± 2170	6490 ± 2360	0.33	7840 ± 2660	7240 ± 2190	0.37	7080 ± 2440	7170 ± 2520	0.91
Ly	1200 ± 570	1660 ± 840	0.26	1350 ± 700	1890 ± 570	< 0.01	1100 ± 630	1660 ± 1090	0.06
$CD3^+$	880 ± 450	1160 ± 680	0.39	1040 ± 660	1340 ± 420	0.05	850 ± 540	1170 ± 830	0.81
$CD4^+$	290 ± 180	640 ± 390	0.06	410 ± 360	760 ± 290	< 0.01	350 ± 270	610 ± 350	0.02
$CD8^+$	350 ± 220	500 ± 390	0.41	390 ± 310	550 ± 200	0.02	310 ± 230	520 ± 470	0.09
$CD38^+$	400 ± 180	500 ± 300	0.48	400 ± 300	550 ± 300	0.08	310 ± 270	420 ± 230	0.22
$CD16^+$	170 ± 80	360 ± 280	0.15	170 ± 100	360 ± 290	< 0.01	130 ± 80	320 ± 230	< 0.01
$CD19^+$	90 ± 50	130 ± 90	0.35	100 ± 70	150 ± 70	0.01	90 ± 60	190 ± 170	0.02
$CD95^+$	660 ± 260	820 ± 470	0.42	600 ± 480	920 ± 330	< 0.01	500 ± 340	790 ± 530	0.06
$CD25^+$	40 ± 30	140 ± 90	0.02	50 ± 40	150 ± 170	< 0.01	40 ± 40	120 ± 80	< 0.01
$CD4^+/CD8^+$	0.9 ± 0.3	1.46 ± 0.69	0.09	1.33 ± 0.85	1.48 ± 0.83	0.52	1.14 ± 0.56	1.24 ± 0.62	0.62

Comparative analysis of cellular immune parameters in Groups I and II was performed dependently of beta-herpevirus infection. Each group was subdivided into three subgroups: renal transplant recipients and GIC patients without, with latent and with active HHV-6 and/or HHV-7 infection. In Group I patients with active viral infection a tendency to decrease all cellular immunological parameters (leukocytes, monocytes, lymphocytes and $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$, $CD95^+$) was observed when compared with the subgroups with latent and without HHV-6 and HHV-7 infection. Although the number of leukocytes and the levels of other immunological parameters ($CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$, $CD95^+$) were increased in both groups with latent beta-herpesvirus infection compared with the subgroups without viral infection, both groups showed significant differences in numbers of $CD4^+$, $CD16^+$, $CD19^+$ and $CD25^+$ subpopulations ($p < 0.05$) between patients with latent and

active beta-herpesvirus infection. In renal transplant recipients with active viral infection mean absolute number of $CD4^+$ lymphocytes was decreased to 14%; in GIC patients to 19% compared with patients with latent infection. The most dramatic decrease was detected in the number of $CD16^+$, where in Group I patients with active viral infection this subpopulation was decreased to 23% and in Group II – to 11%, only comparing both patients groups with latent viral infection (Table 3). Another interesting finding was revealed in both groups with active beta-herpesvirus infection. The number of B lymphocytes ($CD19^+$) had a tendency to decrease in renal transplant recipients (on average to 10%); however, in GIC patients it had a tendency to increase (on average to 26%).

Discussion

This study has revealed interesting results when comparing two different groups of patients with different forms of immunosuppression. Renal transplant recipients have immunosuppression caused by medical treatment (immunosuppressants) and GIC patients immunosuppression – by underlying disease. Immunological parameters show more severe immunosuppression in renal transplant recipients (almost two-fold decrease in all parameters) independently of beta-herpesvirus infection. In this group, there was also a higher incidence of beta-herpesvirus infection compared with GIC patients (89% against 68%). However, comparative analysis of cellular immune parameters dependently of beta-herpevirus infection shows significant changes in numbers of $CD4^+$, $CD16^+$, CD19 and $CD25^+$ subpopulations between both groups with latent and active infection. Decrease in $CD4^+$ could be a cause of higher frequency of HHV-7 and HHV-6 + HHV-7 reactivation (47% in renal transplant recipients and 31% in GIC patients). Although, in renal transplant recipients more severe immunosuppression has been identified, a higher decrease of $CD4^+$ is detected in GIC patients. These results give evidence of HHV-7 involvement in immunosuppression of GIC patients, because $CD4^+$ cells are the main target cells for this virus. Lower level of natural killer cells ($CD16^+$) was detected in renal transplant recipients than in GIC patients. Decrease of $CD16^+$ in both groups could be caused by immunomodulating properties of beta-herpesviruses, because these cells are not the main target cells for HHV-6 or HHV-7. A significant difference in the number of $CD19^+$ cells is also found between groups with active beta-herpesvirus infection. In renal transplant recipients the number of B-lymphocytes ($CD19^+$) is decreased to 10%; however, in GIC patients the number of $CD19^+$ cell has a tendency to increase (on average to 26%). In spite of immunosuppression in GIC patients, their immune system is able to fight with active viral infection compared with renal transplant recipients.

At the moment, there are a lot of researches dedicated to investigate involvement of HHV-6 in different chronic diseases but only a few of them show interactions between other beta-herpesviruses (CMV and HHV-7). For example, Chapenko, et al., (2001) showed that HHV-7 infection has a higher incidence in renal transplant recipients than HHV-6 or CMV and it reactivates first, leading to the reactivation of other beta-herpesviruses. Current data also show a higher incidence of HHV-7 in renal transplant recipients as well as in GIC patients. Interestingly, almost in all cases active HHV-6 infection was found simultaneously with active HHV-7 infection, which coincides with previous published data.

All these results indicate the importance of HHV-6 and also HHV-7 involvement in immunosuppression in both mentioned patient groups; however, additional investigations are required.

Conclusions

A more severe immunosuppression in renal transplant recipients leads to more often reactivation of beta-herpesviruses. The decrease of $CD4^+$ could be caused by HHV-7 reactivation in renal transplant recipients as well as in GIC patients. An increased number of $CD19^+$ cells in GIC patients with active beta-herpesvirus infection shows inability of their immune system to persist with active HHV-6 and HHV-7 infection. Active HHV-7 infection was more prevalent in renal transplant recipients.

References

1. Campadelli-Fiume G., Mirandola P., Menotti L. Human herpesvirus 6: an emerging pathogen // Emerg Infect Dis, 1999; 5 (3): 353–366.
2. Caserta M. T., Mock D. J., Dewhurst S. Human herpesvirus 6 // Clin Infect Dis, 2001; 33 (6): 829–833.
3. Chan P. K., Chan M. Y., Li W. W., et al. Association of human beta-herpesviruses with the development of cervical cancer: bystanders or cofactors // J Clin Pathol, 2001; 54 (1): 48–53.
4. Chapenko S., Folkmane I., Tomsone V., et al. Infection of beta-herpesviruses (CMV, HHV-6, HHV-7): role in postrenal transplantation complications // Transplant Proc. 2001; 33 (4): 2463–2464.
5. Dewhurst S., Skrincosky D., van Loon N. Human herpesvirus 7 // Expert Rev Mol Med, 1997; 1997: 1–10.
6. Dulery R., Salleron J., Dewilde A., et al. Early human herpesvirus type 6 reactivation after allogeneic stem cell transplantation: a large-scale clinical study // Biol Blood Marrow Transplant, 2012; 18 (7): 1080–1089.
7. Kakimoto M., Hasegawa A., Fujita S., Yasukawa M. Phenotypic and functional alterations of dendritic cells induced by human herpesvirus 6 infection // J Virol, 2002; 76 (20): 10338–10345.
8. Kennedy R., Celis E. T helper lymphocytes rescue CTL from activation-induced cell death // J Immunol, 2006; 177 (5): 2862–2872.
9. Krueger G. R., Wassermann K., De Clerck L. S., et al. Latent herpesvirus-6 in salivary and bronchial glands // Lancet, 1990; 336 (8725): 1255–1256.
10. Lusso P. HHV-6 and the immunosystem: mechanisms of immunomodulation and viral escape // J Clin Virol, 2006; 37 (1): 4–10.
11. Quadrelli C., Barozzi P., Riva G., et al. β-HHVs and HHV-8 in lymphoproliferative disorders // Mediterr J Hematol Infect Dis, 2011; 3 (1): e2011043.
12. Santoro F., Kennedy P. E., Locatelli G., et al. CD46 is a cellular receptor for human herpesvirus 6 // Cell, 1999; 99 (7): 817–827.
13. Schonnebeck M., Krueger G. R., Braun M., et al. Human herpesvirus-6 infection may predispose cells to superinfection by other viruses // In Vivo, 1991; 5 (3): 255–263.
14. Smith A., Santoro F., Di Lullo G., et al. Selective suppression of IL-12 production by human herpesvirus 6 // Blood, 2003; 102 (8): 2877–2884.
15. Yoshikawa T. Significance of human herpesviruses to transplant recipients // Curr Opin Infect Dis, 2003; 16 (6): 601–606.

Morphology of Extraoccular Muscles in Case of Strabismus

Anna Valaine, Anna Augule, Mara Pilmane, Sandra Valeina [1]

Rīga Stradiņš University, Institute of Anatomy and Anthropology, Latvia
[1] *Children's Clinical University Hospital, Latvia*

Abstract

Strabismus is a common disorder of children and adults that affects up to 4% of general population. The aim of the present study was to describe the morphologic changes in human strabismic extraoccular muscle.

The histopathological study was conducted on 15 patients with strabismus. Tissues were processed for IGF-1, IGFR-1, TGF-β1, NGFR and PGP 9.5 by means of biotin-streptavidin immunohistochemistry.

In all specimens, atrophic degenerative changes were observed. The muscle fibers were heterogeneous in shape, size and colour. A chronic persistent perivascular inflammation with lymphocyte predominance, but also presence of neutrophils was observed, as well as neoangiogenesis. Also new myotube formation was detected. Numerous to moderate numbers of TGF-β1 positive endothelial cells and fibroblasts were observed. VEGF was seen in endothelium in moderate numbers. IGF-1 was seen occasionally in endothelial and smooth muscle cells. Occasional IGFR1 positive endothelial, inflammation cells and connective tissue cells were observed. Numerous to moderate number of PGP 9.5 positive nerve fibers were seen in nerve bundles, among muscle fibers and endothelial cells. NGFR positive perivascular nerve fibers were mostly seen in low to moderate appearance.

Occasional to few numbers of structures positive for IGF-1 and its receptor indicate possible disturbances in muscle fiber growth and regeneration in extraocular muscles in case of strabismus. Moderate to numerous numbers of TGF-β1 positive structures indicate the continuing regeneration of connective tissue and blood vessels in strabismus affected muscle. Notable numbers of VEGF positive endothelial cells simultaneously to the sclerotic blood vessels and neoangiogenesis proves the tissue ischemia in strabismus affected eye muscle. Presence of PGP 9.5 continuing nerve fibers excludes innervation disorder in strabismus affected eye muscle.

Keywords: strabismus, growth factors, extraoccular muscles.

Introduction

Strabismus is a disorder, which is characterised by misalignment of the eyes and probably even loss of binocular vision and affects up to 4% of the general population, but is more common in children. In addition to genetic predisposition, both ocular and non-ocular factors can also cause strabismus. For some children, treatment with glasses is sufficient. For many children and most adults with strabismus, surgical correction is necessary [4, 23]. Surgical treatment of strabismus entails either resection of

the “underacting” extraocular muscle, recession of the “overacting” muscle, or a combination of both procedures. Surgical success rates vary, and these depend on the type of strabismus. Botulinum toxin injections are also an effective treatment for weakening an “overacting” muscle [41].

Recently, there has been a range of reports about the impact of several growth factors on animal extraocular muscle, that might even become an alternative pharmacological treatment for strengthening an “underacting” or weaken an “overacting” eyeball muscle.

In rabbits one week after a single injection of Transforming growth factor β1 (TGF-β1) treated muscles showed significant decreases in generation of force and mean myofiber cross-sectional area of fast myosin heavy chain-positive myofibers compared with control muscles [3]. TGF-β1 is a family of multifunctional proteins that inhibit the growth of most cell types, and these proteins induce the deposition of extracellular matrix [32]. TGF-β1 is believed to be the most important ligand in the pathogenesis of fibrotic diseases in the eye [38].

In chicken extraocular muscles treatment with Insulin like growth factor 1 (IGF-1), Cardiotrophin 1 (CT1), and combination of IGF-1 and CT1 significantly increased contractile force by 14% to 22%. CT1 and combination treatment significantly increased muscle mass by 10% to 24% [26]. A highly conserved signaling pathway involving IGF-1, and a cascade of intracellular components that mediate its effects, plays a major role in the regulation of skeletal muscle growth [39]. IGF-1 is a promising antiatrophy agent because of its ability to promote hypertrophy [37].

Antibodies to the IGF-1 receptor appear to impact recruitment and activation of T cells and stimulation of hyaluronan production, in processes that play key roles in the development of inflammation and increased orbital tissue swelling [24].

Evidence accumulating over the last decade has established the fundamental role of vascular endothelial growth factor (VEGF) as a key regulator of normal and abnormal angiogenesis [17]. VEGF is aberrantly expressed in several diseases including asthma where it contributes to bronchial vascular remodeling and chronic inflammation [13] as well as cancer, rheumatoid arthritis and diseases of the eye [34].

Vascular endothelial growth factor expression is enhanced in ischemic skeletal muscle and is thought to play a key role in the angiogenic response to ischemia [18]. The temporal and spatial correlation of VEGF overexpression with angiogenesis during tumor growth, inflammation, and wound healing provides strong evidence for a functional role of VEGF as a key regulator of angiogenesis. The growth factor stimulates vascular endothelial proliferation and macrophage migration and increases microvessel permeability to macromolecules [16].

The expression of protein gene product 9.5 (PGP 9.5), a member of the ubiquitin hydrolase family of proteins, is confined to neural and neuroendocrine cells. PGP 9.5 has been claimed as a specific marker of neural and/or nerve sheath differentiation [10].

Nerve growth factor receptor (NGFR) is a receptor of neurotrophins and involved in survival, differentiation and apoptosis of neurons. NGFR is found in the tips of neuronal axons, which, under the influence of nerve growth factor and other trophic factors, guides the direction of axonal growth, by initiating remodeling of the neuron’s cytoskeleton [30].

The aim

The aim of the research was to describe the morphologic changes in strabismic extraocular muscle.

Material and methods

Material. The specimens used for research were obtained from 15 strabismus surgery patients (9 males, 6 females) ranging in the ages from 6 to 30 years (Table 1). In 14 cases, esotropia was diagnosed, and therefore resection of the lateral rectus muscle was performed, in one case, exotropia was adjusted by medial rectus muscle resection. Specimens are the property of the collection of the Institute for Anatomy and Anthropology of Rīga Stradiņš University.

Table 1. Patient information

Specimen No.	Age, years	Gender	Muscle	Diagnosis
1	6	M	*M. rectus lateralis*	Esotropia
2	6	M	*M. rectus lateralis*	Esotropia
3	6	M	*M. rectus lateralis*	Esotropia
4	7	M	*M. rectus lateralis*	Esotropia
5	7	M	*M. rectus medialis*	Exotropia
6	8	F	*M. rectus lateralis*	Esotropia
7	11	M	*M. rectus lateralis*	Esotropia
8	11	F	*M. rectus lateralis*	Esotropia
9	12	M	*M. rectus lateralis*	Esotropia
10	12	F	*M. rectus lateralis*	Esotropia
11	15	M	*M. rectus lateralis*	Esotropia
12	16	F	*M. rectus lateralis*	Esotropia
13	21	F	*M. rectus lateralis*	Esotropia
14	29	M	*M. rectus lateralis*	Esotropia
15	30	F	*M. rectus lateralis*	Esotropia

Abbreviations: M – male, F – female, *M. rectus lateralis* – *Musculus rectus lateralis*, *M. rectus medialis* – *Musculus rectus medialis*.

Methods. The tissue fragments were fixed for 24 hours in a mixture consisting of 2% formaldehyde and 0.2% picric acid in 0.1-M phosphate buffer (pH 7.2) and after that washed for 12 hours in phosphate buffer (pH 7) containing 10% sucrose. Then tissues were embedded in paraffin and the blocks of paraffinized tissues were sectioned into slides 5 µm in thickness by means of a microtome. The slides were deparaffinized and prepared to detect the following growth factors and their receptors: TGF-β1 (code-ab1279, working dilution 1 : 1000, Abcam, England), IGF-1 (code-MAB291, working dilution 1 : 50, R & D Systems, Germany), IGF1R (code-af305NA, working dilution 1 : 100, R & D Systems, Germany), VEGF, (code-M7273, working dilution 1 : 50, Dako Cytomation, Denmark), PGP 9.5 (code-Z5116, working dilution 1 : 600, Dako Cytomation, Denmark), NGFR (code-ab3125, working dilution 1 : 150, Abcam, England).

To obtain an overview picture, the slides were processed for hematoxylin / eosin.

Quantification of structures. For quantification of structures, the semiquantitative counting method was used. The designations were as follows: 0, negative reaction; 0 / + – occasionally marked structures in the view field; + – a few positive structures in the view field; + / ++ – few to moderate positive structures; ++ – a moderate number of marked structures in the view field; ++ / +++ – moderate to numerous positive structures; +++ – numerous marked structures in the view field; ++++ – abundance of marked structures found in the view field [35].

For data storing and processing, Microsoft Office Excel 2010 was used. Statistical analysis was performed with SPSS 20. Pearson's correlation test was used to evaluate the correlation in between growth factors and morphologic changes. The p value < 0.05 was considered statistically significant. Correlation was considered as weak if value R was 0–0.3; moderate if value R was 0.31–0.69 and strong if value R was 0.7–1.

Results

In all specimens, atrophic degenerative changes were observed (Table 2), which was noticeable in changed heterogeneous muscle fiber shape and size, lightened fiber colour, fiber fragmentation, a thickened perimysium and lacking cell nuclei. Regeneration processes with new myotube formation and centrally located nuclei were detected in eight specimens (Figures 1 and 2). In seven tissue specimens, inflammation processes were noticed (Figure 3). Inflammation was chronically persistent and perivascular, with predominantly lymphocytic, but also neutrophilic infiltration. Neoangiogenesis was observed in 11 specimens (Figure 4).

Quantification of structures. For the quantification of structures, the semiquantitative counting method was used. The designations were as follows: 0 – negative reaction; 0/+ – occasionally positive structures in the view field; + – a few positive structures in the view field; + / ++ – few to moderate positive structures in the view field; ++ – moderate count of positive structures in the view field; ++ / +++ – moderate to numerous positive structures in the view field; +++ – numerous positive structures in the view field; ++++ – abundance of positive structures in the view field.

A considerable number of TGF-β1 positive cells (endothelial cells and fibroblasts) was found in two specimens, but a moderate to large number was observed in two specimens. A moderate number of TGF-β1 positive endothelium cells and fibroblasts was observed in four cases; in other four cases few numbers of these cells were positive for TGF-β1. A completely negative reaction was marked in one case (Table 3, Figure 5).

Table 2. Morphologic changes in extraocular muscle in case of strabismus

Specimen No.	Inflammation	Neoangiogenesis	Muscle fiber regeneration	Muscle fiber degeneration
1	0	+	+	+++
2	+ / ++	0	0	+++
3	0	++	0	+++
4	+++	0	+++	++
5	0	0	+	+++
6	++	+	+	+++
7	+	+	+++	+++
8	0	+	0	+++
9	0	++	0 / +	+++
10	0 / +	++	0	+++
11	0	0	0/+	+++
12	0	+++	0	+++
13	+	++ / +++	0 / +	+++
14	0	+	0	+++
15	0 / +	++	0	+++
Total	**0 / +**	**+**	**0 / +**	**+++**

Figure 1. Centrally located nuclei in regenerating muscle fibers and thickening of perimysium in strabismic eyeball muscle (Hematoxylin & eosin, original magnification × 400)

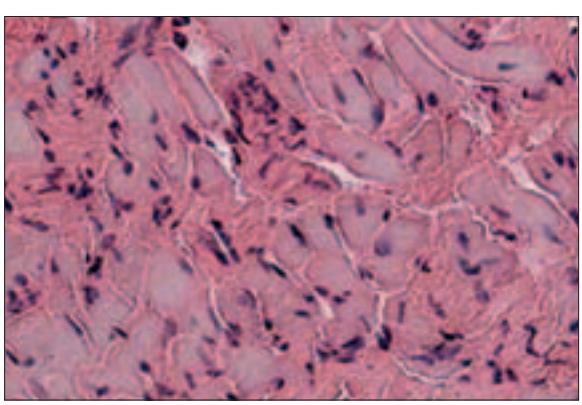


Figure 2. Heterogeneous muscle fiber shape and sharp edged fibers indicating degeneration in eyeball muscles in case of strabismus (Hematoxylin & eosin, original magnification × 400)

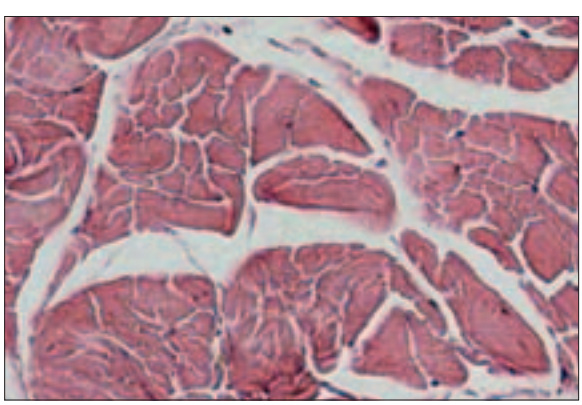


Figure 3. Extravasation of inflammation cells in perimysium around strabismic eyeball muscle (Hematoxylin & eosin, original magnification × 400)

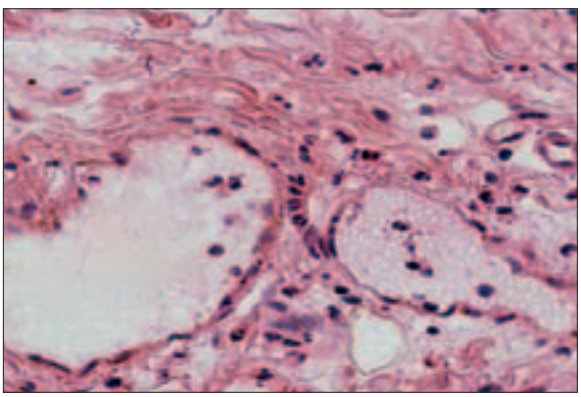


Figure 4. Neoangiogenesis and thickening of blood vessel wall in vascular sclerosis in strabismic eyeball muscle (Hematoxylin & eosin, original magnification × 200)

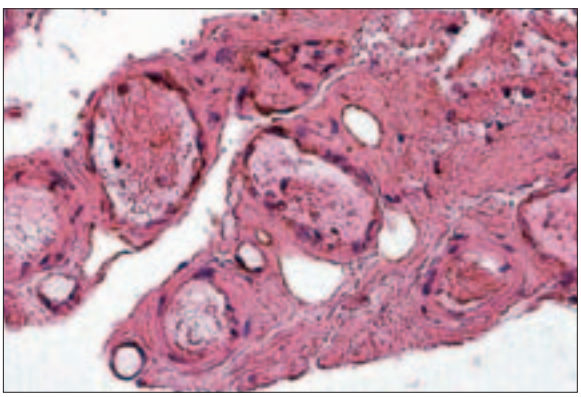


Table 3. Growth factors in extraocular muscle in case of strabismus

Specimen No.	TGF-β1	IGF-1	IGFR-1	VEGF	PGP 9.5	NGFR
1	+ / ++	+	0 / +	++	+ / ++	0
2	+	0	0 / +	++	0	+
3	++	++	0	++++	++	+ / ++
4	+++	+ / ++	++	++ / +++	++ / +++	0 / +
5	+	0	0	++	+	0
6	++	0 / +	+	+ / ++	+	+
7	++	+ / ++	++	+++	+++	0
8	+	0	0	++	+ / ++	0
9	++ / +++	+	0	+++	0 / +	++
10	++ / +++	0	0	+++	0 / +	++
11	+++	++	0 / +	+++	++	+ / ++
12	0 / +	0 / +	0	0	0	0 / +
13	0	0 / +	0	+	0 / +	0
14	+	0	0 / +	++ / +++	++ / +++	+
15	++	0 / +	0 / +	+ / ++	+ / ++	+
Total:	**+ / ++**	**0 / +**	**0 / +**	**++**	**+ / ++**	**+**

Abbreviations: TGF-β1 – Transforming growth factor β1, IGF-1 – Insulin like growth factor 1, IGFR-1 – Insulin like growth factor 1 receptor, VEGF – Vascular endothelial growth factor, PGP 9.5 – Protein gene product 9.5, NGFR – Nerve growth factor receptor.

Quantification of structures. For the quantification of structures, the semiquantitative counting method was used. The designations were as follows: 0 – negative reaction; 0/+ – occasionally positive structures in the view field; + – a few positive structures in the view field; + / ++ – few to moderate positive structures in the view field; ++ – moderate count of positive structures in the view field; ++ / +++ – moderate to numerous positive structures in the view field; +++ – numerous positive structures in the view field; ++++ – abundance of positive structures in the view field.

VEGF was seen exclusively in endothelium. In one case the number of marked cells was abundant, in four cases large, but in two cases moderate to large. Four specimens showed a moderate number of VEGF positive cells. One specimen showed few VEGF positive cells, another – no positive cells (Table 3).

A moderate number of IGF-1 positive endothelium cells was observed two cases. In other two cases a few to moderate number positive of muscle fibers, vascular smooth muscle cells (Figure 7), and fibroblasts were marked. In four cases, occasional positive fibroblasts could be observed. Five specimens showed a completely negative reaction to IGF-1 (Table 3).

In seven specimens IGF-1R reaction was negative, occasional positive endothelial, inflammation cells and connective tissue cells were observed in five cases. There were also moderate numbers of marked smooth muscle cells in sclerotized blood vessels (Table 3, Figure 6).

Numerous to moderate number of PGP 9.5 positive nerve fibers were seen in nerve bundles, among muscle fibers and endothelial cells, also few macrophages and mast cells contained this factor (Table 3, Figure 8).

NGFR positive perivascular nerve fibers were mostly seen in low to moderate appearance. In five cases no NGFR positive structure was observed (Table 3).

Using Pearson's correlation test, following results were obtained: a significant positive correlation was observed between the counts of immunoreactive structures for TGF-β1 and VEGF ($p = 0.005$, $r = 0.684$), TGF and IGF-1 ($p = 0.004$, $r = 0.698$).

The count of IGF-1 positive structures significantly correlates with the formation of new myotubes ($p = 0.044$, $r = 0.526$), positive structures for VEGF ($p = 0.02$, $r = 0.590$), positive structures for PGP 9.5 ($p = 0.029$, $r = 0.564$). A significant positive correlation between structures positive for IGF-R1 and inflammation processes ($p = 0.004$, $r = 0.701$), new myotube formation ($p = 0.935$, $r = 0.001$) was observed.

Figure 5. Endothelial cells showing immunoreactivity for TGFβ in eyeball muscles in case of strabismus (Immunohistochemistry for TGF-β, original magnification × 400)

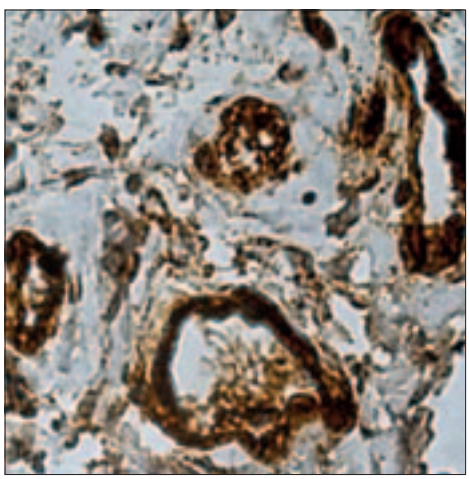


Figure 6. IGF-1R positive vascular smooth muscle cells in blood vessel wall in eyeball muscles in case of strabismus (Immunohistochemistry for IGF-1R, original magnification × 400)

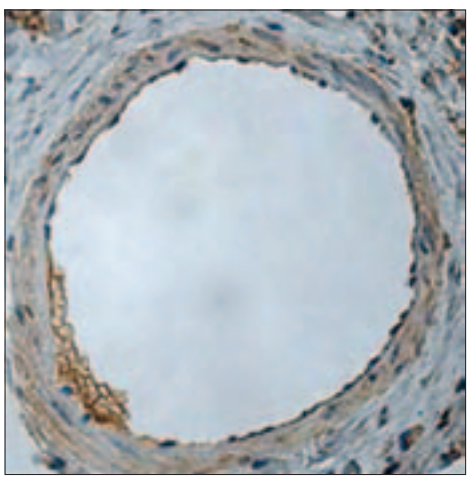


Figure 7. Positive vascular smooth muscle cells for IGF-1 seen in the blood vessel wall in eyeball muscles in case of strabismus (Immunohistochemistry for IGF-1, original magnification × 400)

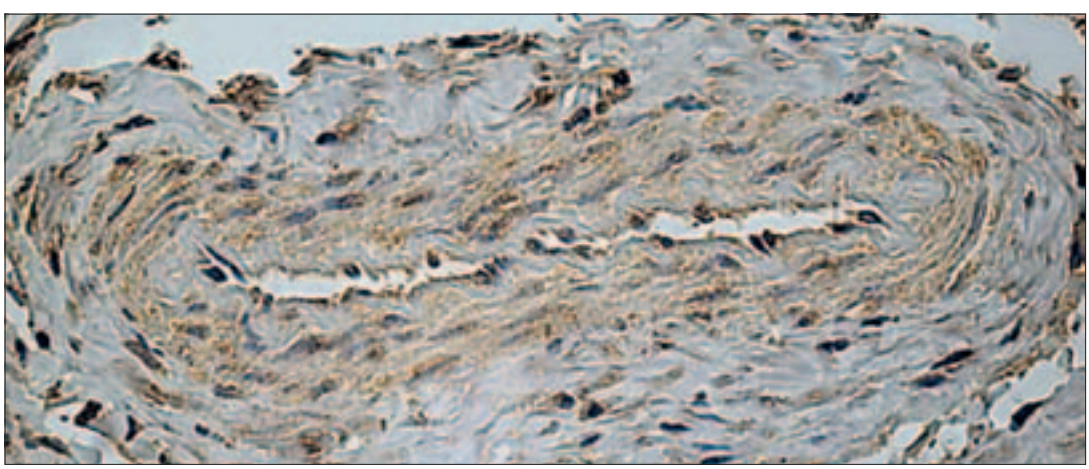
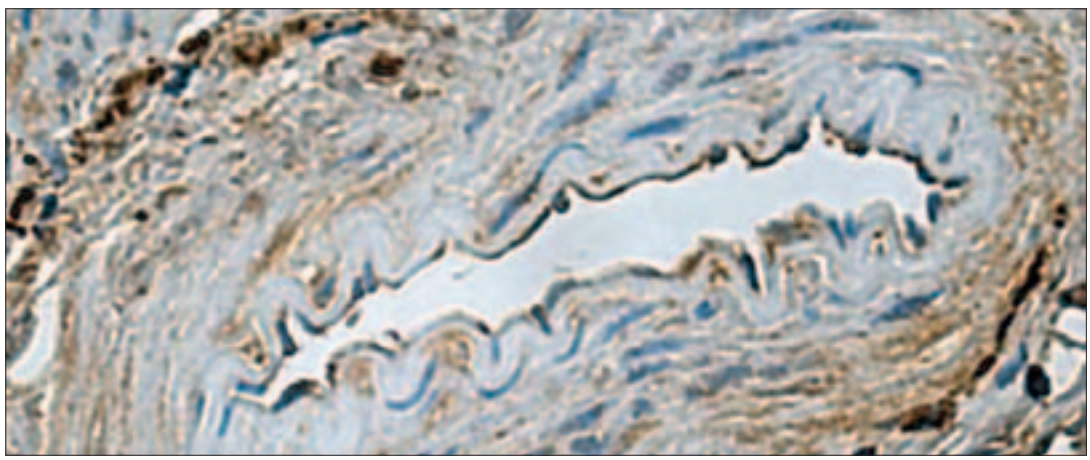


Figure 8. Endothelial cells immunoreactive for PGP 9.5 in eyeball muscles in case of strabismus (Immunohistochemistry for PGP 9.5, original magnification × 400)



The count of VEGF positive structures significantly correlated with structures positive for NGFR ($p = 0.006$, $r = 0.669$). Inflammation processes significantly correlated with new myotube formation $p = 0.02$, $r = 0.590$).

Discussion

The observed degenerative changes in all specimen as well as marked neoangiogenesis and sclerotizing of blood vessels create the subject for discussion.

Other studies of EOM morphology in case of strabismus also showed signs of atrophy, increases in endomysial and perimysial collagen and variation in muscle fiber shape and size [7, 27, 29].

In most of the analyzed specimen numbers of IGF-1, positive structures were low or no positive structure was detected. In contrast to that, other studies show that IGF-1 levels in EOM are higher than in limb skeletal muscle. This underscores the potential relevance of these myogenic growth factors in extraoccular muscle plasticity and force regulation. So do gain-of-function experiments, in which prolonged exposure of adult rabbit EOM to IGF-1 resulted in significantly increased cross-sectional area, increased single-twitch force generation and shortening of half relaxation time [11, 25, 41]. At the same time, blocking IGF-1 by neutralizing antibodies or binding proteins significantly prolonged both twitch contraction time and half relaxation time of EOM [25]. EOMs are believed to be the fastest muscles in the human body, but it has been observed, that contraction speed is slowed and fatigue resistance is reduced in strabismic EOM [2, 25].

IGF-1 is a potent stimulator of protein synthesis in muscle, directs skeletal muscle development, growth and regeneration, and protects the muscle from degeneration [9, 12, 14, 28]. The sparse positive structures for IGF-1 and its receptor may point out disturbed growth and regeneration processes in strabismic eyeball muscle.

Mostly the numbers of TGF-β positive structures in the analyzed specimens were moderate or moderate to large. TGF-β modulates fibroblast phenotype and function; it has the ability to induce the expression of extracellular matrix proteins in mesenchymal cells, and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the extracellular matrix. TGF-β relates to connective tissue remodeling, repair and fibrosis [8, 40]. It has been reported, that anti-TGF-β1 agents were effective against postoperative inflammation and fibrosis following strabismus surgery in rabbits [22].

A significant positive correlation between TGF-β1 and VEGF was detected, which is likely to occur due to TGF-β potency to promote angiogenesis *in vivo* via recruitment of paracrine VEGF-expressing hematopoietic effector cells. This mechanism may activate vascular growth and remodeling during conditions, when TGF-β activity is upregulated, e.g. inflammation or tumor growth [21, 36]. TGF-β signaling is even reported to be required for normal vascular development [26].

VEGF was seen in moderate to large numbers in endothelial cells together with angioneogenesis and blood vessel sclerosis. Low physiological amounts of VEGF seem to be required for blood vascular homeostasis, endothelial cell survival, and production of NO and prostacyclin. Much higher concentrations, induced through hypoxia, inflammation and other growth factors such as TGF caused angiogenesis [1, 33].

Positive structures for the general neuronal marker PGP 9.5 [19, 31] were seen in few to moderate numbers in the described specimens. Rarely specific genetic mutations can be causing structural changes in cranial nerves and thereby resulting in dysinnervation and atrophy of specific extraocular muscles [5, 6, 15], which can be excluded in our case.

Conclusions

Occasional to few numbers of structures positive for IGF-1 and its receptor indicate possible disturbances in muscle fiber growth and regeneration in extraocular muscles in case of strabismus.

Moderate to numerous numbers of TGF-β1 positive structures indicate the continuing regeneration of connective tissue and blood vessels in strabismus affected muscle.

Notable numbers of VEGF positive endothelial cells simultaneously to the sclerotic blood vessels and neoangiogenesis proves the tissue ischemia in strabismus affected eye muscle.

Presence of PGP 9.5 continuing nerve fibers excludes innervation disorder in strabismus affected eye muscle.

References

1. Al-Lathyfeh M., Silva P. S., Sun J. K. et al. Antiangiogenic therapy for ischemic retinopathies // Cold Spring Harb Perspect Med, 2012; 2 (6).
2. Anderson B. C., Christiansen S. P., Grandt S., et al. Increased extraocular muscle strength with direct injection of insulin-like growth factor-I // Invest Ophthalmol Vis Sci, 2006; 46 (6): 2461–2467.
3. Anderson B. C., Christiansen S. P., McLoon L. K. Myogenic growth factors can decrease extraocular muscle force generation: a potential biological approach to the treatment of strabismus // Invest Ophthalmol Vis Sci, 2008; 49 (1): 221–229.
4. Anderson B. C., Daniel M. L., Kendall J. D., et al. Sustained release of bone morphogenetic protein-4 in adult rabbit extraocular muscle results in decreased force and muscle size: potential for strabismus treatment // Invest Ophthalmol Vis Sci, 2011; 52 (7): 4021–4029.
5. Assaf A. A. Congenital innervation dysgenesis syndrome (CID)/congenital cranial dysinnervation disorders (CCDDs) // Eye (Lond), 2011; 25 (10): 1251–1261.
6. Baker R. S., Millett A. J., Young A. B., Markesbery W. R. Effects of chronic denervation on the histology of canine extraocular muscle // Invest Ophthalmol Vis Sci, 1982; 22 (6): 701–705.
7. Berard-Badier M., Pellissier J. F., Toga M., et al. Ultrastructural studies of extraocular muscles in ocular motility disorders // Albrecht von Graefes Arch Klin Exp Ophthalmol, 1978; 208 (1–3): 193–205.
8. Biernacka A., Dobaczewski M., Frangogiannis N. G. TGF-β signaling in fibrosis // Growth Factors, 2011; 29 (5): 196–202.
9. Brisson K. B., Barton R. E. Insuline-like growth factor-I E-peptide activity is dependent on the IGF-I receptor // PLoS One, 2012; 7 (9).
10. Campbell L. K., Thomas J. R., Lamps L. W., et al. Protein gene product 9.5 (PGP 9.5) is not a specific marker of neural and nerve sheath tumors: an immunohistochemical study of 95 mesenchymal neoplasms // Mod Pathol, 2003; 16 (10): 963–969.
11. Chen J., von Bartheld C. S. Role of exogenous and endogenous trophic factors in the regulation of extraocular muscle strength during development // Invest Ophthalmol Vis Sci, 2004; 45 (10): 3538–3545.
12. Clemmons D. R. Metabolic actions of insulin-like growth factor-I in normal physiology and diabetes // Endocrinol Metab Clin North Am, 2012; 41 (2): 425–443.
13. Clifford R. L., John A. E., Brightling C. E., Knox A. J. Abnormal histone methylation is responsible for increased vascular endothelial growth factor 165a secretion from airway smooth muscle cells in asthma // Immunol, 2012; 189 (2): 819–831.
14. Coleman M. E., DeMayo F., Yin K. C., et al. Myogenic vector expression of insulin-like growth factor I stimulates muscle cell differentiation and myofiber hypertrophy in transgenic mice // J Biol Chem, 1995; 270 (20): 12109–12116.

15. Das V. E. Responses of cells in the midbrain near-response area in monkeys with strabismus // Invest Ophthalmol Vis Sci, 2012; 53 (7): 3858–3864.
16. Deindl E., Buschmann I., Hoefer I. E., et al. Role of ischemia and of hypoxia-inducible genes in arteriogenesis after femoral artery occlusion in the rabbit // Circ Res, 2001; 89 (9): 779–786.
17. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis // Physiol Cell Physiol, 2001; 28 (6): 1358–1366.
18. Germani A., Di Carlo A., Mangoni A., et al. Vascular endothelial growth factor modulates skeletal myoblast function // Pathol, 2003; 163 (4): 1417–1428.
19. Habash S. F., Hantash Abu O., Yunis Abu M. Assessment of the innervation pattern of oral squamous cell carcinoma using neural protein gene product (9.5) // J Oral Maxillofac Pathol, 2012; 16 (1): 16–21.
20. Jaffe M., Sesti C., Washington I. M., et al. Transforming growth factor-β signaling in myogenic cells regulates vascular morphogenesis, differentiation, and matrix synthesis // Arterioscler Thromb Vasc Biol, 2012; 32 (1): 1–11.
21. Josifova T., Schneider U., Henrich P. B., Schrader W. Eye disorders in diabetes: potential drug targets // Infect Disord Drug Targets, 2008; 8 (2): 70–75.
22. Jung K. I., Choi J. S., Kim H. K., Shin S. Y. Effects of an anti-transforming growth factor-β agent (Pirfenidone) on strabismus surgery in rabbits // Curr Eye Res, 2012; 37 (9): 770–776.
23. Khan A. O., Shinwari J., Sharif L. A., et al. Infantile esotropia could be oligogenic and allelic with Duane retraction syndrome // Mol Vis, 2011; 17: 1997–2002.
24. Khoo T. K., Bahn R. S. Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies // Thyroid, 2007; 17 (10): 1013–1018.
25. Li T., Feng Ch. Y., von Bartheld C. S. How to make rapid eye movements "rapid": the role of growth factors for muscle contractile properties // Pflugers Arch, 2011; 461 (3): 373–386.
26. Li T., Wiggins L. M., von Bartheld, C. S. Insulin-like growth factor-1 and cardiotrophin 1 increase strength and mass of extraocular muscle in juvenile chicken // Invest Ophthalmol Vis Sci, 2010; 51 (5): 2479–2486.
27. Martinez J. A., Hay S., McNeer W. K. Extraocular muscles light microscopy and ultrastructural features // Acta Neuropathologica, 1976; 34 (3): 237–253.
28. Messi L. M., Delbono O. Target-derived trophic effect on skeletal muscle innervation in senescent mice // The Journal of Neuroscience, 2003; 23 (4): 1351–1359.
29. Meyer E., Ludatscher M., Zonis S. Primary and secondary overacting inferior oblique muscles: an ultrastructural study // Br J Ophthalm, 1984; 68: 416–420.
30. Mills S. E. Histology for pathologists. – 3rd ed. – Lippincott Williams & Wilkins, 2007. – Pp. 242–243.
31. Mitchell B. S., Schumacher U., Kaiserling E. Are tumours innervated? Immunohistological investigations using antibodies against the neuronal marker protein gene product 9.5 (PGP 9.5) in benign, malignant and experimental tumours // Tumour Biol, 1994; 15 (5): 269–274.
32. Miyazono K. Transforming growth factor-beta and its receptors // Nippon Yakurigaku Zasshi, 1996; 107 (3): 133–140.
33. Philippou A., Halapas A., Maridaki M., Koutsilieris M. Type I insulin-like growth factor receptor signaling in skeletal muscle regeneration and hypertrophy // Musculoskelet Neuronal Interact, 2007; 7 (3): 208–218.
34. Pihlmann M., Askou A. L., Aagaard L., et al. Adeno-associated virus-delivered polycistronic microRNA-clusters for knockdown of vascular endothelial growth factor in vivo // Gene Med, 2012; 14 (5): 328–338.
35. Pilmane M., Ozolina L., Abola Z., et al. Growth factors, their receptors, neuropeptide-containing innervation, and matrix metalloproteinases in the proximal and distal ends of the esophagus in children with esophageal atresia // Medicina (Kaunas), 2011; 47 (8): 453–460.
36. Punglia R. S., Lu M., Hsu J., et al. Regulation of vascular endothelial growth factor expression by insulin-like growth factor I // Diabetes, 1997; 46 (10): 1619–1626.
37. Rommel C., Bodine S. C., Clarke B. A., et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways // Nat Cell Biol, 2001; 3 (11): 1009–1013.
38. Saika S., Yamanaka O., Okada Y., et al. TGF beta in fibroproliferative diseases in the eye // Front Biosci, 2009; 1: 376–390.
39. Schiaffino S., Mammucari C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models // Skeletal Muscle, 2011; 1: 4.
40. Verrecchia F., Mauviel A. Transforming growth factor/beta and fibrosis // World J Gastroenterol, 2007; 13 (22): 3056–3062.
41. Willoughby C. L., Christiansen S. P., Mustari M. J., McLoon L. K. Effects of the sustained release of IGF-1 on extraocular muscle of the infant non-human primate: adaptations at the effector organ level // Invest Ophthalmol Vis Sci, 2012; 53 (1): 68–75.

Investigation of B-cell Phenotypes in Epstein–Barr Virus Infected Burkitt's Lymphoma and Lymphoblastoid Cell Lines

Irina Spaka [1, 2], *Svetlana Kozireva* [1], *Jevgenija Osmjana* [1], *Artjoms Spaks* [3], *Ilona Sasoveca* [1], *Elena Kashuba* [4], *Irina Kholodnyuk* [1]

[1] *Rīga Stradiņš University, A. Kirchenstein Institute of Microbiology and Virology, Latvia*
[2] *Rīga Stradiņš University, Doctoral Studies in Medicine, Latvia*
[3] *Pauls Stradiņš Clinical University Hospital, Department of Thoracic Oncology, Latvia*
[4] *Karolinska Institutet, Department of Microbiology, Tumor and Cell Biology (MTC), Sweden*

Abstract

Epstein–Barr virus (EBV) is implicated in the pathogenesis of the endemic Burkitt's lymphoma (BL). BL is listed in the World Health Organization (WHO) classification of lymphoid tumors as an "aggressive B-cell non-Hodgkin's lymphoma", characterized by a high degree of proliferation of the malignant cells and deregulation of the c-MYC gene [Bellan, et al., 2010]. BL-derived cell lines are commonly used as model systems to study biology and evolution of cancer [reviewed in Klein, et al., 1985; Klein, et al., 2007]. The EBV-positive BL-derived cell lines initially maintain the original tumor phenotype of EBV infection (latency I, Lat I), but most of them drift toward a lymphoblast phenotype of EBV latency III (Lat III) during *in vitro* culturing. The aim of the present work was to characterize B-cell subsets in EBV-positive BL and lymphoblastoid (LCL) cell lines and to verify whether a particular cell subset correlates with the type of EBV infection. The phenotype analysis of three EBV-negative and nine EBV-positive (four of Lat I and five of Lat III) BL cell lines and five EBV-positive of Lat III LCL cell lines was performed by polychromatic flow cytomery, based on the expression pattern of CD19, CD10, CD38, CD27, and CD5 markers. Two cell subsets – $CD19^+CD10^+$ and $CD19^+CD10^-$ – were defined in Lat III BL cell lines.

The respective research lead to the following conclusion that EBV infection of the latency III is associated with the $CD10^-$ phenotype of B cells as in Burkitt's lymphoma as in lymphoblastoid cell lines.

Keywords: Burkitt's lymphoma (BL) cell lines, B-cell subsets, Epstein–Barr virus (EBV), EBV latency, phenotype profiles.

Introduction

Burkitt's lymphoma (BL) is an aggressive B-cell lymphoma, characterized by a high degree of proliferation of malignant cells and deregulation of the c-MYC gene. BL can be classified into three forms, which differ in geographic distribution and Epstein–Barr virus (EBV) association: endemic (eBL), sporadic (sBL) and HIV-associated BL. There is a low background incidence of BL worldwide (sBL), which is rarely associated with EBV and accounts for 1–2% of adult lymphoma in Western Europe and America, but eBL is associated with EBV in over 95% of cases and is predominant in the equatorial Africa and other parts of the world where malaria is hyperendemic. Endemic EBV-associated BL has an incidence of

5–10/100 000 children and accounts for up to 74% of childhood malignancies in the equatorial African [van den Bosch, 2004; Brady, et al., 2007; Bellan, et al., 2010]. Detection of somatic mutations in the rearranged immunoglobulin variable region genes suggested that both sporadic and endemic BL represent a B-cell malignancy originating from germinal center (GC) B-cells or their descendants [Klein, et al., 1995; Klein, 2009]. BL tumor cells usually express IgM, B-cell markers such as CD19, CD20 and CD22, and markers of GC centroblasts, such as CD10, BCL6 and the GC-expressed protein GCSAM [Brady, et al., 2007].

EBV is implicated in pathogenesis of endemic BL and post-transplant lymphomas in immunosuppressed individuals [Young, et al., 2004; Klein, et al., 2010]. The EBV-carrying posttransplant and AIDS-associated lymphoma cells express the full set of EBV latent proteins (the latency III form of infection, Lat III): six EBV nuclear antigens (EBNA 1–6) and three latent membrane proteins (LMP1, 2A, and 2B). The vast majority of EBV-positive BL tumors display a restricted latency I (Lat I express only EBNA-1) form of infection. EBV has the unique ability to transform normal resting B-lymphocytes *in vitro* into permanent, latently infected lymphoblastoid cells, in which every cell constitutively expresses viral latent proteins, six EBNAs and three LMPs (Lat III) [Young L. et al., 2004; Klein E. et al., 2007].

With rare exceptions, all humans harbor the EBV. EBV shows a high degree of B-cell tropism. It binds to a B lymphocyte specific surface molecule, CD21 (receptor for the C3d fragment of complement) [Rickinson, 2001]. The EBV infection leads to B-cell activation, inducing the interferon pathway, cell surface adhesion molecules and receptors (such as CD23, CD40, and CD38), and chemokines (CXCL8/IL8, CXCL10/IP10, CCL5/RANTES, CCL17/TARC, and CCL22/MDC) [Nicholas, 2005]. The upregulation of CCR6 and CCR10 was observed in B-cells immortalized with EBV *in vitro* [Nakayama, et al., 2002]; the increase of mRNA expression for CCR2 and CCR9 was detected in tonsil B-cells upon EBV infection [Ehlin-Henriksson, et al., 2009]. One proposed model suggests that the infected B-cell, in the establishment of EBV latency *in vivo*, follows the same path as the antigen-activated B-cell, which proliferates, enters the follicle and expands to form a germinal centre [Thorley-Lawson, 2001]. Latently infected virus-carrying B-cells in healthy individuals are found in the peripheral blood (PB) resting, memory compartment in a silent state [Chen, et al., 1995; Babcock, et al., 1998].

The expression of EBV-encoded proteins differs depending on the type, differentiation and activation status of the target cell. The B-cell growth transformation program is based on the expression of Lat III six nuclear and three membrane proteins. Infected cells can enter into the mitotic cycle, leading to the emergence of proliferating lines. BL-derived cell lines are commonly used as model systems to study the biology of cancer [Klein, et al., 2007]. It is known that EBV-positive BL cell lines initially maintain the original tumor phenotype of EBV Lat I, but most of them drift toward a lymphoblastoid phenotype during *in vitro* culturing and express all nine EBV latent proteins (Lat III) [Rowe, et al., 1987; Rickinson, et al., 1996].

Flow cytometry is a technique allowing rapid and qualitative analysis of multiple characteristics of a single cell [Carter, et al., 2000]. One of the fields, where flow cytometry analysis is extensively employed, is diagnostics of hematologic neoplasms.

Recently, a comprehensive polychromatic flow cytometry (pFC) study of 106 healthy adults [Caraux, et al., 2010] and a population-based cohort study of 600 healthy adults [Perez-Andres, et al., 2010] has shown that CD19, CD10, CD27 and CD38 is the minimal combination of subsetting markers, which allows unequivocal identification of immature ($CD10^+$, $CD38^+$, $CD27^-$), naïve ($CD10^-$, $CD38^-/^+$, $CD27^-$), memory B lymphocytes ($CD10^-$, $CD38^-/^+$, $CD27^+$), and plasma cells ($CD10^-$, $CD38^{++}$, $CD27^{++}$) within PB circulating $CD19^+$ cells. In this study, this combination of CD markers for the immunophenotyping of BL cell lines to separate two main B lymphocyte populations – immature and mature – has been applied.

The aim

The aim of the research was to characterize by pFC the phenotypes of cells in EBV-positive BL cell lines and EBV-positive lymphoblastoid (LCL) cell lines in order to verify whether a particular cell subset correlates with the type of EBV infection (Lat I and Lat III).

Material and methods

Cell cultures. B-cell lines that were analyzed in the study are listed in Table 1. The study includes nine EBV-positive and three EBV-negative BL cell lines and five EBV-positive LCL cell lines. The original characteristics of the cell lines have been previously described and verified [Lenoir, et al., 1985; Pokrovskaja, et al., 1996; Maeda, et al., 2001].

Cell lines analysis by polychromatic flow cytometry. Cells of BL lines were immunofluorescently stained for cell surface markers using the standard protocol "Cell Surface Staining of Human PBMCs and Suspension Cell Lines" (BD Biosciences, San Jose, CA, USA). Fluorochrome-conjugated mouse anti-human monoclonal antibodies (mAbs) CD19–PE-Cy5 (clone HIB19) or CD19–PerCP-Cy5.5 (clone HIB19), CD10–PE (clone HI10a), CD38–Horizon V450 (clone HB8), CD27–FITC (clone M-T271), and CD5–PE-Cy7 (clone L17F12), and nonspecific mouse IgG1 and IgG2b, matching the respective fluorochrome and Ig isotype of CD19, CD10, CD38, CD27, and CD5, were purchased from BD Biosciences. Autofluorescent cells were excluded from each analysis in 2 ways: using unstained cells and using an empty fluorescence channel in each staining experiment. Five-color polychromatic flow cytometry (pFC) was performed, cells were scored using a FACSAriaII analyzer equipped with 3 lasers (BD; Becton, Dickinson and Company, NJ, USA), and the data were processed using FACS Diva 6.2 software (BD).

Negative controls included substitution of the relevant mAb with a murine Ig of the same isotype and showed no significant positivity. In the fluorescence-activated cell analysis, 3000–9000 events were acquired. The threshold line was based on the staining obtained with irrelevant isotype-matched mAb; the cell line was defined as negative if < 3% of cells stained positive with mAbs.

RT-PCR analysis. Reverse transcription RT-duplex-PCR analysis was carried out as reported by us previously; duplex-PCR was performed using two pairs of primers simultaneously (30 cycles, 55 °C of the annealing) with the one pair of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) [Kholodnyuk, et al., 2006]. The primers used to define the EBV EBNA1 transcripts derived from Qp and from Wp/Cp promoters were 5›-gtgcgctaccggatggcg-3› (Q), 5›-cgtgtgacgtggtgtaaagt-3› (Y), and 5›-ggtctccggacaccatctct-3› (K1) [Maeda, et al., 2001]. The EBNA2 primers that recognize the EBV type-1 sequences and the LMP1 primers were described by Tierney, et al. in 1994.

Table 1. The list of analyzed Burkitt's lymphoma and lymphoblastoid cell lines

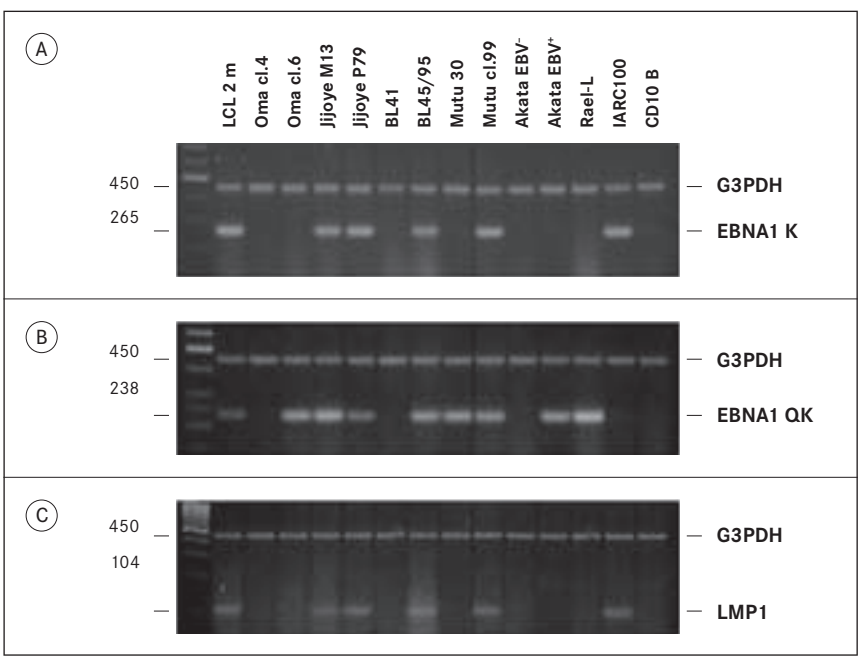
Cell line	EBV latency	Origin
Originally EBV-negative		
Mutu cl.30	EBV^-	BL
Oma cl.4	EBV^-	BL
BL 41	EBV^-	BL
EBV-positive		
Oma cl.6	Lat I	BL
$Akata^+$	Lat I	BL
Mutu cl.148	Lat I	BL
Jijoye M13	Lat I	BL
Raji	Lat III	BL
Akuba	Lat III	BL
BL16	Lat III	BL
BL41/95	Lat III	BL
BL41/95	Lat III	BL
LCL 05-18	Lat III	LCL
LCL 07-08	Lat III	LCL
IARC99	Lat III	LCL
IARC171	Lat III	LCL
IARC174	Lat III	LCL

Results

We analyzed B cell phenotypes in twelve BL cell lines, three EBV-negative and nine EBV-positive (three of Lat I and six of Lat III) and five LCL cell lines (all EBV-positive of Lat III) by pFC. The cell lines are well characterized by the EBV latency form of infection. Six BL cell lines in the study were established by a single cell cloning of the original EBV-positive BL-tumor derived cell lines: Oma cl.4 (EBV-negative) and Oma cl.6 (Lat I); Mutu cl.30 (EBV-negative), Mutu cl.148 (Lat I) and Mutu cl.99 (Lat III); Jijoye M13 (Lat I/III) [Pokrovskaja, et al., 1996]. We confirmed the presence of EBV and the latency type in all cell lines, using RT-duplex-PCR of EBV genes, EBNA1 (derived from Qp and from Wp/Cp promoters) and LMP1. The results are represented in Figure 1.

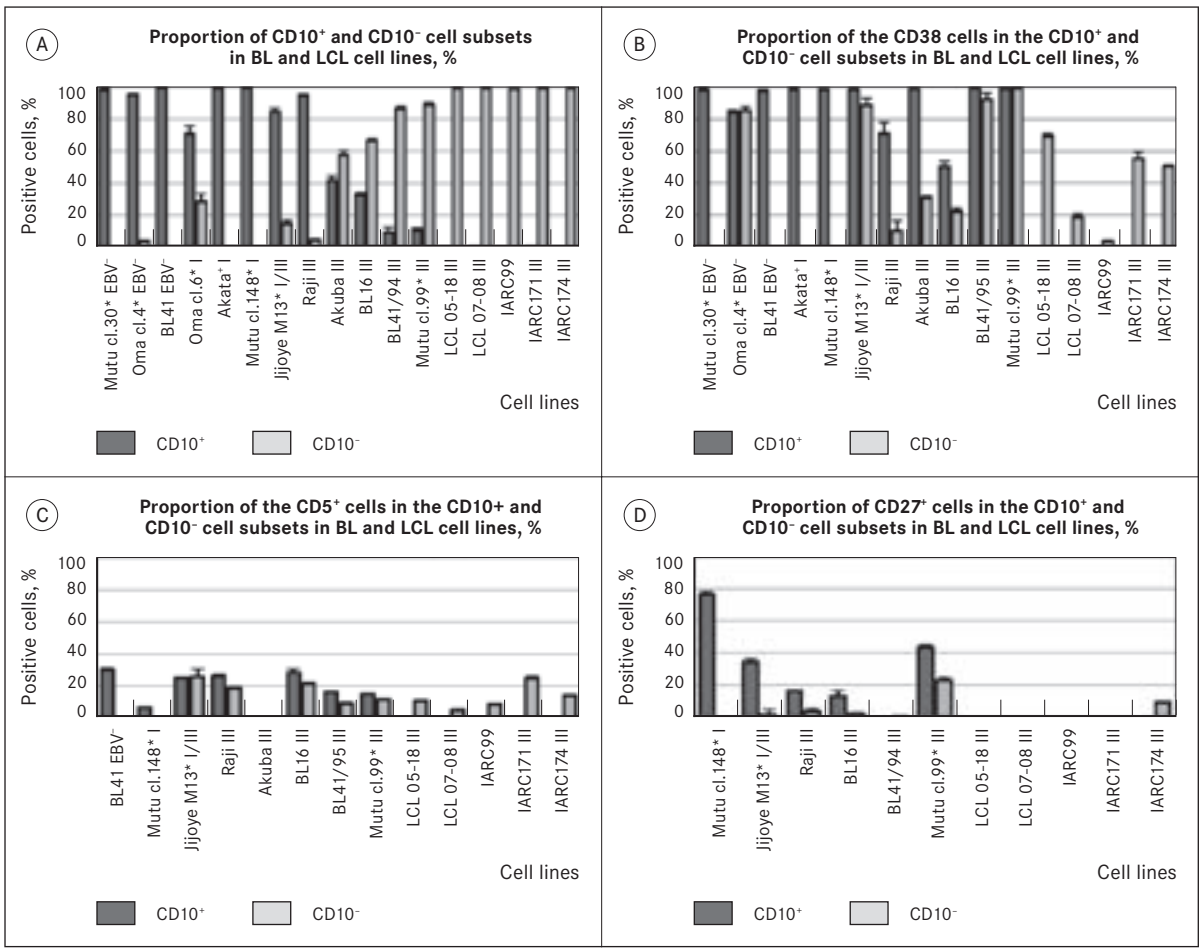
For a phenotype profiling of BL and LCL cell lines, analysis of cell surface expression of CD19, CD10 and CD38; CD27 and CD5 assayed in the Lat III BL cell lines and LCL cell lines was carried out. Presence of CD19 and CD10 was determined to characterize the major subsets of the cell lines: $CD19^+CD10^+$ and $CD19^+CD10^-$. The CD38 cell surface expression was examined, because of CD38 involvement in the B-cell activation. The results are presented in Figure 2. In EBV-negative and Lat I BL cell lines, all cells showed the $CD19^+CD10^+CD38^+$ phenotype (more than 84%). Only a small fraction of the $CD19^+CD10^-$ cells (about 4%) was found in EBV-negative cell line Oma cl.4. In contrast, more than 90% of the cells were $CD19^+CD10^-$ $CD38^+$ in two Lat III cell lines: Mutu cl.99 that was established by a single cell cloning, and BL41/95 that was generated by the EBV (B95-8) infection of EBV-negative BL41 cells (Figure 2). The rest of the cells were $CD19^+CD10^+CD38^+$. In all BL cell lines, the $CD19^+CD10^+$ cells expressed CD38. There were also five $CD10^-$ negative LCL cell lines: LCL 05-18, LCL 07-08, IARC 99, IARC 171, IARC 174 (all EBV-positive of Lat III). Surprisingly, in two of these EBV-positive CD10-negative LCL cell lines (LCL 07-08 and IARC 99) the cell surface expression of CD38 was less than 20%, but in other three – above 50%.

Figure 1. RT-duplex-PCR analysis of EBV latent genes transcripts in Burkitt's lymphoma cell lines and lymphoblastoid cell lines (A, B, C)*



* The EBNA1 and LMP1 RT-PCR products in the representative cell lines. The EBNA1 transcripts derived from Qp and from Wp/Cp promoters, respectively, are referred to as QK and YK splices. G3PDH, the PCR product of the glyceraldehyde-3-phosphate dehydrogenase gene; LCL, the Lat III LCL cell lines; BL41 and Rael, the Lat I BL cell lines used as the controls. Duplex-PCR was conducted using two pairs of primers simultaneously. Representative data from two independent experiments are shown.

Figure 2. B cell phenotype profiles in Burkitt's lymphoma and lymphoblastoid cell lines. Bars show the percentage of positive cells



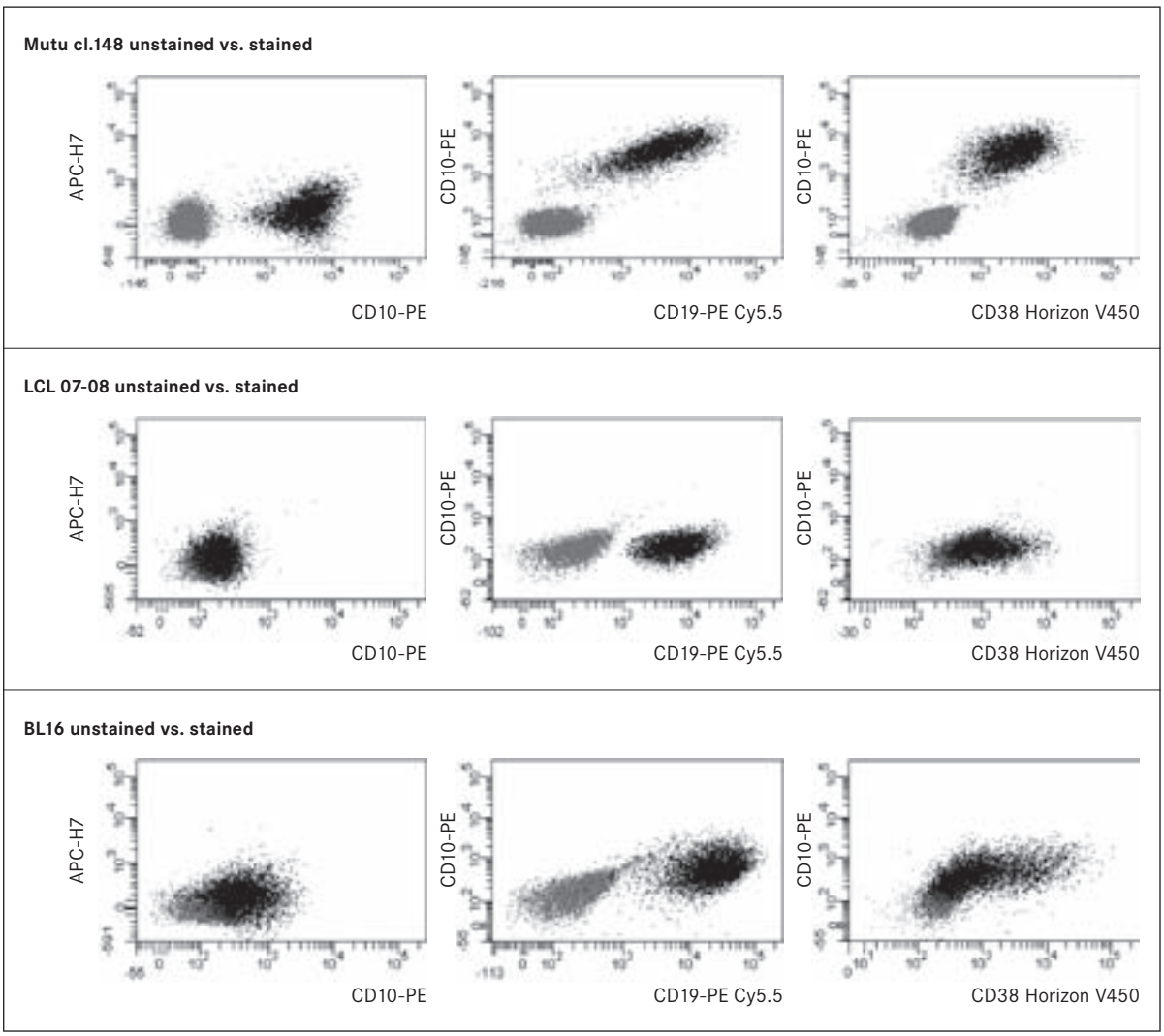
The CD38, CD27, and CD5 stained cells were defined in 2 cell subsets, $CD19^+CD10^+$ (black bars) and $CD19^+CD10^-$ (grey bars) for each BL cell line and LCL cell line.

* Not applicable, the EBV^+ and EBV^- cell lines were established by a single cell cloning of the original EBV^+ BL-derived cell lines; Mutu cl.30$^-$ and Oma cl.4$^-$, the EBV^- BL cell lines. BL41/95 was generated by infection of the EBV^- BL41 cell line cells with B95-8 EBV; Jijoye M13 I/III, BL cell line of EBV Lat I originally that acquired the phenotype of EBV Lat III during the *in vitro* culturing: the mRNA expression of EBV latent genes LMP1 and EBNA1 Qp was detected (Figure 1); LCL 05-18, the LCL cell line generated by B95-8 EBV infection of B-cells from PB of EBV^- healthy adult donors. The percentage of cells expressing the respective B-cell surface CD marker is averaged over both experiments with mean ± SD shown. The percentage of positive cells based on quadrant encompassing 3% of isotype control-stained cells in dot blots (0 < 3% of cells). Data are representative of two independent experiments with two tubes per experiment.

BL cell lines of Lat III, i.e. Jijoye M13, Raji, BL18, Akuba, BL16, BL 41/95, and Mutu cl.99 consisted of the $CD19^+CD10^+$ and $CD19^+CD10^-$ cell subsets in proportion –2.6–95.0% and 5.6–97.5% of the cells, respectively (Figure 2). The $CD19^+CD10^-$ cells expressed the cell surface CD38 in twelve out of the sixteen cell lines that contained the $CD19^+CD10^-$ cell subset.

It is noteworthy that CD27, the marker of B-cell maturation, was expressed on the $CD19^+CD10^+$ cells in four Lat III BL cell lines. Moreover, in one Lat III BL cell line (Mutu cl.99), the CD27 cell surface expression was detected in 19.5% of the $CD19^+CD10^-$ cells. But in one BL Lat I cell line (Mutu cl.148) the expression of CD27 was found in 77.1% of the $CD19^+CD10^+$ cells (Figure 2). In only one LCL cell line IARC 174, the expressed CD27 protein was found in 9.8% of the cells.

Figure 3. Flow cytometry analysis of two BL derived cell lines and one LCL cell line*



The CD5 marker was analyzed in eight out of twelve BL cell lines: one of Lat I (Mutu cl.148), one EBV-negative (BL41) and six of Lat III (with an exception of Akuba cell line); and in all LCL cell lines. The cell surface expression was revealed in both $CD10^+$ and $CD10^-$ B-cell subsets. However, in $CD19^+CD10^+$ cell lines Jijoye M13, Raji and BL 16, the expression of CD5 was higher than in $CD19^+CD10^+$ cell lines Mutu cl.148, BL 41.95 and Mutu cl.99: in the range of 25.6–30.7% and 7.3–17.1%, respectively. For $CD19^+CD10^-$ EBV-positive Lat III LCL cell lines the low expression under 15% was revealed in four out of the five cell lines.

Discussion

Continuous cell lines represent potent objects for the investigation of genetic and epigenetic changes and identification of genes that play a role in the pathogenesis of the most aggressive and intractable human malignancies. It has been shown that the spectrum of aberrations in each cell line correlated with the spectrum of aberrations seen in the corresponding primary tumor, suggesting that cell lines represent a faithful model of cancer [Roschke, et al., 2003; Karpova, et al., 2006].

Epstein–Barr virus (EBV), a ubiquitous B-lymphotropic human gamma-herpesvirus, infects nearly all human populations. Moreover, a great majority of adults produce antibodies to the virus. EBV in a form of episome persists in peripheral blood memory B cells [Young, et al., 2004; Klein, et al., 2007].

It was demonstrated that EBV can establish infection even in the absence of a classical GC memory B-cell population, in the small proportion of GC-independent memory B cells (IgD^+, $CD27^+$) [Conacher, et al., 2005].

It is important that EBV has the unique ability to transform resting B-cells into permanent, latently infected lymphoblastoid cell lines (LCLs), an *in vitro* model of the lymphomagenic potential of the virus. In EBV-transformed LCLs, every cell carries multiple extrachromosomal copies of the viral episome and constitutively expresses a limited set of viral gene products, the so-called latent proteins, which comprise six EBV nuclear antigens (EBNAs 1–6) and three latent membrane proteins (LMPs 1, 2A and 2B). The pattern of latent EBV gene expression, which appears to be activated only in B-cell infections, is referred to as "latency III" (Lat III). EBNA1 is expressed in all virus-infected cells, in which its role in the maintenance and replication of the episomal EBV genome is achieved through sequence-specific binding to the plasmid origin of viral replication, OriP. EBNA1 can also interact with certain viral promoters, thereby contributing to the transcriptional regulation of the EBNAs (including EBNA1 itself) and of LMP1 [reviewed in Rickinson, 2001; Young, Rickinson, 2004].

LMP1 is the main transforming protein of EBV; it functions as a classic oncogene in rodent-fibroblast transformation assays and is essential for EBV-induced B-cell transformation *in vitro* [Kaye, et al., 1993]. LMP1 has pleiotropic effects when it is expressed in cells, resulting in the induction of cell-surface adhesion molecules and activation antigens [Wang, et al., 1990], and upregulation of anti-apoptotic proteins (for example, BCL2 and A20) [Eliopoulos, et al., 1999]. The ability of EBNA2, EBNA3C and LMP1 to induce LCL-like phenotypic changes when expressed individually in human B-cell lines indicates these viral proteins as key effectors of the immortalization process [Wang, et al., 1990]. The role of EBV latent genes in the *in vitro* transformation of B-cells has been confirmed more recently by the generation of recombinant forms of EBV that lack individual latent genes. Studies using these viruses have confirmed the absolute requirement for EBNA2 and LMP1 in the transformation process [reviewed in Young, Rickinson, 2004].

Conclusions

The phenotype analysis of cells in EBV-negative and EBV-positive (both of Lat I and Lat III) Burkitt's lymphoma derived cell lines defined two major cell subsets present in BL Lat III cell lines – $CD19^+CD10^+$ and $CD19^+CD10^-$. In both subsets, the CD27 and the CD5 cell surface expression was detected in a proportion of the cells only. Moreover, the Epstein–Barr virus infection of the latency III was associated with the $CD10^-$ negative phenotype of B-cells as in Burkitt's lymphoma as in lymphoblastoid cell lines.

The biological characterization of B-cell lymphoma cell subclones could facilitate the definition of high-risk patients, the prediction of response to therapy and the selection of most appropriate therapy strategies.

Acknowledgements

This research was supported by the European Social Fund grant Nr. 2009/0221/1DP/1.1.1.2.0/09/APIA/VIAA/074, Latvia (SK, JO, AS, IS, IKH), by Latvian Council of Science (LZP) grant Nr. 09.1392 (IS, IKH), and in part by The Swedish Institute, Stockholm, Sweden (SK, EK, IKH).

References

1. Bellan C., Stefano L., Giulia de F., Rogena E. A., et al. Burkitt lymphoma versus diffuse large B-cell lymphoma: a practical approach // Hematol Oncol, 2010; 28 (2): 53–56.
2. Brady G., MacArthur G. J., Farrell P. J. Epstein–Barr virus and Burkitt lymphoma // J Clin Pathol, 2007; 60 (12): 1397–1402.
3. Caraux A., Klein B., Paiva B., Bret C., et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138– and CD138+ plasma cells // Haematologica, 2010; 95: 1016–1020.

4. Carter N. P., Ormerod M. G. Introduction to the principles of flow cytometry // Flow Cytometry – 3rd ed. – Oxford University Press – 2000. – 275 p.
5. Conacher M., Callard R., McAulay K., Chapel H., et al. Epstein–Barr virus can establish infection in the absence of a classical memory B-cell population // J Virol, 2005; 79: 11128–11134.
6. Ehlin-Henriksson B., Liang W., Cagigi A., Mowafi F., et al. Changes in chemokines and chemokine receptor expression on tonsillar B-cells upon Epstein–Barr virus infection // Immunology, 2009; 127: 549–557.
7. Eliopoulos A. G., Gallagher N. J., Blake S. M., Dawson C. W., et al. Activation of the p38 mitogen-activated protein kinase pathway by Epstein–Barr virus-encoded latent membrane protein 1 coregulates interleukin-6 and interleukin-8 production // J Biol Chem, 1999; 274: 16085–16096.
8. Karpova M. B., Schoumans J., et al. Combined spectral karyotyping, comparative genomic hybridization, and in vitro apoptyping of a panel of Burkitt's lymphoma-derived B-cell lines reveals an unexpected complexity of chromosomal aberrations and a recurrence of specific abnormalities in chemoresistant cell lines // International Journal of Oncology, 2006; 28 (3): 605–617.
9. Kaye K. M., Izumi K. M., Kieff E. Epstein–Barr virus latent membrane protein 1 is essential for B-lymphocyte growth transformation // Proc Natl Acad Sci USA, 1993; 90: 9150–9154.
10. Kholodnyuk I. D., Kozireva S., Kost-Alimova M., Kashuba V., et al. Down regulation of 3p genes, LTF, SLC38A3 and DRR1, upon growth of human chromosome 3-mouse fibrosarcoma hybrids in severe combined immunodeficiency mice // Int J Cancer, 2006; 119: 99–107.
11. Klein U., Klein G., Ehlin-Henriksson B., Rajewsky K., et al. Burkitt's lymphoma is a malignancy of mature B-cells expressing somatically mutated V region genes // Mol Med, 1995; 1: 495–505.
12. Klein U., Rajewsky K., Küppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B-cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B-cells // J Exp Med, 1998; 188: 1679–1689.
13. Klein E., Kis L. L., Klein G. Epstein–Barr virus infection in humans: from harmless to life endangering virus-lymphocyte interactions // Oncogene, 2007; 26: 1297–1305.
14. Klein G. Burkitt lymphoma – a stalking horse for cancer research // Semin Cancer Biol, 2009; 19 (6): 347–350.
15. Klein G., Klein E., Kashuba E. Interaction of Epstein–Barr virus (EBV) with human B-lymphocytes // Biochem Biophys Res Commun, 2010; 396: 67–73.
16. Lenoir G. M., Vuillaume M., et al. The use of lymphomatous and lymphoblastoid cell lines in the study of Burkitt's lymphoma // IARC Scientific Publications, 1985; 60: 309–318.
17. Maeda A., Kiss C., Chen F., Ehlin-Henriksson B., et al. EBNA promoter usage in EBV-negative Burkitt lymphoma cell lines converted with a neomycin-resistant EBV strain // Int J Cancer, 2001; 93: 714–719.
18. Nakayama T., Fujisawa R., Izawa D., Hieshima K., et al. Human B-cells immortalized with Epstein–Barr virus upregulate CCR6 and CCR10 and downregulate CXCR4 and CXCR5 // J Virol, 2002; 76: 3072–3077.
19. Nicholas J. Human gammaherpesvirus cytokines and chemokine receptors // J Interferon Cytokine Res, 2005; 25: 373–383.
20. Perez-Andres M., Paiva B., Nieto W. G., Caraux A., et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic // Cytometry B Clin Cytom, 2010; 78 (1): S47–S60.
21. Pokrovskaja K., Ehlin-Henriksson B., Bartkova J., Bartek J., et al. Phenotype-related differences in the expression of D-type cyclins in human B-cell-derived lines // Cell Growth Differ, 1996; 7: 1723–1732.
22. Rickinson A. B., Lee S. P., Steven N. M. Cytotoxic T lymphocyte responses to Epstein–Barr virus // Curr Opin Immunol, 1996; 8 (4): 492–497.
23. Rickinson A. B. Fields Virology. – 4th ed. – Philadelphia: Lippincott Williams & Wilkins, 2001.
24. Roschke A. V., Tonon G., et al. Karyotypic complexity of the NCI-60 drug-screening panel // Cancer Research, 2003; 63: 8634–8647.
25. Rowe M., Rowe D. T., Gregory C. D., Young L. S., et al. Differences in B-cell growth phenotype reflects novel patterns of Epstein–Barr virus latent gene expression in Burkitt's lymphoma cells // EMBO J, 1987; 6: 2743–2751.
26. Tierney R. J., Steven N., Young L. S., Rickinson A. B., et al. Epstein–Barr virus latency in blood mononuclear cells: analysis of viral gene transcription during primary infection and in the carrier state // J Virol, 1994; 68: 7374–7385.
27. Thorley-Lawson D. A. Epstein–Barr virus: exploiting the immune system // Nat Rev Immunol, 2001; 1: 75–82.
28. Van den Bosch C. A. Is endemic Burkitt's lymphoma an alliance between three infections and a tumor promoter? // Lancet Oncol, 2004: 5738–5746.
29. Wang F., Gregory C. D., Rowe M., Rickinson A. B., et al. Epstein–Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23 // J Virol, 1990; 64: 2309–2318.
30. Young L. S., Rickinson A. B. Epstein–Barr virus: 40 years on // Nat Rev Cancer, 2004; 4: 757–768.

Association of Age with Sexual Dysfunctions in Gynecological Patients of Reproductive Age

Ieva Briedite, Gunta Ancane [1], *Irena Rogovska* [2]

Rīga Eastern Clinical University Hospital "Gailezers", Department of Gynecology, Latvia
Rīga Stradiņš University, Department of Obstetrics and Gynecology, Latvia
[1] *Rīga Stradiņš University, Department and Clinic of Psychosomatic Medicine and Psychotherapy, Latvia*
[2] *Rīga Stradiņš University, Division of Doctoral Studies, Latvia*

Abstract

Sexuality is an integral part of human expressions regardless of age. Female sexual dysfunction is highly prevalent, occurring in 25–63% of women of all ages, but the prevalence tends to increase with age. Since sexual dysfunction affects a significant number of female patients, it could be diagnosed during annual physical examination or gynecology related visits; however, studies indicate that doctors are unlikely to ask about their patient's sexual function or offer appropriate advice, and that people tend to be reluctant to seek medical help.

The aim of the study was to assess possible relation of the age of gynecological patients to the prevalence of various forms of female sexual dysfunction. *FSFQ28* patient inquiry form was used to evaluate eight main fields of female sexual function. As a result, it was concluded that, although rates of patients' sexual function in most of the domains decline with age, female sexual dysfunctions are widespread in all age groups. Health care providers should refrain from holding preconceived notions based on age, gender or any other factors that might lead them to assume that sex is not an important health issue for their patient.

Keywords: sexual dysfunctions, gynecology, female sexuality, aging and sexuality.

Introduction

Sexuality at all ages, including the older adult, is a vital aspect of life satisfaction [Woloski-Wruble, et al., 2010]. Female sexual function is multifaceted and complex, and depends on biological, psychological, and sociocultural contexts, as well as possible interactions between them [Valadares, et al., 2011]. Overall, the state of knowledge is less advanced regarding female sexual physiology in comparison with male sexual function. Female sexual dysfunction has received little clinical and basic research attention and remains a largely untapped field in medicine [Azadzoi, Siroky, 2010] although sexual health has been recognized as an integral part of overall health [WHO, 2004]. The pathophysiology of female sexual dysfunction appears more complex than that of males, involving multidimensional hormonal, neurological, vascular, psychological, and interpersonal aspects. Organic female sexual disorders may include a wide variety of vascular, neural, or neurovascular factors that lead to problems with libido, lubrication, and orgasm [Azadzoi, Siroky, 2010].

Sexual problems may be warning signs of as-yet-undiagnosed health problems such as vascular disease, diabetes, infections, or adverse effects of medication that interfere with sexual functioning [Langer-Most, Langer, 2010]. However, the precise etiology and mechanistic pathways of age-related female sexual arousal disorders are yet to be determined [Azadzoi, Siroky, 2010]. Endocrine changes during aging as well as endocrine disorders directly or indirectly may modulate sexual function by altering sex hormones, or by affecting vascular, neurogenic and/or psychological factors. Problems of sexual function of short duration may create frustration and anguish, and when chronic, may lead to anxiety and depression and may damage relationships or create problems in other areas of a patient's life [Wierman, et al., 2010]. In women, psychological factors (history of sexual abuse, depression, anxiety, obsessive-compulsive disorders), sociocultural issues (beliefs regarding sexual activity), and interpersonal issues (partner availability, partner function, relationship with partner, communication with partner) affect sexual function in all age groups. With aging, additional psychological stresses may come to the fore, particularly loss of fertility [Azadzoi, Siroky, 2010].

Gynecologic pathology, specifically cancer, is associated with significant impairment in physical and psychophysiological sexual arousal. These problems can lead to decreased self-esteem, depression, and relationship distress [Ratner, et al., 2011]. Diagnosis and treatment of female sexual dysfunction are currently based on subjective reporting by the woman and physical examination [Graziottin, et al., 2009]. Sexual health and physical health are often closely related. Gynecologists are both primary care providers and specialists. As primary care providers with a special focus and knowledge, they provide continuity of care often throughout the life cycle of the female patient for a wide variety of problems, while addressing preventative care and health maintenance [Langer-Most, Langer, 2010]. Sexual response is as much a function of the brain as it is of the genitals [Karatafl, et al., 2009]. Not a single existing speciality or discipline has been able to cover all aspects of sexual function and dysfunction [Wagner, 2005]. Structured, multidisciplinary and integrative approach is fundamental to the evaluation and management of female sexual dysfunctions [Graziottin, et al., 2009].

The number of women who seek medical treatment for sexual dysfunction is lower than those who actually have a sexual dysfunction [Feldhaus-Dahir, 2009]. Unfortunately, studies show low rates of sexual health assessment by primary care providers; many may underestimate patient risk, and/or lack of the knowledge and comfort to discuss questions about sexual dysfunction or satisfaction with their patients [Langer-Most, Langer, 2010]. Normal physiological changes with aging and other medical conditions, which increase with age and their treatments, have an impact on sexuality and the way it can be expressed [Yee, 2010]. Sexual problems are frequent among adults, but these problems are infrequently discussed with physicians [Langer-Most, Langer, 2010]. Significant barriers to seeking medical help for sexual difficulties and concerns have been identified. These have been shown to be connected to a number of factors, some of which relate to the patient (e.g., embarrassment), some to the physician (e.g., ageist attitudes), and others to the geographical or cultural location of the individual (e.g., difficulty in accessing services). Clearly, women experience age-related sexual difficulties [Hinchliff, Gott, 2011]. Sexual problems are often the first symptoms of a disease to such an extent, that many diseases and drug therapies can increase the prevalence of sexual problems [Lauman, Waite, 2008; Palacios, et al., 2009]. However, it is important to recognize that they can also encounter the same sexual problems that younger adults face (e.g., those related to relationship difficulties), which are not connected with aging [Hinchliff, Gott, 2011].

Gynecologic pathology is prevalent in older women. However, such pathology should not be the indicative of the demise of their sexual activities. Communication with a woman before any intervention should be thorough, and sexual issues should be always brought up [Ratner, et al., 2011]. By addressing issues of sexuality, a gynecologist informs a patient that it is appropriate to discuss sexual problems. Gynecologists play an instrumental role in assessing and managing normal and pathological aging changes to improve the sexual health of women and their partners by providing guidance, education, and acceptance [Langer-Most, Langer, 2010]. Woman's sex life does not have to end in old age. This is in part up to her, but also in large part related to her relationship status as well as her health status [Katz, 2012].

The aim

The aim of this study was to assess possible relation between the age of gynecological patients and prevalence of various forms of female sexual dysfunction, and to find out whether there is a particular age group with a higher prevalence of sexual dysfunctions.

Material and methods

The study population was directed to analyze different gynecological (except oncological) patients independently of reasons for being in gynecological clinic in order to see an average patient and to apply conclusions to ordinary gynecological care visits. A central wide-spectrum gynecology clinic, representing patients from all over the country, was chosen for this study. The study group consisted of gynecological patients from Gynecology Department of Rīga Eastern Clinical University Hospital "Gaiļezers". Inclusion criteria were: age 18–50 years, patients who had had sexual activity during the last four weeks, patients who voluntary agreed to participate in the study and were able to fill in the questionnaire. Exclusion criteria: age under 18 years, age above 50 years, patients who had not had sexual activity during the last four weeks, patients who did not agree to participate in the study. Age restriction 18 years was related to the study interest in adult sexuality, but age restriction 50 years was related to a highly possible and well-known impact of menopause on sexuality. The study lasted for six months, the participation in the study was offered to all patients of appropriate age.

Questionnaire method was used to survey patients in gynecological clinic. Standardized and validated *Female Sexual Function Questionnaire 28* [Quirk, et al., 2002; 2005] with 28 questions to calculate the function of the main domains of female sexuality (desire, arousal sensations, lubrication, cognitive excitement, orgasm, pain, satisfaction, partner) and to evaluate the level of sexual function of sexually active patients was used. The validity of the *Female Sexual Function Questionnaire 28* at both the item level and the domain level supports the use of individual domains as primary endpoints. The *Female Sexual Function Questionnaire 28* can detect both – the presence of sexual dysfunction and the specific components of sexual function affected, both – the physical and the cognitive aspects of sexual response are evaluated within the items, cut-scores for function of each domain have been generated.

In compliance with *Female Sexual Function Questionnaire 28* calculations, results of patient's sexual function for all parameters (desire, arousal sensations, lubrication, cognitive excitement, orgasm, pain, satisfaction, partner) were divided into three function levels – normal sexual function, borderline function and sexual dysfunction. Borderline means the tendency to and probability of sexual dysfunction, but additional information is required before making diagnosis. In the section about a partner, there is no part of sexual dysfunction. There is only normal function versus borderline, which indicates a possible sexual problem because of relationship. In total, 209 correctly completed questionnaires were obtained and used for data analysis. Comfortable conditions and privacy were provided, as well as enough time to complete the questionnaire accurately. Each questionnaire got a code and no private data were used. Prior to participation, patients were not screened to rule out any particular medical conditions or medications or any reasons for being hospitalized. The study was approved by the Ethics Committee of Rīga Stradiņš University. *Female Sexual Function Questionnaire 28* calculation and interpretation was performed in compliance with standardized scoring system.

Patients were divided in three age groups: 18–30 years (Group 1); 31–40 years (Group 2); 41–50 years (Group 3). The distribution of sexual function by the age group (and by domain) was assessed using rxc frequency tables. Statistical significance of differences was tested by Pearson chi-square (χ^2) test for categorical variables and by one-way analysis of variance with Bonferroni correction (in order to control overall type II error). The relationship between assessment score and age was assessed by means of regression analysis. P values less than 0.05 was chosen as a level of statistical significance.

Results

Patient distribution by age groups was 45.93% (n = 96) in Group 1, 33.01% (n = 69) in Group 2, 21.05% (n = 44) in Group 3. The analysis of the levels of sexual function of each domain in all age groups together showed that the percentage of patients reporting normal function was the highest in partner domain (88.04%), pain domain (80.86%), satisfaction domain (44.50%) and orgasm domain (33.97%), and the lowest rates were reported in lubrication domain (18.66%), arousal sensations and cognitive excitement domains (19.14% each), and desire domain (22.01%). The percentage of patients reporting borderline function was the highest in desire domain (51.20%), cognitive excitement domain (42.11%), lubrication domain (40.67%), satisfaction domain (40.19%), and the lowest in pain domain (12.92%), arousal sensations domain (34.45%) and orgasm domain (36.36%). The prevalence of sexual dysfunctions in all study groups is shown in Figure 1.

In the desire domain, the highest proportion of patients reporting normal function was observed in Group 1 (30.21%), and the lowest in Group 3 (13.64%). Reporting of borderline function decreased with ageing (56.25% in Group 1 vs. 53.62% in Group 2 vs. 36.36% in Group 3). Differences among age groups were statistically significant ($p < 0.01$). In the arousal sensation domain, reporting of normal function decreased with ageing (27.08% in Group 1 vs. 15.94% in Group 2 vs. 6.82% in Group 3). The same trend was observed also regarding reporting of borderline function (36.46% in Group 1 vs. 34.78% in Group 2 vs. 29.55% in Group 3). Differences among age groups were statistically significant ($p < 0.05$). In the lubrication domain, reporting of normal function was similar in Group 2 (11.59%) and Group 3 (11.36%) and it was lower in comparison to Group 1 (27.08%). The highest proportion of patients reporting borderline function was observed in Group 2 (46.38%); it was lower in Group 1 (42.71%) and Group 3 (27.27%). Differences among age groups were statistically significant ($p < 0.01$). In the domain of cognitive excitement, the proportion of normal functioning was the highest in Group 1 (28.13%) and lower in Group 2 (11.59%) and Group 3 (11.36%). The highest proportion of patients reporting borderline function was observed in Group 2 (47.83%), it was lower in Group 1 (46.88%) and the lowest in Group 3 (22.73%). Differences among age groups were statistically significant ($p < 0.01$). In the orgasm domain, reporting of normal function decreased with aging (40.63% in Group 1 vs. 30.43% in Group 2 vs. 25.00% in Group 3). The same trend was observed also regarding reporting of borderline function (40.63% in Group 1 vs. 37.68% in Group 2 vs. 25.00% in Group 3). Differences among age groups were statistically significant ($p < 0.01$). In the satisfaction domain, proportion of patients reporting normal function decreased with ageing (56.25% in Group 1 vs. 37.68% in Group 2 vs. 29.55% in Group 3). The highest proportion of patients reporting borderline function was observed in Group 2 (47.83%), it was lower in Group 1 (37.50%) and Group 3 (34.09%). Differences among age groups were statistically significant ($p < 0.01$). There were no statistically significant differences among age groups found in the analysis of sexual function in pain and partner domains. Figure 2 illustrates comparative levels of sexual dysfunction of each domain in all three age groups.

Figure 1. Prevalence of sexual dysfunctions in each domain, %

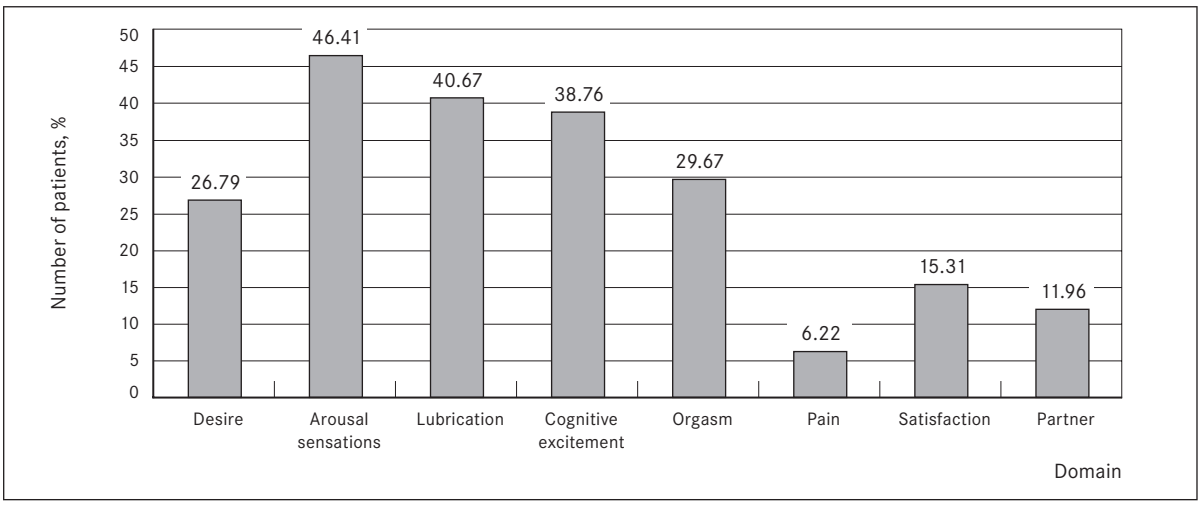
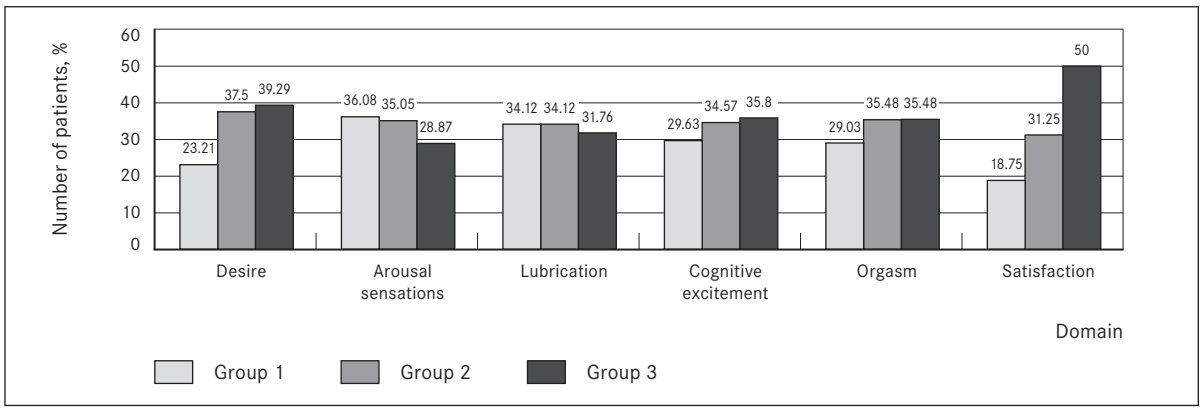


Figure 2. Distribution of sexual dysfunction of each domain in three age groups, %



The analysis of the changes in each separate domain of sexual function in context of increasing age showed gradual regression of function in several domains – desire, arousal sensations, lubrication, cognitive excitement, orgasm, satisfaction (Table 1). There was no statistically significant association between increasing age and function found in the analysis of pain and partner domains.

Table 1. Regression analysis of relationship between assessment scores of sexual function and age

Domain	Regression coefficient	Standard error	p value	95% Confidence interval
Desire	–0.1935873	0.0334008	< 0.01	–0.2594366 — –0.1277379
Arousal sensations	–0.0979765	0.024767	< 0.01	–0.1468045 — –0.0491485
Lubrication	–0.0562878	0.0148132	< 0.01	–0.0854919 — –0.0270838
Cognitive excitement	–0.0780445	0.0137022	< 0.01	–0.1050582 — –0.0510308
Orgasm	–0.1074342	0.0290351	< 0.01	–0.1646767 — –0.0501917
Satisfaction	–0.1725717	0.0328791	< 0.01	–0.2373925 — –0.1077509

The regression analysis of assessment scores in Group 2 and Group 3 versus Group 1 showed faster decline of assessment points in older age groups in comparison to the youngest age group. Major negative relationship between the age and sexual function was observed in domains of desire, satisfaction and orgasm.

Discussion

Medical and mental health providers need to be aware of the importance of sexuality in the population and the relationship to physical, emotional and social aspects of a woman's life [Ratner, et al., 2011]. Although theoretically gynecological disorders could possibly increase occurrence of sexual disorders, frequency of sexual dysfunctions among gynecological patients in this study in general corresponds with the prevalence in common population [Marnach, Casey, 2008; Shifren, et al., 2008; Zakhari, 2009], nonetheless showing a higher prevalence of arousal dysfunction in all three parameters describing arousal (arousal sensations, lubrication, cognitive excitement). This respective study indicated gradual regression of sexual assessment points in almost all domains characterizing female sexual function. Although sexual function in women declines with age [Ratner, et al., 2011], aging needs not necessarily cause asexuality, poor health, and loss of productivity [Azadzoi, Siroky, 2010]. Even having lower assessment points of sexual function, women still continue to have enjoyable sexual relationships in middle and later age. There was not statistically reliable negative association between increasing age and function found in the analysis of pain and partner domains. Probably, the population of gynecological patients in this case was the reason of irregular distribution of pain assessment, because some of the patients could have had some extra pain

because of having some gynecological problem lately, not because of their age. Moreover, partner problems potentially are more associated with social, emotional and psychological factors of the particular couple, not with the age of a person. The major regression of assessment points of the desire with increasing age could be explained with gradual decline of testosterone levels in women after 20 years until menopause.

Sexual activity may change with normal aging but it is still within the context of sexual health. There is clear evidence in literature and research [Merkatz, 2002; Zakhari, 2009; Sobczak, 2009] that sexual health is a part of overall health. Consequently, it cannot be ignored in the system of education or system of health care and daily health care visits. Empowering women to find sexual expression and sexual satisfaction is part of health promotion. Sexual satisfaction is correlated with life satisfaction, which is an important contribution to achieving successful aging [Woloski-Wruble, et al., 2010]. This study did not show any particular age group as a special target group for screening of sexual dysfunctions. Some domains showed lower assessment points in younger patients (arousal sensations, lubrication), but other domains showed lower assessment points in older patients (desire, orgasm, satisfaction).

Wide interpretation of the study results and generalization to all population of gynecological patients is restricted due to a relatively small study group, but it gives an opportunity to see and analyze tendencies and actualize the problem. Intrinsic disadvantage of questionnaire method is a subjective conception of questions, remembrance failures, because questions covered four weeks of the past time period, as well as impossible verification or particularization of answers.

Overall, this study gives the evidence that sexual dysfunction is a significant problem of gynecologic patients in all age groups. Gynecologists' discussion of sexuality with all their patients would improve patient education and counseling as well as the ability to clinically identify a highly prevalent spectrum of health-related and potentially treatable sexual problems [Langer-Most, Langer, 2010].

Conclusions

Although rates of patients' sexual function in most of the domains decline with age, female sexual dysfunction is widespread in all age groups. All female patients, irrespective of age, should be screened for sexual disorders during health care visits.

References

1. Azadzoi K. M., Siroky M. B. Neurologic factors in female sexual function and dysfunction // Korean Journal of Urology, 2010; 51: 443–449.
2. Feldhaus-Dahir M. Female sexual dysfunction: barriers to treatment // International Journal of Urological Nursing, 2009; 29 (2): 81–86.
3. Graziottin A., Serafini A., Palacios S. Aetiology, diagnostic algorithms and prognosis of female sexual dysfunction // Maturitas, 2009; 63 (2): 128–134.
4. Hinchliff S., Gott M. Seeking medical help for sexual concerns in mid- and later life: a review of the literature // Journal of Sex Research, 2011; 48 (2–3): 106–117.
5. Karatafl O. F., Ozcan M. E., Gümüfl E.I., et al. The impact of performance status on sexual functions in healthy young women // Bulletin of Clinical Psychopharmacology, 2009; 19 (1): 119–120.
6. Katz A. Sex, health and aging // Nursing for Women's Health, 2012; 15 (6): 519–521.
7. Langer-Most O., Langer N. Aging and sexuality: how much do gynecologists know and care? // Journal of Women & Aging, 2010; 22: 283–289.
8. Laumann E. O., Waite L. J. Sexual dysfunction among older adults: prevalence and risk factors from a nationally representative U.S. probability sample of men and women 57–85 years of age // The Journal of Sexual Medicine, 2008; 5 (10): 2300–2311.
9. Marnach M. L., Casey P. M. Understanding women's sexual health: a case-based approach // Mayo Clinic Proceedings, 2008; 83 (12): 1382–1387.
10. Merkatz R. B. Female sexual dysfunction // Journal of Women's Health & Gender-based Medicine, 2002; 11 (4): 331–333.

11. Palacios S., Castanoa R., Grazziotin A. Epidemiology of female sexual dysfunction // Maturitas, 2009; 63: 119–123.
12. Quirk F. H., Haughie S., Symonds T. The use of the sexual function questionnaire as a screening tool for women with sexual dysfunction // The Journal of Sexual Medicine, 2005; 2 (4): 469–477.
13. Quirk F. H., Heiman J. R., Rosen R. C., et al. Development of a sexual function questionnaire for clinical trials of female sexual dysfunction // Journal of Women's Health & Gender-based Medicine, 2002; 11 (3): 277–289.
14. Ratner E. S., Erekson E. A., Minkin M. J., Foran-Tuller K. A. Sexual satisfaction in the elderly female population: a special focus on women with gynecologic pathology // Maturitas, 2011; 70 (3): 210–215.
15. Shifren J. L., Monz B. U., Russo P. A., et al. Sexual problems and distress in United States women: prevalence and correlates // Obstetrics & Gynecology, 2008; 112: 970–978.
16. Sobczak J. A. Female sexual dysfunction: knowledge development and practice implications // Perspectives in Psychiatric Care, 2009; 45 (3): 161–172.
17. Valadares A. L. R., Pinto-Neto A. M., Souza M. H., et al. The prevalence of the components of low sexual function and associated factors in middle-aged women // The Journal of Sexual Medicine, 2011; 8: 2851–2858.
18. Wagner G. Sexual medicine in the medical curriculum // International Journal of Andrology, 2005; 28 (2): 7–8.
19. Wierman M. E., Nappi R. E., Avis N., et al. Endocrine aspects of women's sexual function // The Journal of Sexual Medicine, 2010; 7: 561–585.
20. Woloski-Wruble A. C., Oliel Y., Leefsma M., Hochner-Celnikier D. Sexual activities, sexual and life satisfaction, and successful aging in women // The Journal of Sexual Medicine, 2010; 7: 2401–2410.
21. World Health Organization. Sexual health – a new focus for WHO // Progress in Reproductive Health Research, 2004; 67: 1–8.
22. Yee L. Aging and sexuality // Australian Family Physician, 2010; 39 (10): 718–721.
23. Zakhari R. Female sexual dysfunction: A primary care perspective // Journal of the American Academy of Nurse Practitioners, 2009; 21: 498–505.

PCR Based Detection of Microsatellite Instability in Colorectal Cancer

Zanda Daneberga, Inese Eglite, Monta Ustinova, Dace Berzina, Ilze Strumfa, Janis Gardovskis, Edvins Miklasevics

Rīga Stradiņš University, Institute of Oncology, Latvia

Abstract

Colorectal cancer (CRC) is a heterogeneous disease caused by distinctive molecular mechanisms. In two of the so far described molecular pathways, microsatellite instability (MSI) in tumour has been involved. Analysis of MSI is considered to be clinically important to discriminate hereditary cases as well as in therapy choice.

The purpose of this study was to assess the set of available methods for detection of MSI in formalin-fixed, paraffin-embedded (FFPE) tissue blocks of CRC tumour and normal tissue. The evaluation of quantity, quality and integrity for DNA isolated from FFPE tissue blocks in respect to storage life of blocks and validity of DNA samples for multiplex fluorescent PCR with subsequent fragment length analysis using capillary electrophoresis as well as interpretation of results was carried out.

49 patient samples (DNA isolated from histological sections of FFPE normal and tumour tissue blocks) were analyzed in this study. Three patient samples were eliminated from the sample set due to the low DNA concentration and poor purity ratio. Out of 46 patients, 10 (21.74%) were detected to be MSI-H (with high microsatellite instability).

The DNA isolation method used for FFPE blocks proved to be reliable in MSI detection regardless the storage life of blocks. For a successful MSI detection, the optimization of PCR protocols appears to be necessary.

The acquired results of the study are feasible and applicable for further study of CRC as well as hereditary cases of CRC.

Keywords: CRC, microsatellite, MSI, polymerase chain reaction (PCR).

Introduction

Every year around 1000 new cases of colorectal cancer (CRC) have been diagnosed in Latvia [Borosenko, et al., 2009] (based on the data from Central Statistical Bureau of Latvia 690 newly diagnosed cases of CRC were registered in 2012). The estimated age-standardised rates per 100 000 (ASRs) of CRC incidences by sex in Latvia in 2012 were 45.5 in males and 30.0 in females (in comparison, ASR of CRC incidences by sex in Europe in 2012 were 55.7 and 34.6, respectively) [Ferlay, et al., 2013].

CRC is a heterogeneous disease caused by distinctive molecular mechanisms. The genomic instability plays an important role in the multistep progression of colorectal carcinogenesis. Three major molecular pathways to develop CRC have been described. The first and most common pathway

is successive inactivation of tumour-suppressor genes causing chromosomal instability (CIN). This pathway accounts up to 85% of CRC cases. The second pathway is based on CpG island methylator phenotype (CIMP) including sporadic microsatellite instability (MSI) high cancers. The third pathway accounts to pure MSI caused by germ-line cells mutation in a DNA mismatch repair (MMR) gene. The MMR system is based on four MMR proteins (MLH1; MSH2; MSH6; PMS2) which are responsible for the repair of errors during DNA synthesis [Goel, et al., 2003; Jass, 2007; Worthley, Leggett, 2010; Wu, Bekaii-Saab, 2012].

CIN pathway without MSI accounts to around 85% of all CRC cases. The rest of the cases are associated with MSI, approximately 12% to 15% are sporadic cancers, but 3% to 5% belong to hereditary cancer (Lynch syndrome) [de la Chapelle, Hampel, 2010].

Defective MMR genes do not affect all microsatellites in tumour. The Bethesda guideline for detection of MSI was proposed in 2002. This suggests a panel of five microsatellite markers – D2S123, D5S346, D17S250, Bat25 and Bat26 for the detection of MSI in tumour cells [de la Chapelle, Hampel, 2010; Umar, et al., 2004]. These guidelines also set the classification of MSI degree. Tumours with detected instability in ≥ 40% of microsatellite markers (≥ 2/5 in Bethesda panel) are considered to be highly instable (MSI-H). Tumours with < 40% instability (one marker in five-marker panel) are classified as low instability (MSI-L). MSI-L is considered as indeterminate relevance to the clinical condition. Tumours without evidence of MSI are considered microsatellite stable (MSS) [de la Chapelle, Hampel, 2010].

Many publications have shown clinical importance of MSI status detection in CRC. CRCs with MSI have typical pathological features and usually are diagnosed at an earlier stage. The better prognosis and better progression-free survival for patients with MSI-H cancer were confirmed by meta-analysis of 32 studies [de la Chapelle, Hampel, 2010; Vilar, Gruber, 2010]. Moreover, the difference in response to adjuvant chemotherapy has been noticed. The better response to the adjuvant chemotherapy with 5-fluorouracil/Leucovorin was recognised in patients with MSS cancer compared with those who have MSI [de la Chapelle, Hampel, 2010; Wu, Bekaii-Saab, 2012].

There are recommendations to use *BRAF* (v-raf murine sarcoma viral oncogene homolog B) gene mutation testing for differentiation of microsatellite instability high (MSI-H) in hereditary non-polyposis colon cancer (HNPCC) cases and sporadic MSI-H CRC in clinical practice. MSI is commonly detected in ~ 90% of patients with HNPCC and just 15% of sporadic CRC. Moreover, it is recommended that patients at increased risk for Lynch syndrome undergo pre-screening with microsatellite instability analysis [Zhang, Li, 2013].

The aim

The main goal of this study was to assess the methods used in laboratory for the detection of MSI in formalin-fixed, paraffin-embedded (FFPE) tissue blocks of CRC tumour and normal tissue. The project tasks included the evaluation of quantity, quality and integrity of DNA isolated from FFPE tissue blocks in respect to storage life of blocks and validity of DNA samples for multiplex fluorescent PCR with subsequent fragment length analysis using capillary electrophoresis, as well as the interpretation of the obtained results.

Material and methods

Patients. The analyzed group consisted of 49 patients (51% females, 49% males) with stage II and stage III (TNM classification [Edge, et al., 2010]) colon (C18) or rectal cancer (C20) diagnosed between years 2007–2011 in Pauls Stradiņš Clinical University Hospital.

The age of the patients at diagnose set up varied from 34 up to 75 years. All patients underwent surgically radical tumour resection and the majority of them (74%) received an adjuvant chemotherapy

course afterwards, mainly with 5FU (5-fluorouracil) and Leucovorin, Xeloda, Tegafur or FOLFOX in standard doses. After the two-year therapy, in two cases tumour recurrence was revealed.

According to morphological structure of tumours, low or average-differentiated adenocarcinoma classified by Bethesda criteria is considered as the dominant [Umar, et al., 2004]. In most cases tumour invades through the *muscularis propria* into the pericolorectal tissues, or even to the surface of the visceral peritoneum which indicates the depth of tumour invasion and may be as a poor prognostic feature in spite of less detected regional lymph nodes metastasis.

In 8 cases, there was registered lethal outcome of the disease, on average within 3 years after diagnosis. The case-record data for some of the patients were incomplete, with missing information about the outcome of the disease.

Tumour and normal tissues from each patient were obtained during radical tumour resection.

The Latvian Central Committee of Medical Ethics and the Pauls Stradiņš Clinical University Hospital Department of Science approved this study.

DNA isolation. DNA was isolated from histological sections of formalin-fixed, paraffin-embedded tissue blocks by using "QIAamp DNA FFPE Tissue Kit" (Qiagen, Germany), according to the manufacturer protocol. DNA concentration was measured with UV-VIS spectrophotometer Nanodrop ND-1000 (Thermo Scientific, USA). The purity of isolated DNA was evaluated by the ratio of absorbance at 260/280 nm and 260/230 nm.

The integrity of the isolated DNA of tumour and normal tissue was checked by the amplification of 50 ng/µl DNA under standard PCR conditions for 272 bp long and 173bp long random fragments. Length of fragments was chosen based on the microsatellite panel amplicon length (100–250 bp). 10 µl of PCR products were separated on 2.0% agarose gel in 0.5X TBE at 5.5V/cm for 30min. GeneRuler 100 bp (Thermo Scientific, USA) was used. The gel was stained with ethidium bromide (0.5 µg/ml), and visualised with UV.

PCR and microsatellite analysis. Five microsatellites (the Bethesda panel) for the MSI detection were chosen. The monoplex PCR reaction was performed in 16 µl reaction volume with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5U HotStart Taq polymerase (Qiagen, Germany), each primer at concentration as described before [Berg, et al., 2000] and 50 ng of DNA template. "Touch-down" PCR was performed under the following cycling conditions: initial denaturation and enzyme activation at 95 °C followed by 5 cycles at 94 °C for 45 seconds, at 58 °C for 45 seconds and at 72 °C for 1 minute; 5 cycles at 94 °C for 45 seconds, at 57 °C for 45 seconds and at 72 °C for 1 minute; 25 cycles at 94 °C for 45 seconds, at 55 °C for 45 seconds and at 72 °C for 1 minute. The final extension at 60 °C for 45 minutes (to aid non-template adenine addition). The negative control (DNA substituted with nuclease free H_2O) was included in every sample set.

Capillary electrophoresis was performed on ABI 3130 Genetic analyser (Applied Biosystems, USA). 2 µl of PCR product were mixed with 9 µl of formamide and 0.5 µl of LIZ500 size standard (Applied Biosystems, USA). Before the run denaturation at 95 °C for 5 minutes was performed. The run parameters were as follow: capillary length 36 cm with POP7 polymer (Applied Biosystems, USA), injection voltage 1.6 kV, injection time 16 seconds, and run voltage 14.0 kV. Genotyping results were analysed by GeneMapper™ v4.0 software (Applied Biosystems, USA). The analysis parameters were: dye set G5, light peak smoothing, local Southern size calling method. The corresponding peaks length was calculated according to the calibration curve of the LIZ500 size standard.

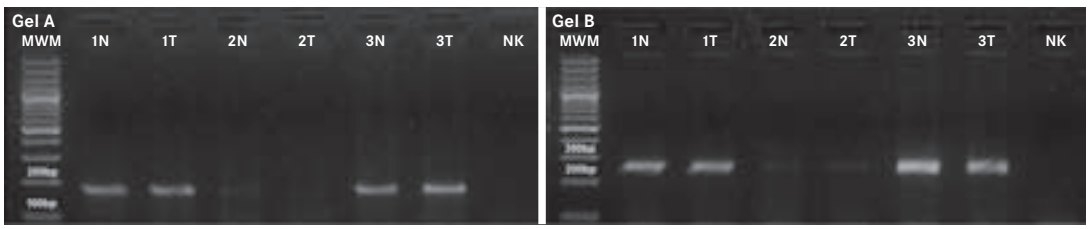
Results

DNA isolation. 98 paired (normal and tumour) DNA samples were isolated and checked for DNA quantity and purity. The concentration of DNA varied between 14.40 ng/µl and 1239.60 ng/µl with the average concentration 374.82 ng/µl. The ratio of absorbance at 260/280 nm for the samples were

calculated between 1.99 and 1.66 (according to the manufacturer protocol the ratio 1.7–1.9 is acceptable). The ratio of absorbance at 260/230 nm varied from 0.71 to 2.32 (according to the manufacturer protocol the ratio 2.0–2.2 is acceptable). Based on the low DNA concentration and poor purity ratio, six paired samples were eliminated from the sample set.

The integrity of DNA was evaluated by visual inspection of 173 bp and 272 bp long amplified fragments on agarose gel (Figure 1). Samples with weak band on agarose gel or with lack of PCR product was considered to be with low integrity. 66 samples out of 92 gave poor results. Although to test the validity of samples for the microsatellite assay, samples with low DNA integrity were not excluded from the sample set.

Figure 1. Example of DNA integrity evaluation:
Gel A – visualisation of the 173 bp long PCR product,
Gel B – visualisation of the 272 bp long PCR product



Note: N – PCR product from normal tissue; T – PCR product from tumour tissue; NK – negative control

The analysed quality indicators of isolated DNA and storage life of FFPE blocks did not show any correlation.

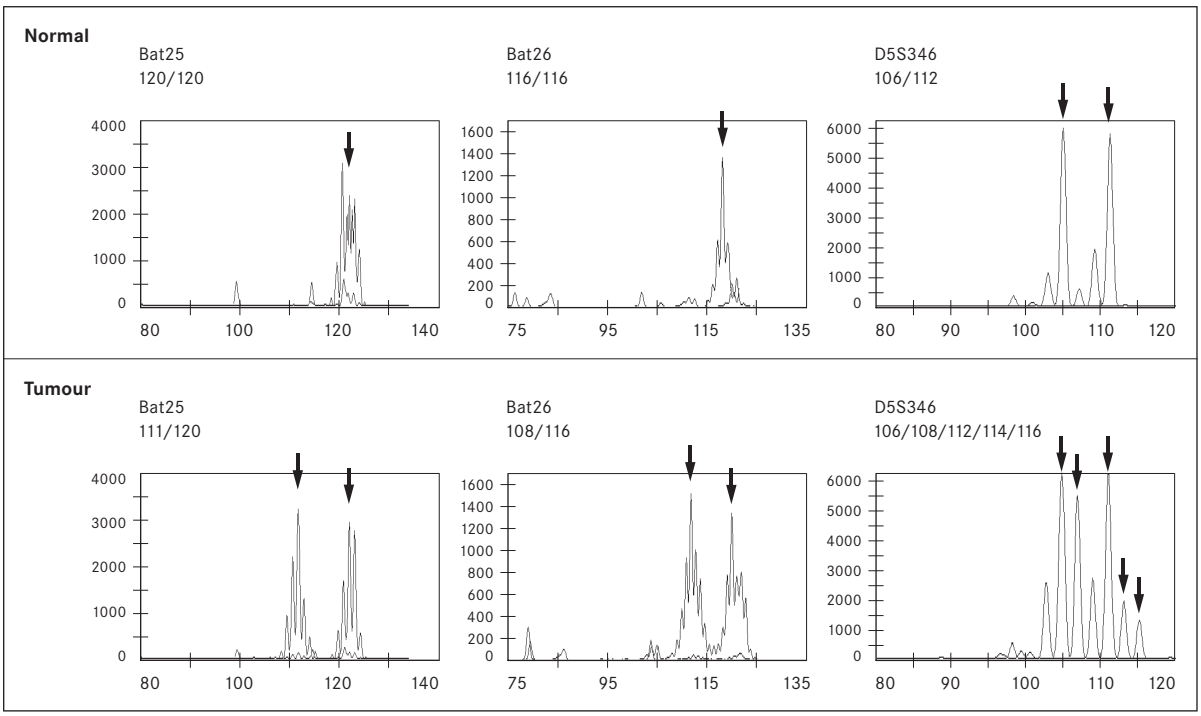
Microsatellite analysis. 92 paired (normal cell line and tumour) DNA samples were amplified and analysed according to the protocol described by Berg, et al. (2000). Multiplex reaction of five microsatellite markers did not give satisfactory results. The overlap of PCR products and missing amplification product of D17S250 were observed. Thus, the assay optimization involved monoplex PCR for each microsatellite marker and "Touch-down" PCR cycling conditions.

The analysis of microsatellite markers for each patient was performed by precise sizing of marker repeats and by comparison of corresponding alleles in normal and tumour. One repeat difference from true allele peak was interpreted with caution, taking into account the formation of well-known artefacts in microsatellite PCR amplification, such as stutter peaks and non-templated 3`-A nucleotide additions. Most of the patient samples (33 out of 46) failed to amplify one of the markers. Two patient samples failed to amplify two markers and these patient samples were excluded from the sample set as samples without detected status. Tumour was considered to be MSI-H if in ≥ 40% of microsatellite markers deviation from normal tissue alleles was found [de la Chapelle, Hampel, 2010]. The results of MSI detection are summarized in Table 1. Figure 2, however, demonstrates MSI-H status for the patient with detected instability of three microsatellite markers.

Table 1. Results of MSI analysis

Instability status	Number of patients, n	Proportion of patients, %
MSI-H	10	21.74
MSI-L	8	17.39
MSS	26	56.52
No status	2	4.35
Total	46	100

Figure 2. Electropherograms of Bat 25, Bat 26 and D5S346 for normal and tumour tissue samples of the patient, demonstrating MSI-H status with instability of three markers



Discussion

Although, MSI status detection has been described in many different publications in recent years, PCR conditions for the same primer sets and markers varied by article [Berg, et al., 2000; Drobinskaya, et al., 2005; Goel, et al., 2010; Nguyen, Lakhan, Finette, 2013]. Success to amplify microsatellite markers is restricted to DNA quality and integrity. Formalin fixation normally results in a DNA fragmentation and cross-linking. These factors can cause low PCR efficiency in microsatellite analysis [Drobinskaya, et al., 2005].

We analysed the quality and integrity of DNA samples isolated from formalin-fixed, paraffin-embedded tissue blocks. The results revealed sufficient amount of DNA, regardless the storage life of FFPE blocks. The evaluation of DNA integrity resulted in much lower proportion of good quality samples. However, we were able to use these samples for MSI detection.

The microsatellite analysis results confirmed feasibility of using the chosen method for MSI detection even in low quality DNA samples and regardless the storage life of FFPE blocks. Unspecific PCR products were observed in the experiments carried, and the necessity of PCR protocol optimization was defined.

Data analysis parameters using automated data analysis software and interpretation of results are an important clue for good quality results. The quantity of analysed PCR products should be evaluated and optimized according to the relative fluorescence units (RFU). Interpretation of monomorphic alleles (Bat25 and Bat26) was the most ambiguous due to +1 and –1 stutter peaks. ± 1 bp deviation was considered as acceptable, the interpretation of results was stressed on the comparison of normal cell line and tumour results. Although, monomorphic markers are more complicated for interpretation, they have advantages over dinucleotide markers. Bat25 and Bat26 are quasi-monomorphic markers with rare polymorphisms in the germ-line [Loukola, Eklin, Laiho, et al., 2001].

According to Zhang and Li (2013), dinucleotide instability is usually observed for most MSI-L cases. The same trend was observed in our samples.

Conclusions

The DNA isolation method used for FFPE blocks proved to be reliable in microsatellite instability detection regardless the storage life of blocks. DNA with low integrity is possible to be used for MSI detection.

A number of factors influenced the efficiency of PCR protocol used for microsatellite analysis. The optimization of PCR protocols appears to be necessary.

Analysis and interpretation of our results proved validity of chosen methods for MSI detection.

The acquired results of the study are feasible and applicable for further study of CRC as well as hereditary cases of CRC. We plan to continue this work by analyzing larger group of patients, as well as to improve the used laboratory methods for implementation in routine practice.

Acknowledgments

This study was supported by The National Research Programme "Development of new prevention, treatment, diagnostics means and practices and biomedicine technologies for improvement of public health".

References

1. Berg K. D., Glaser L. C., Thompson R. E., et al. Detection of microsatellite instability by fluorescence multiplex polymerase chain reaction // JMD, 2000; 2 (1): 20–28.
2. Borosenko V., Irmejs A., Melbarde-Gorkusa I., et al. Initial results of colorectal polyposis in Latvia // Anticancer Research, 2009; 29: 711–716.
3. De la Chapelle A., Hampel H. Clinical relevance of microsatellite instability in colorectal cancer // Journal of Clinical Oncology, 2010; 28 (20): 3380–3387.
4. Drobinskaya I., Gabbert H. E., Moeslein G., Mueller W. A new method for optimizing multiplex DNA microsatellite analysis in low quality archival specimens // Anticancer Research, 2005; 25: 3251–3258.
5. Edge S. B., Byrd D. R., Compton C. C., et al. Colon and rectum // AJCC Cancer Staging Manual. – 7th ed. – New York: Springer, 2010. – Pp. 143–164.
6. Ferlay J., Steliarova-Foucher E., Lortet-Tieulent J., et al., Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012 // European Journal of Cancer, 2013; 49: 1374–1403.
7. Goel A., Arnold C. N., Niedzwiecki D., et al. Characterization of sporadic colon cancer by patterns of genomic instability // Cancer Research, 2003; 63: 1608–1614.
8. Goel A., Nagasaka T., Hamelin R., Boland C. R. An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers // Plos ONE, 2010; 5 (2): e9393. doi:10.1371/journal.pone.0009393.
9. Jass J. R. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features // Histopathology, 2007; 50: 115–130.
10. Loukola A., Eklin K., Laiho P., et al. Microsatellite marker analysis in screening for hereditary nonpoliposis colorectal cancer (HNPCC) // Cancer Research, 2001; 61: 4545–4549.
11. Nguyen T. T. M., Lakhan S. E., Finette B. A. Development of a cost-effective high-throughput process of microsatellite analysis involving miniaturized multiplexed PCR amplification and automated allele identification // Human Genomics, 2013; 7 (6): 1–12.
12. Umar A., Boland C. R., Terdiman J. P., Syngal S., et al. Revised Bethesda guidelines for hereditary nonpolyposis colorectal cancer (Lynch Syndrome) and microsatellite instability // Journal of National Cancer Institute, 2004; 96 (4): 261–268.
13. Vilar E., Gruber S. B. Microsatellite instability in colorectal cancer – the stable evidence // Nat Rev Clin Oncol, 2010; advance on-line publication doi: 10.1038/nrclinonc.2009.237.
14. Worthley D. L., Leggett B. A. Colorectal cancer: molecular features and clinical opportunities // Clin Biochem Rev, 2010; 31: 31–38.
15. Wu C., Bekaii-Saab T. CpG island methylation, microsatellite instability, and BRAF mutations, and their clinical application in the treatment of colon cancer // Chemotherapy Research and Practice, 2012; on-line publication doi:10.1155/2012/359041.
16. Zhang X., Li J. Era of universal testing of microsatellite instability in colorectal cancer // World J Gastrointestinal Oncol, 2013; 5 (2): 12–19.

Products of Homeobox Gene HoxB3 in Placentas of Various Gestational Ages

Ilze Kreicberga [1], *Mara Pilmane* [2], *Dace Rezeberga* [1]

[1] *Rīga Stradiņš University, Department of Obstetrics and Gynecology, Latvia*
[2] *Rīga Stradiņš University, Institute of Anatomy and Anthropology, Latvia*

Abstract

Growth and development of human foetus is determined by the status of maternal-foetal unit; processes in this unit, having impact on the foetal well-being and outcome of pregnancy, are not always clinically evident. Understanding of molecular processes in placenta, maternal-foetal interface, can possibly unhide those processes and witness presence of undetected events, possibly causing unexpected outcome.

Hox genes are known to play a significant role in the establishment of pregnancy, maintenance and development of trophoblastic cells and development of human embryo; gestation dependent variations have not been established. The aim of the study was to assess presence of HoxB3 gene products in cells of placentas of different gestational ages and look for correlations with the gestational age and maternal and neonatal anthropometrical parameters.

Samples from different parts of placenta were taken immediately after delivery, placed into picric acid-formaldehyde fixation and brought to the Institute of Anatomy and Anthropology of Rīga Stradiņš University. Samples were prepared and processed with polyclonal rabbit antibody, manufactured by Santa Cruz, in the working dilution of 1 : 100. Amount of HoxB3 positive cells was detected visually and ranked from 0 to 4 (abundance). As the distribution was close to normal, Pearson correlation was used for statistical evaluation, significance determined at $p < 0.05$. HoxB3 positive cells were found in all the samples with no correlations with the gestational age. In pre-term placentas appeared a negative correlation of HoxB3 positive cells with ponderal index of the neonate, and in term placentas positive correlation with the maternal body height. We concluded that HoxB3 gene has an impact on longitudinal growth of foetus throughout the second and the third trimesters of pregnancy.

Keywords: placenta, immunohistochemistry, HoxB3 products.

Introduction

Foetal growth and development is determined by the interaction of mother and foetus by the means of interface placenta throughout the pregnancy. Transport of all the essential nutrients is provided by the placental blood flow; placenta produces and transports hormones, promoting foetal growth. Disturbances in regulation of foetal growth and development can result in adverse outcomes for the neonate, and these adverse outcomes may persist into adult life. Placenta ensures circumstances for foetal growth and development [Bauer, et al., 1998; Murphy, et al., 2006, Jansson, et al., 2007] and findings in its tissues reveal processes, which have determined the outcome of pregnancy; they have special clinical significance

in cases of high-risk pregnancies or unexpected complications. Understanding the physiological and pathological processes in placenta disclose the roads of problem solving with a possibly better outcome for the foetus, leading to long-term health benefits for the human child.

Antenatal care as well as post-delivery examination of placenta has become a routine part of the perinatal (obstetrical and neonatal) care for the assessment of pathways, possibly leading or having led to an unwanted outcome. Even routine examination of a post-delivery placenta provides significant information on the foetal environment [Fox, Sebire, 2007; Tomas, et al., 2010; Roescher, et al., 2011; Roje, et al., 2011] having possible impact on the child's health status and maternal well-being.

Due to the complexity of those processes, routine praxis does not answer many questions. The present study shows identification of the molecular processes in the post-delivery placentas of different gestational ages for the development of clinically applicable knowledge of molecular processes to create opportunities for the improvement of perinatal outcome in high-risk situations.

Morphogenesis of the developing human embryo is determined by a large variety of homeobox genes, a large family of similar genes that direct the formation of many body structures during early embryonic development; presently the most researched part of homeobox containing genes are Hox genes. First found in *Drosophila melanogaster* as determinants of anterior-posterior axis, they have been proven to determine development of vertebrates, their nervous system [Mc Ginnis, Krumlauf, 1992; Krumlauf, 1994; Schneider-Maunoury, et al., 1998; Carapuço, et al., 2005; Tümpel, et al., 2009] and blood, in altered cases leading to defective haematopoiesis [Lawrence, et al., 1997]. Hox gene products or Hox proteins work as transcription factors, activating or inhibiting the transcription process to RNA. Hox gene products have been researched in different fields of human medicine. The largest number of studies on Hox genes and their products are in oncology, as any alteration in the transcription processes can contribute to the development of cancer [Cillo, et al., 1992; Cillo, 1994–1995; Goodman, Scambler, 2001; Grier, et al., 2005] and its activation [Chen, et al., 2012]. A large number of animal studies show the impact of Hox genes on the development of leukemia [Lawrence, et al., 1996; Magli, et al., 1997; Thorsteinsdottir, et al., 1997; Chiba, 1998; Shimamoto, et al., 1998; Abramovich, Humphries, 2005; Argiropoulos, Humphries, 2007; Fröhling, et al., 2007]. Researches on humans confirm the importance of Hox genes on normal haematopoiesis as well as the development of malignancies [Celetti, et al., 1993; Peschle, et al., 1993; Giampaolo, et al., 1994; Sauvageau, et al., 1995; Thorsteinsdottir, et al., 1997; Van Oostveen, et al., 1999; Chung et al., 2009; Alharbi, et al, 2012].

Hox gene products are highly associated with human reproduction. They are influencing the development of female genital tract [Taylor, 2000]; their altered expression leads to endometriosis and its caused infertility [Taylor, et al., 1999; Kim, et al., 2007; Cakmak, et al., 2010; Zanatta, et al., 2010]. Hox gene products ensure implantation and decidualization [Taylor, et al., 1999; Eun Kwon, Taylor, 1999; Daftary, Taylor, 2000; Vitiello, et al., 2007; Lu, et al, 2008].

Taking into account the abovementioned data, within the cause of the research it was assumed that the evaluation of Hox gene product positive cells in human placentas and correlation of the results with gestational time at delivery and pregnancy outcomes could be interesting and clinically useful.

The aim

The aim of the respective study is to ascertain presence of Hox gene products in cells of post-delivery placentas of various gestational ages and to look for correlations between those factors of molecular processes in the placentas and pregnancy risk factors, gestational age of placenta, anthropometrical parameters and clinical indices of the mothers, placentas and newborns.

Material and methods

On the 12th of March 2009, the study was approved by the Ethics Committee of Rīga Stradiņš University. 53 patients admitted for the delivery care to Rīga Maternity Hospital, having signed informed consent and not meeting exclusion criteria, were included in the study. Exclusion criteria were age lower than 18 years (under-age), no antenatal care, severe systemic disease, positive status for human immunodeficiency virus (HIV).

Patient groups:

- Group 1 (healthy term): 14 patients of term deliveries from 37 gestational weeks with uncomplicated clinical courses and healthy children;
- Group 2 (pre-term): 25 patients of pre-term deliveries with premature children between 22 and 36 weeks of gestation;
- Group 3 (distress): 14 deliveries of various gestational ages (either pre-term or term) with documented foetal distress antenatally or during labour and delivery.

Immediately after the delivery by a cut of a single use surgical knife, there were taken two 1 cm × 1 cm samples from symmetrically located places of the chosen placentas through all the layers of the placental tissues and placed into picric acid-formaldehyde fixation, having been originally described almost 50 years ago [Stefanini, et al., 1967]. The samples were taken to the Institute of Anatomy and Anthropology of Rīga Stradiņš University for further processing. Patient data were acquired from the medical records of Rīga Maternity Hospital and the study survey.

Maternal and placental anthropometrical data were obtained from the medical record of Childbirth of Rīga Maternity Hospital:

1) maternal age, body weight before pregnancy and height, calculated body mass index (BMI = body mass (kg) / height $(m)^2$), weight gain during pregnancy;
2) gestational weeks at delivery;
3) placental weight.

Neonatal data were obtained from the medical record of Neonatal development: birth weight, body length, head and chest circumferences, calculated ponderal index (PI = 100 × body mass (g)/height $(cm)^3$.

For the identification of HoxB3 positive cells in the placental preparations polyclonal rabbit antibody was used, manufactured by Santa Cruz, in the working dilution of 1 : 100. The preparation of the samples for IHC processing was provided in accordance with the Dako REAL™ EnVision Detection System protocol (3rd edition, 2005).

IHC findings were evaluated semi-quantitatively by the amount of the indicator positive cells in the visual field (Pilmane, et al., 1998): none 0, occasional 0 / +, few +, moderate ++, numerous +++ and abundant ++++. The evaluation was done after a complete observation of both samples of each placenta. IHC findings were ranked in the ascending order by modified competition ranking method [Pozzi, 2008]:

0 – none (0) indicator positive cells or ECM structures (in the visual field);
0.5 – occasional (0 / +) indicator positive cells or ECM structures;
1 – few (+) indicator positive cells or ECM structures;
2 – moderate amount (++) of indicator positive cells or ECM structures;
3 – numerous (+++) indicator positive cells or ECM structures;
4 – abundance (++++) of indicator positive cells or ECM structures.

Descriptive statistics were performed. For cross-sample mean comparison, inferential statistical Student's t-tests were performed. This method was chosen due to the approximate normality of the data analyzed.

Pearson product-moment correlation was used to inspect the correlation of mother and neonate specific data with their respective indicators, as well as between the indicators themselves. This measure was chosen due to the linear relationships observed between the correlated data, the strong tendency of the mother, neonate and indicator data to follow normal distribution, as well as the homoscedastic nature of the data analyzed. Pearson correlation and Mann-Whitney U test were considered and tested for correlation analysis, and Pearson correlation was chosen due to the normal distribution of the data analyzed.

Data processing software Microsoft Excel 2013 Preview and IBM SPSS 19.0 were used; the statistical significance was set at $p < 0.05$.

Results

The amount of HoxB3 positive cells did not correlate with the gestational age of placenta, and occasional (0 / +) to numerous (+++) modifications in cells in the visual field were seen in all the placentas of the study. Different types of placental cells were HoxB3 positive: cytotrophoblast, syncytiotrophoblast, extra villous trophoblastic and Höfbauer cells (Figures 1–4).

Figure 1. Study group G2 (pre-term): Maternal part of a 31 gestational week placenta with moderate (++) HoxB3 containing extravillous trophoblastic and Höfbauer cells (HoxB3 IHC, × 250)

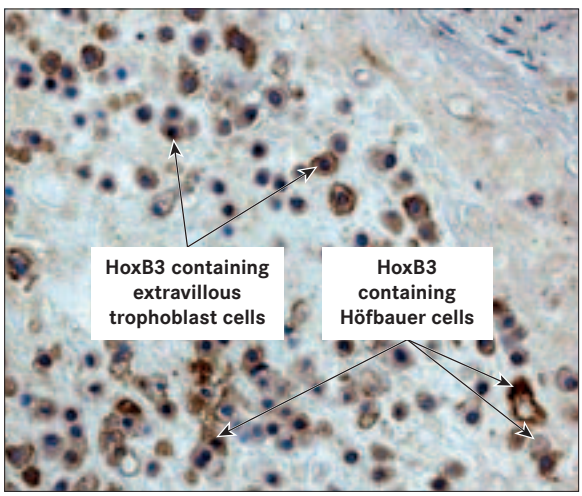


Figure 2. Study group G3 (distress): Maternal part of a 32 week gestational placenta with numerous (+++) HoxB3 containing Höfbauer cells (HoxB3 IHC, × 250)

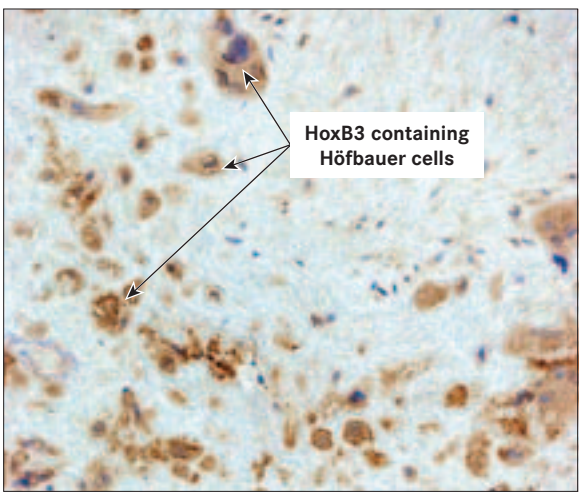


Figure 3. Study group G3 (distress): Maternal part of a tertiary villi of a young p with placenta of 31 gestational week with numerous (+++) HoxB3 containing extravillous trophoblastic, cytotrophoblastic and syncytiotrophoblastic cells (HoxB3 IHC, × 250)

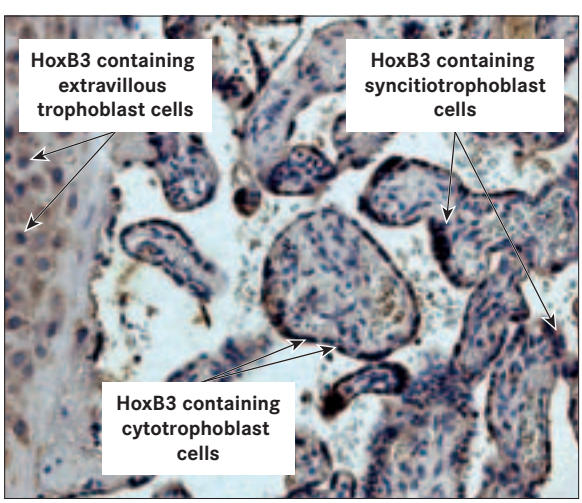
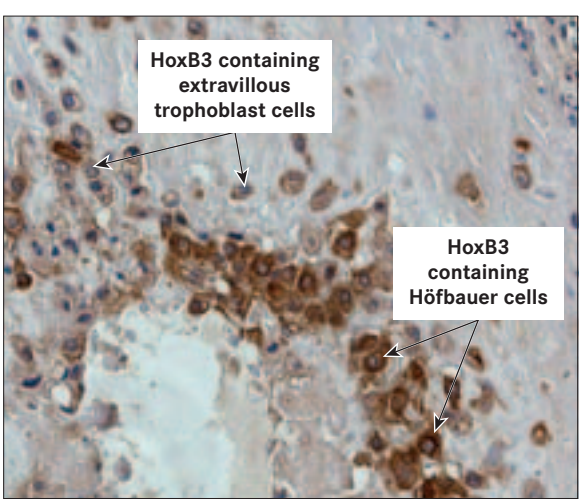


Figure 4. Study group G3 (distress): Maternal part of a 40 week gestational placenta with numerous (+++) HoxB3 containing extravillous trophoblastic and Höfbauer cells. Mother 25 years of age, vaginal delivery of a boy 4330 g (HoxB3 IHC, × 250)



For a more detailed evaluation of the acquired data and more advanced interpretation of the results, epidemiological evaluation and correlation analysis were done in the following sections:

1) in the whole study, including 53 patients;
2) in three study groups: 14 patients with healthy term (G1), 25 patients pre-term (G2) and 14 term or pre-term foetal distress patients (G3);
3) in gestation dependent groups: 19 term and 34 pre-term patients;
4) in specific patient groups, determined by the expected impact of the molecular event.

By the means of modified competition ranking method (Pozzi, 2008) ranks of HoxB3 positive cells were estimated; mean rank values were calculated (Table 1).

Differences between the mean rank values of HoxB3 positive cells in the placentas of various groups were not statistically significant.

Correlating the rank values of HoxB3 products in placentas with maternal parameters appeared to be a statistically significant correlation with the maternal body height in the study group G3 (distress) (Table 2).

Correlating the rank values of HoxB3 products in the placentas with neonatal parameters appeared a statistically significant negative correlation with the ponderal index of the neonate in the whole study and in pre-term placentas of the whole study (Table 3) and a positive correlation with the body length of neonate in the study group G1 (healthy term).

Table 1. Rank values of HoxB3 positive cells in different divisions of the study

Group	The mean ± SD
The whole study	1.44 ± 1.20
Study group G1 (healthy term)	1.25 ± 0.27
Study group G2 (normal pre-term)	1.36 ± 0.71
Study group G3 (distress)	1.39 ± 0.76
Term placentas of the whole study	1.16 ± 0.71
Pre-term placentas of the whole study	1.44 ± 0.72

Table 2. Correlations between rank values of HoxB3 products with maternal parameters

Parameter	G3 (distress) group	HoxB3 products
Maternal body height	Pearson Correlation	0.573*
	Sig. (2-tailed)	0.032

* Correlation is significant at the 0.05 level (2-tailed).

Table 3. Correlations between rank values of HoxB3 products with neonatal parameters

Parameter	Study groups	HoxB3 products
The whole study group		
Ponderal index	Pearson Correlation	–0.323*
	Sig. (2-tailed)	0.018
G1 (healthy term) group		
Body length	Pearson Correlation	0.541*
	Sig. (2-tailed)	0.046
All pre-term in the whole study group		
Ponderal index	Pearson Correlation	–0.461**
	Sig. (2-tailed)	0.008

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

Discussion

We found HoxB3 products in all the placentas of the study in various amounts, meaning that they play a certain role in the development of the maternal-foetal unit. Evaluating the rank values of HoxB3 product positive cells in the placentas of our study groups, we did not find any significant differences between any of them as well as between the pre-term and term placentas of the whole study; in pre-term placentas there were slightly more HoxB3 positive cells. The findings correspond with other studies,

suggesting the influence of Hox gene products on the maintenance of certain type of trophoblastic cells [Amesse, et al., 2003] or differentiation of cytotrophoblastic cells into syncytiotrophoblast [Zhang, et al., 2002].

Evaluating the rank values of HoxB3 structure positive cells in the placentas of small (SGA), appropriate (AGA) and large for the gestational age (LGA) neonates, a higher score was observed in SGA placentas, but the differences were not statistically significant.

Looking for correlations between the rank values of HoxB3 positive cells in the placentas of the study groups with maternal and neonatal parameters we found some statistically significant correlations. In the study group G3 (distress) the rank values of HoxB3 products showed positive correlation with the maternal body height meaning more HoxB3 product positive cells in taller women. This might have been treated as coincidence if we had not seen other correlations. In the whole study as well as in pre-term cases of the whole study, the rank values of HoxB3 products in placentas presented a significantly negative correlation with the ponderal indices of neonates, suggesting its impact on the longitudinal growth of a foetus. This was strengthened by a finding in the study group G1 (healthy term) where the rank values of HoxB3 products showed positive correlations with the neonatal body length. As there are no studies describing correlation of Hox gene products in placenta with foetal anthropometrical parameters, such findings could be perceived as unique. This may suggest a more significant impact of Hox genes than have already been described having an impact on the growth and development of placental tissues *per se* [Zhang, et al., 2002; Amesse, et al., 2003] as well as embryonic development [McGinnis, Krumlauf, 1992; Lappin, et al., 2006].

Conclusions

The amount of HoxB3 positive cells, present in all the post-delivery placentas from 22 weeks of gestation, does not correlate with its gestational age, but with some neonatal anthropometrical indices, indicating HoxB3's role in the placental development itself and also longitudinal growth of a foetus.

References

1. Abramovich C., Humphries R. K. Hox regulation of normal and leukemic hematopoietic stem cells // Curr Opin Hematol, 2005; 12 (3): 210–216.
2. Alharbi R. A., Pettengell R., Pandha H. S., Morgan R. The role of HOX genes in normal hematopoiesis and acute leukemia // Leukemia, 2013; 27 (5): 1000–1008.
3. Amesse L. S., Moulton R., Zhang Y. M., Pfaff-Amesse T. Expression of HOX gene products in normal and abnormal trophoblastic tissue // Gynecol Oncol, 2003; 90 (3): 512–518.
4. Argiropoulos B., Humphries R. K. Hox genes in hematopoiesis and leukemogenesis // Oncogene, 2007; 26 (47): 6766–6776.
5. Bauer M. K., Harding G. E., Bassett N. S., et al. Fetal growth and placental function // Mol Cell Endocrinol, 1998; 140 (1–2): 115–120.
6. Carapuço M., Nóvoa A., Bobola N., Mallo M. Hox genes specify vertebral types in the presomitic mesoderm // Genes Dev, 2005; 15; 19 (18): 2116–2121.
7. Cakmak H., Taylor H. S. Molecular mechanisms of treatment resistance in endometriosis: the role of progesterone-hox gene interactions // Semin Reprod Med, 2010; 28 (1): 69–74.
8. Celetti A., Barba P., Cillo C., et al. Characteristic patterns of HOX gene expression in different types of human leukemia // Int J Cancer, 1993; 21; 53 (2): 237–244.
9. Chen J., Zhu S., Jiang N.. et al. Hox-B3 promotes prostate cancer cell progression by transactivating CDCA3 // Cancer Lett, 2013; 330 (2): 217–224.
10. Chiba S. Homeobox genes in normal hematopoiesis and leukemogenesis // Int J Hematol, 1998; 68 (4): 343–353.
11. Chung N., Jee B. K., Chae S. W., et al. HOX gene analysis of endothelial cell differentiation in human bone marrow-derived mesenchymal stem cells // Mol Biol Rep, 2009; 36 (2): 227–235.
12. Cillo C., Barba P., Freschi G., et al. HOX gene expression in normal and neoplastic human kidney // Int J Cancer, 1992; 30; 51 (6): 892–897.

13. Cillo C. HOX genes in human cancers // Invasion Metastasis, 1994–1995; 14 (1–6): 38–49.
14. Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse Code K5007 // 3rd edition, K5007/EFG/HGW, 2005; 1–19.
15. Daftary G. S., Taylor H. S. Implantation in the human: the role of HOX genes // Semin Reprod Med, 2000; 18 (3): 311–320.
16. Eun Kwon H., Taylor H. S. The role of HOX genes in human implantation // Ann N Y Acad Sci, 2004; 1034: 1–18.
17. Fox H., Sebire N. J. Pathology of the placenta: an introduction and overview // Pathology of the Placenta – 3rd ed. – Saunders Elsevier, 2007. – P. 1.
18. Fröhling S., Scholl C., Bansal D., Huntly B. J. HOX gene regulation in acute myeloid leukemia: CDX marks the spot? // Cell Cycle, 2007; 15; 6 (18): 2241–2245.
19. Giampaolo A., Sterpetti P., Bulgarini D., et al. Key functional role and lineage-specific expression of selected HOXB genes in purified hematopoietic progenitor differentiation // Blood, 1994; 1; 84 (11): 3637–3647.
20. Goodman F. R., Scambler P. J. Human HOX gene mutations // Clin Genet, 2001; 59 (1): 1–11.
21. Grier D. G., Thompson A., Kwasniewska A., et al. The pathophysiology of HOX genes and their role in cancer // J Pathol, 2005; 205 (2): 154–171.
22. Jansson T., Powell T. L. Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches // Clin Sci (Lond), 2007; 113 (1): 1–13.
23. Kim J. J., Taylor H. S., Lu Z., et al. Altered expression of HOXA10 in endometriosis: potential role in decidualization // Mol Hum Reprod, 2007; 13 (5): 323–332.
24. Krumlauf R. Hox genes in vertebrate development // Cell, 1994; 78: 191–201.
25. Lappin T. R., Grier D. G., Thompson A., Halliday H. L. HOX genes: seductive science, mysterious mechanisms // Ulster Med J, 2006; 75 (1): 23–31.
26. Lawrence H. J., Sauvageau G., Humphries R. K., Largman C. The role of HOX homeobox genes in normal and leukemic hematopoiesis // Stem Cells, 1996; 14 (3): 281–291.
27. Lu Z., Hardt J., Kim J. J. Global analysis of genes regulated by HOXA10 in decidualization reveals a role in cell proliferation // Mol Hum Reprod, 2008; 14 (6): 357–366.
28. Magli M. C., Largman C., Lawrence H. J. Effects of HOX homeobox genes in blood cell differentiation // J Cell Physiol, 1997; 173 (2): 168–177.
29. McGinnis W., Krumlauf R. Homeobox genes and axial patterning // Cell, 1992; 68: 283–302.
30. Murphy V. E., Smith R., Giles W., Clifton V. L. Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus // Endocrine Reviews, 2006; 27 (2): 141–169.
31. Peschle C., Testa U., Valtieri M., et al. Stringently purified human hematopoietic progenitors/stem cells: analysis of cellular/molecular mechanisms underlying early hematopoiesis // Stem Cells, 1993; 11 (5): 356–370.
32. Pilmane M., Rumba I., Sundler F., Luts A. Patterns of distribution and occurrence of neuroendocrine elements in lungs of humans with chronic lung diseases // Proc Latv Acad Sci Sect B, 1998; 52: 144–152.
33. Pozzi F. // http://www.mathworks.com/matlabcentral/fileexchange/19496-rankings/content/ModifiedCompetitionRankings.m, 2008.
34. Roescher A. M., Hitzert M. M., Timmer A., et al. Placental pathology is associated with illness severity in preterm infants in the first twenty-four hours after birth // Early Hum Dev, 2011; 87 (4): 315–319.
35. Roje D., Tomas S. Z., Prusac I. K., et al. Trophoblast apoptosis in human term placentas from pregnancies complicated with idiopathic intrauterine growth retardation // J Matern Fetal Neonatal Med, 2011; 24 (5): 745–751.
36. Sauvageau G., Thorsteinsdottir U., Eaves C. J., et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo // Genes Dev, 1995; 15; 9 (14): 1753–1765.
37. Schneider-Maunoury S., Gilardi-Hebenstreit P., Charnay P. How to build a vertebrate hindbrain. Lessons from genetics // C R Acad Sci III, 1998; 321 (10): 819–834.
38. Shimamoto T., Ohyashiki K., Toyama K., Takeshita K. Homeobox genes in hematopoiesis and leukemogenesis // Int J Hematol, 1998; 67 (4): 339–350.
39. Stefanini M., De Martino C., Zamboni L. Fixation of ejaculated spermatozoa for electron microscopy // Nature, 1967; 216: 173–174.
40. Taylor H. S., Bagot C., Kardana A., et al. HOX gene expression is altered in the endometrium of women with endometriosis // Hum Reprod, 1999; 14 (5): 1328–1331.
41. Taylor H. S., Igarashi P., Olive D. L., Arici A. Sex steroids mediate HOXA11 expression in the human peri-implantation endometrium // J Clin Endocrinol Metab, 1999; 84 (3): 1129–1135.
42. Taylor H. S. The role of HOX genes in the development and function of the female reproductive tract // Semin Reprod Med, 2000; 18 (1): 81–89.

43. Thorsteinsdottir U., Sauvageau G., Humphries R. K. Hox homeobox genes as regulators of normal and leukemic hematopoiesis // Hematol Oncol Clin North Am, 1997; 11 (6): 1221–1237.
44. Tomas S. Z., Prusac I. K., Roje D., Tadin I. Trophoblast apoptosis in placentas from pregnancies complicated by preeclampsia // Gynecol Obstet Invest, 2011; 71 (4): 250–255.
45. Tümpel S., Wiedemann L. M., Krumlauf R. Hox genes and segmentation of the vertebrate hindbrain // Curr Top Dev Biol, 2009; 88: 103–137.
46. Van Oostveen J., Bijl J., Raaphorst F., et al. The role of homeobox genes in normal hematopoiesis and hematological malignancies // Leukemia, 1999; 13 (11): 1675–1690.
47. Vitiello D., Kodaman P. H., Taylor H. S. HOX genes in implantation // Semin Reprod Med, 2007; 25 (6): 431–436.
48. Zanatta A., Rocha A. M., Carvalho F. M., et al. The role of the Hoxa10/HOXA10 gene in the etiology of endometriosis and its related infertility: a review // J Assist Reprod Genet, 2010; 27 (12): 701–710.
49. Zhang Y. M., Xu B., Rote N., et al. Expression of homeobox gene transcripts in trophoblastic cells // Am J Obstet Gynecol, 2002; 187 (1): 24–32.

The Effect of Polymorphisms HLA-DR Gene and Associations with Tick-borne Diseases

Lilija Kovalchuka [1], ***Jelena Eglite*** [1], ***Mara Zalite*** [2, 3], ***Irina Lucenko*** [4], ***Inara Logina*** [5], ***Guntis Karelis*** [5], ***Ludmila Viksna*** [2, 3], ***Angelika Krumina*** [2]

[1] *Rīga Stradiņš University, Clinical Immunology and Immunogenetic Laboratory, Latvia*
[2] *Rīga Stradiņš University, Infectology and Dermatology Department, Latvia*
[3] *Infectology Center of Latvia*
[4] *Centre for Disease Prevention and Control, Latvia*
[5] *Rīga Stradiņš University, Neurology and Neurosurgery Department, Latvia*

Abstract

The level of tick-borne disease in Latvia is one of the highest in Europe [1]. Compared to previous years, tick activity and incidence have increased in 2012 [1, 2]. The purpose of this study was to determine HLA-DR alleles in two groups of patients in Latvia: in patients with *Lyme borreliosis* (LB) and patients with tick-borne encephalitis (TBE). The study included 38 patients with clinical stage – *erythema migrans*, 60 patients with TBE and 100 control (healthy) persons. All patients and healthy persons are residents of Latvia. HLA genotyping was performed by PCR with sequence-specific primers.

The frequency of HLA-DRB1*17(03) (15 percent vs. 4 percent; odds ratio, 4.06; pc = 0.003) was significantly higher in patients with LB. The HLA-DRB1*10 (2 percent vs. 8 percent; odds ratio, 0.16; pc = 0.044) was smaller in TBE patients and significantly higher in controls. Among TBE patients the HLA-DRB1*04 and DRB1*17(03) alleles were increased, but the HLA-DRB1*01 was lower in patients, these differences were not significant after Bonferroni correction.

The results of the study suggest that the high risk for tick-borne disease in Latvia is associated with the HLA-DRB1*17(03) allele (pc = 0.003). As the frequency of HLA-DRB1*10 allele (pc = 0.044) were significantly higher in healthy persons, these alleles could be associated with a potential protective effect.

Keywords: tick-borne diseases, HLA alleles, marker, PCR.

Introduction

Today ticks inhabit almost every continent, with the number of species worldwide topping 850 [3, 4, 5]. The recognized number of important diseases transmitted by ticks has been growing over the past 30 years [4, 10, 11]. *Lyme borreliosis* in recent years has been a very topical disease. What is more, the disease level in Latvia is one of the highest in Europe [1, 2, 11]. There are some similarities between the bacterial agents, and HLA molecules, because one or another way of immune response is developed in an organism [6, 7, 8]. There are many hypotheses about the direct role of HLA molecules in the pathogenesis of infection [9, 10, 11, 12]. Clarifying polymorphism of HLA immunogenetic molecular markers helps to identify regularities in the development and pathology to develop a new approach to treating these diseases [11, 13]. While the idea of HLA-related genes being involved in the control of the clinical progression of Lyme arthritis has been well documented [14, 17], the possible role of the HLA region in susceptibility to disease *per se* has also been suggested [15, 18, 21]. Some results have already been found for class I HLA alleles [16, 19]; however,

a greater number of studies have reported increased frequencies of class II alleles in Lyme arthritis patients in several populations [16, 17, 20]. The prevalence of tick-borne encephalitis virus in 2012 exceeded 10%, while in 2011 it was 5.7% and 4.1% in 2010 [1, 10, 11].

The aim

In the present study, the aim was to investigate the HLA DR alleles in two groups of patients: patients with *Lyme borreliosis* and patients with tick-borne encephalitis to identify genetic markers.

Material and methods

Characteristic of the studied patients. The study included 38 patients with clinical stage – *erythema migrans*, 60 patients with tick-borne encephalitis and 100 control (healthy) persons. The included patients' ages ranged from 18 to 62 years. The majority of patients were between 22–45 years of age, representing 60.4% of the total studied. All patients and healthy persons were residents of Latvia. The clinical diagnosis was confirmed at Infectology Center of Latvia. Immunogenetic examinations were performed in Rīga Stradiņš University, Clinical Immunology and Immunogenetic laboratory. The approval from the Ethics Committee of Rīga Stradinš University was obtained. The written informed consent for participation in the study from participants was received.

HLA typing. Blood samples (5 mL) were collected from the subjects in tubes containing anticoagulant (EDTA) and centrifuged at 2500 rpm for 15 minutes, and the Buffy-coat was conserved at – 20 °C until use. The genomic DNA was extracted from proteinase-K-treated peripheral blood leukocytes using the routine "salting-out" method [24, 25]. The DNA was stored in TE buffer (10 ml Tris-HCl, pH 7.5, and 2 ml 0.5 M Na2 EDTA per litter of distilled water). The DNA concentration, around 100–200 µg/ml was determined by fluorescence with a DNA fluorimeter.

HLA-DR genotyping by PCR low-resolution for DRB1*01 to DRB1*18; was performed by PCR with sequence-specific primers (PCR–SSP) [22, 24, 25]. The reaction mixture (15 µl) included 1.0 µl DNA, 1.5 µl PCR buffer [50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.3)], 0.6 µl dNTPs (25 mmol/l), 1.0 µl specific primers (0.2 mmol/l), and 0.5 U of the *Taq* DNA polymerase (Promega). The reaction mixture was subjected to 35 amplification cycles, each consisting of denaturation cycle at 94 °C (60 s), annealing cycles at 67 °C (40 s), and extension cycles at 65 °C (10 s). PCR products were visualized by agarose-gel electrophoresis [23, 25]. After addition of 2 M loading buffer, the PCR reaction mixtures were loaded in agarose gels pre-stained with ethidium bromide (0.5 µk/ml gel). Gels were run for 15 min at 10V/cm gel in 0.5 mM TBE (0.89 M Tris, 0.89 M boric acid and 0.02 M EDTA in aqueous solution) buffer and then examined under UV illumination and recorded [25].

Statistical analysis. The significance of differences in individual subtypes between patients and controls was performed using the Chi square test, with the Bonferroni correction or Fisher's Exact Test when necessary [26]. Data were considered statistically significant when p value was less than or equal to 0.05. However, to account for multiple comparisons, the observed p values were corrected (pc) for the number of alleles when one locus was considered alone. The odds ratios (OR), with 95% confidence intervals (95% CI), were calculated using SISA statistics online http://home.clara.net/sisa/ website, to evaluate the risk of the individual developing the disease while having a particular HLA type.

Results

The frequency of DRB1* alleles of the LB patients and control group are shown in Table 1.

There were differences between LB patients and the control group for HLA-DRB1*17(03) and HLA-DRB1*04, which appeared with greater frequency in the respective patient groups (15 percent vs. 4 percent; odds ratio, 4.06; p = 0.002; and 13 percent vs. 5 percent; odds ratio, 3.22; p = 0.011); respectively. HLA-DRB1*10 (2 percent vs. 8 percent; odds ratio, 0.16; p = 0.036) frequencies were lower in LB patients (Table 1). We also detected a difference in frequency of HLA-DR*13. The frequency of allele DRB1*13

(13 percent vs. 6 percent; odds ratio, 2.37; p = 0.049) was higher in *Lyme borreliosis* patients and lower in controls (Table 1). This difference was a "borderline", and after applying the Bonferroni correction was not statistically significant (Table 1).

The frequency of DRB1* alleles of the TBE patients and control group are represented in Table 2.

Table 1. The frequency of DRB1* alleles studied in-patients with LB and healthy controls from Latvia

Allele DRB1	Patients, n / % (n = 38) 76 alleles	Controls, n / % (n = 100) 200 alleles	OR (95% CI)	p value	pc value
*01	2 (3%)	12 (6%)	0.42 (0.06–2.07)	0.207	ND
*02	2 (3%)	12 (6%)	0.42 (0.06–2.07)	0.207	ND
*03	3 (4%)	9 (5%)	0.87 (0.18–3.65)	0.569	ND
*04	10 (13%)	9 (5%)	**3.22** (1.15–9.07)	**0.011**	0.162
*07	3 (4%)	15 (8%)	0.51 (0.11–1.94)	0.217	ND
*08	2 (3%)	10 (5%)	0.51 (0.08–2.58)	0.311	ND
*09	2 (3%)	16 (8%)	0.31 (0.05–1.46)	0.083	ND
*10	1 (2%)	15 (8%)	**0.16** (0.01–1.22)	0.036	0.044
*11	8 (11%)	21 (11%)	1.00 (0.39–2.53)	0.831	ND
*12	4 (6%)	17 (9%)	0.60 (0.16–1.98)	0.364	ND
*13	10 (13%)	12 (6%)	**2.37** (0.90–6.23)	**0.049**	0.055
*14	6 (8%)	13 (7%)	1.23 (0.40–3.66)	0.682	ND
*15	4 (6%)	12 (6%)	1.87 (0.23–3.04)	0.537	ND
*16	2 (3%)	11 (6%)	0.46 (0.07–2.30)	0.255	ND
*17 (03)	11 (15%)	8 (4%)	**4.06** (1.44–11.65)	**0.002**	0.003
*18 (03)	6 (8%)	8 (4%)	2.06 (0.61–6.83)	0.155	ND

Abbreviations: ND – not defined; OR – odds ratio; CI – confidence interval; p value – probability; pc value – after Bonferroni adjustment.

Table 2. The frequency of DRB1* alleles studied in-patients with TBE and healthy controls from Latvia

Allele DRB1	Patients, n / % (n = 60) 120 alleles	Controls, n / % (n = 100) 200 alleles	OR (95% CI)	p value	pc value
*01	1 (2%)	12 (6%)	0.13 (0.01–0.99)	0.017	0.240
*02	8 (7%)	12 (6%)	1.12 (0.40–3.05)	0.811	ND
*03	5 (4%)	9 (5%)	0.92 (0.26–3.11)	0.887	ND
*04	13 (11%)	9 (5%)	2.58 (0.99–6.80)	0.030	0.386
*07	7 (6%)	15 (8%)	0.76 (0.27–2.07)	0.568	ND
*08	4 (4%)	10 (5%)	0.66 (0.17–2.34)	0.480	ND
*09	9 (8%)	16 (8%)	0.93 (0.37–2.33)	0.871	ND
*10	8 (7%)	15 (8%)	0.88 (0.33–2.30)	0.779	ND
*11	8 (7%)	21 (11%)	0.61 (0.24–1.51)	0.247	ND
*12	9 (8%)	17 (9%)	0.87 (0.35–2.16)	0.751	ND
*13	6 (5%)	12 (6%)	0.82 (0.27–2.45)	0.707	ND
*14	9 (8%)	13 (7%)	1.17 (0.44–3.03)	0.732	ND
*15	2 (2%)	12 (6%)	0.27 (0.04–1.28)	0.066	ND
*16	8 (7%)	11 (6%)	1.23 (0.44–3.41)	0.668	ND
*17(03)	12 (10%)	8 (4%)	2.67 (0.98–7.40)	0.031	0.396
*18(03)	11 (9%)	8 (4%)	2.42 (0.87–6.83)	0.058	ND

Abbreviations: ND – not defined; OR – odds ratio; CI – confidence interval; p value – probability; pc value – after Bonferroni adjustment.

Among TBE patients the DRB1*04 (11 percent vs. 5 percent; odds ratio, 2.58; p = 0.03) and DRB1*17(03) (10 percent vs. 4 percent; odds ratio, 2.67; p = 0.031) alleles, had the greatest frequency (Table 2). While DRB1*01 frequency was lower (1 percent vs. 6 percent; odds ratio, 0.13; p = 0.017) in these patients.

Although, in patients the HLA-DRB1*04 and -DRB1*17(03) were increased, but the HLA-DRB1*01 was lower, these differences were not significant after Bonferroni correction (Table 2).

Therefore, the frequency of HLA-DRB1*04 was significantly greater in patients with *Lyme borreliosis* than in the control group (odds ratio, 3.22; p = 0.011); and in patients with tick-borne encephalitis comparing with the normal subjects (odds ratio, 2.58; p = 0.03) (Tables 1 and 2). Eight patients with *Lyme borreliosis* who had HLA-DRB1*04 were heterozygous at the DR locus. Four had HLA-DRB1*04 and HLA-DRB1*17(03), two had HLA-DRB1*04 and HLA-DRB1*15, one had HLA-DRB1*04 and HLA-DRB1*18(03), and one HLA-DRB1*04 and HLA-DRB1*11.

Of the 13 patients with TBE, who had HLA-DRB1*04, only two were homozygous and had severe meningoencephalitis. The other 11 patients were heterozygous at the DR locus. Four had HLA-DRB1*04 and HLA-DRB1*18(03), three had HLA-DRB1*04 and HLA-DRB1*17(03) and remaining had HLA-DRB1*04 in association with different DR alleles. A secondary association was noted with the HLA-DRB1*17(03) allele. This allele was found in 15 percent of the patients with *Lyme borreliosis* and in 10 percent of the patients with TBE, but in only 4 percent in controls group (Table 1 and Table 2). The frequency of HLA-DRB1*17(03) allele was significantly higher among *Lyme borreliosis* patients than among TBE (odds ratio, 4.06; p = 0.002 and odds ratio, 2.67; p = 0.031, respectively). Also, the frequency of HLA-DRB1*18(03) tended to be higher among Lyme borreliosis and TBE patients (8 and 9 percent vs. 4 percent; odds ratio, 2.06 and 2.42, respectively) but this difference was not statistically significant (Tables 1 and 2).

Of interest, the allele DRB1*10 (odds ratio, 0.16; p = 0.036) was smaller in *Lyme borreliosis* patients and significantly higher in controls (Table 1). Moreover, a different allele – DRB1*01 (odds ratio, 0.13; p = 0.017) was smaller in TBE patients and significantly higher in controls (Table 2). Probably, HLA-DRB1*10 showed a tendency to protection against *Lyme borreliosis*, bet DRB1*01 showed a tendency to protection against TBE in Latvian patients.

Discussion

Many studies have tried to identify genetic markers for infectious diseases; some of them have focused on HLA [4–6, 27, 28]. The products of HLA genes interact with surface-specific receptors of T lymphocytes, resulting in activation of the host's immune response. The association of TBD infections with the host's HLA has been partially investigated [29, 30]. The type and strength of this association differs among distinct populations, as well as among racial and/or ethnic groups [31].

In our HLA study, a strong association was confirmed between *Lyme borreliosis* and the HLA-DRB1*17(03). Although, the frequency of HLA-DRB1*04 allele was increased in patients with *Lyme borreliosis* and tick-borne encephalitis, after applying the Bonferroni correction, these differences were not significant.

One more statistically significant difference was found in patients with *Lyme borreliosis*. The frequency of HLA-DRB1*10 allele was significantly lower in *Lyme borreliosis* patients compared with the control group. These results suggest the high risk for tick-borne disease in Latvia associated with the HLA-DRB1*17(03), and perhaps, HLA-DRB1*04 alleles. The HLA-DRB1*10 allele seems to have a protective effect in patients in Latvia with *Lyme borreliosis*.

Although this series of 60 patients with tick-borne encephalitis is the largest tested to date, the number of patients was not large enough to show significant differences between TBE patients and control group in the frequencies of individual alleles.

There are several hypotheses about the HLA/disease association mechanism, and it is possible that this mechanism varies for different diseases. One of the hypotheses attributes a greater or less affinity of HLA for the disease-causing peptide [6, 12]. Thus, the HLA antigens function as receptors for some

etiological agents, by facilitating their entry into the cell or by making such entry difficult. Another possibility would be the early intervention of HLA in the thymic selection of lymphocytes, by determining which antigens will be presented to the T lymphocytes [20, 33]. There is also the hypothesis that there may be a mechanism of tolerance of T cells to these pathogens, through molecular mimicking between antigens of the infectious microorganisms and antigens of the host, thus providing susceptibility or protection against these diseases [32, 33]. The study reviewed the main associations of the HLA-DR alleles with *Lyme borreliosis* and tick-borne encephalitis.

The HLA alleles vary in ethnically different populations [2, 8, 9]. Studies suggest that the alleles that confer resistance to certain pathogens are prevalent in areas where they cause endemic diseases. Greater resistance to infectious diseases occurs in persons that are heterozygote for specific HLA alleles, because a heterozygous person would have a broader spectrum of peptides to link to the T lymphocytes [11, 13, 35]. These alleles also vary from one disease to another, due to the differences in their pathogenesis [34]. In our study, only two patients with TBE who had HLA-DRB1*04, were homozygous and had severe meningo-encephalitis. The diversity of these results is probably due to environmental influences; in addition, possible differences among ethnic group's populations [9].

Genetic studies of infectious diseases not only help us gain a better understanding of the pathogenic mechanisms of diseases, they may also help with the development of vaccines.

One of the advantages of polymorphism of the HLA region apparently is the ability to avoid deficiencies in efficient immune response against a specific infectious agent [34].

Susceptibility to an infectious disease may be due to imperfections in some stages of this system. A person that has a certain combination of HLA alleles that do not link in an appropriate manner to the peptide, or whose HLA-peptide link does not elicit an adequate response from the lymphocytes, will be less apt to resist the invasion of the infectious agent than a person who does not have these deficiencies [34]. In patients in whom HLA provides protection, these genes probably select and stimulate T cells that multiply and eliminate the invading agent, through the production of inflammatory cytokines or by destroying the infected cells themselves [35].

Conclusions

These results suggest that the inflammatory events of the subacute arthritis can set the stage for the development of chronic disease in individuals possessing an HLA susceptibility allele. In particular, the HLA-DRB1*17(03), and perhaps, HLA-DRB1*04 alleles contribute significantly to a genetic predisposition *Lyme borreliosis* in the population of Latvia, but the HLA-DRB1*10 allele could be associated with a potential protective effect. Knowledge of the mechanisms of genetic protection against, and susceptibility to infectious diseases is one of the important steps towards controlling them in endemic areas, and contributes to our understanding of both the pathogenic and protective mechanisms of these processes. The mechanisms of immune response to infection that are influenced by the HLA genes may be the key to future vaccines using the peptides of organisms that mimic the HLA antigens.

Acknowledgements

This research work was supported by Rīga Stradiņš University grant 09.1604.

References

1. Statistical data on incidence of infectious diseases in Latvia // http://www.ecdc.europa.eu/en/publications/publications/tbe-in-eu-efta.pdf (accessed on 25.01.2013).
2. Arsa F., Bormane A., Krumina A., Tick-borne encephalitis and Lyme borreliosis in Latvia: epidemiological situation in 2007–2009 // Abstract book ESCAIDE, 2010; p. 173.

3. Klein J., Sato A. The HLA system: second of two parts // N Engl J Med, 2000; 343 (11): 782–786.
4. Critical needs and gaps in understanding prevention, amelioration, and resolution of Lyme and other tick-borne diseases: the short-term and long-term outcomes // http://www.ncbi.nlm.nih.gov/books/NBK57024/ (accessed on 20.01.2013).
5. Steere A. C., Glickstein L. Elucidation of Lyme arthritis // Nat Rev Immunol, 2004; 4: 143–152.
6. Steere A. C., Klitz W., Drouin E. E., et al. Antibiotic-refractory Lyme arthritis is associated with HLA-DR molecules that bind a Borrelia burgdorferi peptide // J Exp Med, 2006; 203: 961–971
7. Jones E. Y., Fugger L., Strominger J. L., Siebold C. MHC class II proteins and disease: a structural perspective // Nat Rev Immunol, 2006; 6: 271–282.
8. Furusho J. K. Y. Papel de los genes del complexo principal de histocompatibilidad en los processos infecciosos // Rev Invest Clin, 2000; 52 (4): 461–466.
9. Probst C. M., Bompeixe E. P., Pereira N. F., et al. HLA polymorphism and evaluation of European, African, and Amerindian contribution to the white and mulatto populations from Paraná, Brazil // Hum Biol, 2000; 72: 597–617.
10. Jinam T. A., Saitou N., Edo J., Mahmood A., et al. Molecular analysis of HLA Class I and Class II genes in four indigenous Malaysian populations // Tissue Antigens, 2010; 75: 151–158.
11. Kovalchuka L., Eglite E., Lucenko I., et al. Associations of HLA-DR and DQ molecules with *Lyme borreliosis* in Latvian patients // BMC Res Notes, 2012; 5 (1): 438.
12. Steere A. C., Falk B., Drouin E. E., et al. Binding of outer surface protein A and human lymphocyte function-associated antigen 1 peptides to HLA–DR molecules associated with antibiotic treatment-resistant Lyme arthritis // Arthritis Rheum, 2003; 48: 534–540.
13. Gross D. M., Forsthuber T., Tary-Lehmann M., et al. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis // Science, 1998; 281: 703–706.
14. Tang W. M., Pulido J. S., Eckles D. D., et al. The association of HLA-DR15 and intermediate uveitis // Am J Ophthalmol, 2002; 123: 70–75.
15. Guerau-de-Arellano M., Huber B. T. Development of autoimmunity in Lyme arthritis // Curr Opin Rheumatol, 2002; 14: 388–393.
16. Steere A. C., Falk B., Drouin E. E., et al. Binding of outer surface protein A and human lymphocyte function-associated antigen 1 peptides to HLA-DR molecules associated with antibiotic treatment-resistant Lyme arthritis // Arthritis Rheum, 2003; 48: 534–540.
17. Chen J., Field J. A., Glickstein L., et al. Association of antibiotic treatment-resistant Lyme arthritis with T cell responses to dominant epitopes of outer surface protein A of Borrelia burgdorferi // Arthritis Rheum, 1999; 42: 1813–1822.
18. Iliopoulou B. P., Alroy J., Huber B. T. Persistent arthritis in Borrelia burgdorferi-infected HLA-DR4-positive CD28-negative mice post-antibiotic treatment // Arthritis Rheum, 2008; 58: 3892–3901.
19. Carter J. D., Espinoza L. R., Inman R. D., et al. Combination antibiotics as a treatment for chronic Chlamydia-induced reactive arthritis: a double-blind, placebo-controlled, prospective trial // Arthritis Rheum, 2010; 62: 1298.
20. Fernando M. M., Stevens C. R., Walsh E. C., et al. Defining the role of the MHC in autoimmunity: a review and pooled analysis // PLoS Genet, 2008; 4: e1000024.
21. Marrack P., Kappler J., Kotzin B. L. Autoimmune disease: why and where it occurs // Nat Med, 2001; 7: 899–905.
22. Shen C., Zhu B., Liu M., Li S. Genetic polymorphisms at HLA-A, -B, and -DRB1 loci in Han population of Xi'an city in China // Croat Med J, 2008; 49: 476–482.
23. Jones E. Y., Fugger L., Strominger J. L., Siebold C. MHC class II proteins and disease: a structural perspective // Nat Rev Immunol, 2006; 6: 271–282.
24. Klitz W., Maiers M., Spellman S. New HLA haplotype frequency reference standards: high-resolution and large sample typing of HLA DR-DQ haplotypes in a sample of European Americans // Tissue Antigens, 2003; 6: 296–307.
25. Olerup O., Aldener A., Fogdell A. HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) // Tissue Antigens, 1993; 41 (3): 119–134. 26. Categorical data analysis using the SAS system // www.google.com/books?hl=lv&lr=&id=JcOcTfRYCn4C&oi=fnd&pg=PA1&dq=assessed+by+Mantel+%E2%80%93+Haenszel+test+and+Fisher+exact+correction (accessed on 20.03.2012).
26. Crandall H., Dunn D. M., Ma Y., et al. Gene expression profiling reveals unique pathways associated with differential severity of Lyme arthritis // J Immunol, 2006; 177: 7930–7942.
27. Gross D. M., Steere A. C., Huber B. T. T helper 1 response is dominant and localized to the synovial fluid in patients with Lyme arthritis // J Immunol, 1998; 160: 1022–1028.
28. Iliopoulou B. P., Huber B. T. Emergence of chronic Lyme arthritis: putting the breaks on CD28 costimulation // Immunopharmacol Immunotoxicol, 2008; 5: 1–12.

29. Kotsch K., Wehling J., Blasczyk R. Sequencing of HLA class II genes based on the conserved diversity of the non-coding regions: sequencing based typing of HLA-DRB genes // Tissue Antigens, 1999; 53: 486–497.
30. Lee K. W., Oh D. H., Lee C., Yang S. Y. Allelic and haplotypic diversity of HLA-A, -B, -C, -DRB1, and -DQB1 genes in the Korean population // Tissue Antigens, 2005; 65: 437–447.
31. Shin J. J., Glickstein L. J., Steere A. C. High levels of inflammatory chemokines and cytokines in joint fluid and synovial tissue throughout the course of antibiotic-refractory Lyme arthritis // Arthritis Rheum, 2007; 56: 1325–1335.
32. Yssel H., Shanafelt M. C., Soderberg C., et al. Borrelia burgdorferi activates a T helper type 1-like T cell subset in Lyme arthritis // J Exp Med, 1991; 174: 593–601.
33. Alves C., Souza T., Meyer I., et al. Immunogenetics and infectious diseases: special reference to the mayor histocompatibility complex // Braz J Infect Dis, 2006; 10: 122–131.
34. Drouin E. E., Glickstein L., Kwok W. W., et al. Searching for borrelial T cell epitopes associated with antibiotic-refractory Lyme arthritis // Mol Immunol, 2008; 45: 2323–2332.

Authors