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Dagnija Kalniete

MICRORNA EXPRESSION AS A PROGNOSTIC INDICATOR FOR BREAST CANCER DEVELOPMENT

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Speciality – Molecular Biology

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The Doctoral Thesis is available at RSU Library and RSU website: www.rsu.lv

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LIST OF ABBREVATIONS

BRCA1 – Breast cancer susceptibility gene 1

BRCA2 – Breast cancer susceptibility gene 2

ER – Estrogen receptor

H – Hereditary

HER2 – Human Epidermal Growth Factor Receptor 2

LA – Luminal A

LB – Luminal B

MicroRNA – Microribonucleicacids

Mrna – messenger RNA

PTEN – Phosphate and tensin homolog

PR – Progesterone Receptor

RNA – Ribonucleicacid

S – Sporadic

TN – Triple-negative

TN – H Triple-negative hereditary

TN – S Triple-negative sporadic

1. INTRODUCTION

Breast cancer is one of the most common malignancy and the most frequent cause of death among woman worldwide [*Jemal*, 2011]. More than 1000 new cases each year are diagnosed in Latvia [*SKPSC, 2013*]. Breast cancer is clinically, morphologically and genetically heterogeneous disease and the pace of the disease, response to therapy, side effects and the outcome depends on the heterogeneous nature of the disease. To decrease mortality rate and reduce possible side effect prognostic and predictive biomarkers must be used. These biomarkers potentially could predict the pace of the disease, possible side effects and provided possibly best outcome. Currently used imunohistochemical biomarkers are not enough informative to predict the outcome of the disease or efficacy of the therapy hence new, more informative biomarkers are required. Such potential biomarkers could be microRNAs- in a length of approximately 18–20 nucleotides noncoding molecules that regulate gene expression at the post transcriptional level. [*Calin*, 2002]. These molecules are involved in such critical cell events as differentiation, growth and apoptosis hence these molecules play an important role in the pathogenesis of the cancer [*Calin*, 2002; *Cannell*, 2008]. Expression of the different microRNAs in cancer tissues is altered and this changed expression correlates with clinical and pathophysiological features of cancer. MicroRNAs can act similarly as oncogenes or tumor suppressor genes and expression is either up-regulated or down-regulated [*Iorio*, 2005; *Heneghan*, 2010]. One of the most studied microRNA with the oncogenic potential which consistently is up-regulated in wide variety of cancers, including breast cancer, is miR-21. Up-regulated expression of miR-21 correlates with more advanced breast cancer stage, positive lymph node status, and high proliferation index Ki67 and overall bad prognosis for the patient [*Yan*, 2008; *Huang*, 2009]. By inhibiting the tumor suppressor tropomyosin-1 gene (*TPM1*) and programmed cell death gene-4

(*PDCD4*), miR-21 is directly involved in the growth, proliferation, and invasion of the tumor cells [*Li*, 2012]. Another target of miR-21 is phosphatase and tensin homolog gene (*PTEN*) that is involved in the PI3K/Akt pathway [*Li*, 2012]. Some of the miRNAs have shown different expressions not only within the specific subtype of breast cancer but among distinct subtypes of breast cancer, as well. MiR-210 is differently expressed between TN and ER positive/HER2 negative breast cancers: higher expression is in TN breast cancers than in ER positive/HER2 negative breast cancers [*Radojicic*, 2011]. A non-significant tendency of high expression level of miR-210 and other miRNAs (miR-21, miR-221, and miR-222) is related to worse overall and disease-free survival of TN breast cancer patients [*Radojicic*, 2011].

The triple-negative (TN) morphology of breast tumor is found in the 57% of patients with the *BRCA1* gene mutations and 23% of patients with the *BRCA2* gene mutations [*Atchley*, 2008]. TN breast cancers are referred to estrogen receptor (ER) negative, progesterone receptor (PR) negative, and human epidermal growth factor receptor (HER2) negative tumors and they have tendency to be more aggressive than other subtypes [*Dent*, 2007; *Bayraktar*, 2011]. TN breast cancer patients harboring the *BRCA1* mutations at the time of the diagnosis are younger, have smaller tumor size, and have significantly better recurrence-free and disease-specific survival than TN breast cancer patients with no mutations in the *BRCA1* gene [*Gershoni-Baruch*, 1999; *Frankel*, 2008; *Heneghan*, 2010; *Gonzalez-Angulo*, 2011]. In the basal type of breast cancers two signaling pathways- JUN/MAPK and PI3K/AKT are altered [*Guille*, 2013]. Mutations in the *PTEN*, *PIK3CA*, *AKT*, and *MAGI3-AKT3* gene activate PI3K/AKT signaling pathway whereas mutations in the *MAP3K1*, *MAP3K13*, and *MAP2K4* gene inactivate JUN/MAPK signaling pathway [*Banerji*, 2012; *Elis*, 2012; *Shah*, 2012, *The Cancer Genome Atlass Network*, 2012].

The aim of this study was to look for the miRNA that differs in expression between TN hereditary and sporadic tumors and associate expression level of some miRNA to overall survival of TN breast cancer patients.

1.1. The aim of the study

To identify microRNAs that are differently expressed between triple-negative hereditary and triple-negative sporadic breast cancers and find correlation between microRNAs and gene expression.

1.2. The tasks of the study

1) Determine miRNA (miR-10b, miR-21, miR-29a, miR-31, and miR-214) expression levels in breast cancer and in normal control tissues.
2) Determine miRNA level of expression of different types of breast tumors.
3) Compare miRNA expression level of hereditary and sporadic breast cancer tissues.
4) Determine the relationship between miRNA expression levels and overall survival of breast cancer patients.
5) Identify the differences between genes expressed in TN-H and TN-S breast tumors.
6) Identify the differently expressed genes in association with miRNA expression.

1.3. Hypothesis

MicroRNA expression is a prognostic indicator for breast cancer development.

1.4. Novelty of this study

In this study between TN-H and TN-S breast cancer tissue differently expressed microRNAs are found. In addition, high expression of miR-214 in TN tumor tissues is associated with worse overall survival of breast cancer patients. In this study, 22 genes that are expressed differently between TN-H and TN-S breast cancer tissues are found. In addition, three of the genes: *C12ORF23*, *C1ORF19* and *AMMECR1*L are regulated by miR-21 and miR-214.

2. MATERIAL AND METHODS

2.1. The study design

The study was based on retrospective microRNA (miR-10b, miR-21, miR-29a, miR-31 and miR-214) and 25000 different gene expression analysis in the tumor tissues of the breast cancer patients, who had signed the informed consent forms.

In this study a correlation between microRNA expression in tumor tissue and overall survival in patients whose clinical data were available was determined by retrospective analysis.

2.2. The study group

In this study 72 breast cancer patients from the 2004 to 2011 that were hospitalized in P. Stradins Clinical University Hospital and had signed informed consent forms were included.

The study group consisted of two subgroups: hereditary breast cancer patients and sporadic breast cancer patients. This study included patients who met the criteria which are presented in the table 2.2.1. Exclusion criteria are presented in table 2.2.2.

The study group consisted of 72 tumor and 57 lines (breast epithelial) tissue samples. Tumor group consisted of 24 hereditary breast cancers and 48 sporadic breast cancer tissues. Hereditary breast cancer group consisted of 4 LA, 1 LB, 1 HER2, and 18 TN breast cancer tissue. Sporadic breast cancer group was selected according to the molecular subtypes of hereditary breast cancer group. Sporadic breast cancer group consisted of 9 LA, 6 LB, 1 HER2, and

32 TN tumor tissues. Resection line was used as a control group to evaluate whether expression of microRNAs are altered.

Table 2.2.1.

Including Criteria

Hereditary breast cancer patients	Sporadic breast cancer patients
Mutation in the *BRCA1* or *BRCA2* gene.	No mutation in the *BRCA1* or *BRCA2* gene.
	In the family history no data about HBC/HBOC syndrome.
Available cancer FFPE tissues.	Available cancer FFPE tissues
FFPE tissues younger than 7 years	FFPE tissues younger than 7 years
Contains more than 50% of cancer cells per sample.	Contains more than 50% of cancer cells per sample.
Available healthy epithelial FFPE tissues.	Available healthy epithelial FFPE tissues.
Available clinical data and information about molecular subtype.	Available clinical data and information about molecular subtype.
From the year 2004 to 2011 hospitalized in Pauls Stradins Clinical University Hospital.	From the year 2004 to 2011 hospitalized in Pauls Stradins Clinical University Hospital.
Signed informed consent forms.	Signed informed consent forms.
	Match molecular subtypes of hereditary group.

Table 2.2.2.

Excluding Criteria

Hereditary breast cancer patients	Sporadic breast cancer patients
No mutation in the *BRCA1* or *BRCA2* gene.	In the family history data about HBC/HBOC syndrome.
Contains less than 50% of cancer cells per sample.	Contains less than 50% of cancer cells per sample.
FFPE tissues older than 7 years	FFPE tissues older than 7 years

2.3. Clinical data

The clinical data were obtained from P. Stradins Clinical University Hospital medical records and PREDA database. Immunohistochemical data: PR, ER, HER2, Ki67 expression, histological/ molecular subtypes and differentiations (G) were obtained from the medical records. Information TNM stage, clinical stage (I to IV) at the time of the diagnosis and tumor dynamics (relapse and death) was obtained from the PREDA database. Tumor size was

determined by the T stage according to the AJCC (American Joint Committee on Cancer) guidelines. Tumors, which at the time of diagnosis was T1 and T2 were defined as less than 5 cm, while the tumors, which at the time of diagnosis was T3 and T4 was defined more than 5 cm.

2.4. RNA extraction from formalin fixed and paraffin embedded tissues

RNAs from the formalin-fixed and paraffin tissues were extracted with the Total Nucleic Acid Isolation Kit (Life Technologies) according to the manufacturer's instructions.

2.5. RNA reverse transcription

RNA reverse transcription (RT) was carried out with the TaqMan MicroRNA Reverse Transcription Assays (Life Technologies) according to the manufacturer's instructions.

RT reaction mixture per reaction (total volume 15 µl) was prepared according to the following protocol:

10 mM dNTP (with dTTP)	0.15 µl
MultiScribe reverse transcriptase (50 u/ µl)	1.00 µl
10X reverse transcriptase buffer	1.50 µl
RNase inhibitor (20u/µl)	0.19 µl
Water	4.16 µl
RNA	5.00 µl
RT Primer * ……..	3.00 ml

RT* primers are listed in the table 2.5.1.

RT reaction mixture was placed in the thermal cycler and incubated at the conditions specified in the table 2.5.2.

Table 2.5.1.

RT Primers

Primer	Type
RNU6B reference	TaqMan MicroRNA RT Assays
hsa-miR-10b	TaqMan MicroRNA RT Assays
hsa-miR-21	TaqMan MicroRNA RT Assays
hsa-miR-29a	TaqMan MicroRNA RT Assays
hsa-miR-31	TaqMan MicroRNA RT Assays
hsa-miR-214	TaqMan MicroRNA RT Assays

Table 2.5.2.

RT Conditions

Type of the step	Time	Temperature
Incubation	30 min	16 ^{o}C
Incubation	30 min	42 ^{o}C
Incubation	5 min	85 ^{o}C
Incubation	Pause	4 ^{o}C

2.6. Quantitative analysis of microRNAs using real-time polymerase chain reaction

MicroRNAs (miR-10b, miR-21, miR-29a, miR-31 and miR-214) quantity in the tumor tissues and resection line of tissues was determined by TaqMan MicroRNA TaqMan Universal PCR Assays (Life Technologies) using real-time polymerase chain reaction (RT-PCR). RT-PCR mixture per reaction (total volume 20 µl) was prepared according to the following protocol:

RT-PCR primer *	1.00 µl
RT product	1.30 µl
2X TaqMan Universal PCR Master Mix	10.00 µl
Water	7.70 µl

* RT-PCR primers listed in table 2.6.1.

Table 2.6.1.

RT-PCR Primers

Primer	Type
RNU6B reference	TaqMan MicroRNA RT-PCR Assays
hsa-miR-10b	TaqMan MicroRNA RT-PCR Assays
hsa-miR-21	TaqMan MicroRNA RT-PCR Assays
hsa-miR-29a	TaqMan MicroRNA RT-PCR Assays
hsa-miR-31	TaqMan MicroRNA RT-PCR Assays
hsa-miR-214	TaqMan MicroRNA RT-PCR Assays

The quantitative amount of RNU6B, miR-10b, miR-21, miR-29a, miR-31 and miR-214 in tumor and resection line tissues was determined using the reaction conditions shown in the table 2.6.2. Each sample was performed in three replicates. Relative expression of each microRNAs was determined by comparative quantification method.

Table 2.6.2.

RT-PCR Conditions

Type of the step	Time	Temperature
Incubation	10 min	95 ^{o}C
Amplification	40x	
Denaturation	15 sec	95 ^{o}C
Elongation	60 sec	60 ^{o}C*

* Signal was captured in the FAM channel at the wavelength of 494 to 518 nm

2.7. Gene expression profile

Gene expression profile of TN breast cancer tissues were determined by Whole-Genome Gene Expression DASL HT chip (Illumina) according to the manufacturer's instructions.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad InStat 3 statistical software and GraphPad Prism 6 software.

Each sample was performed in three repeats. The expression levels were determined with the Rotor-Gene Q Series Software 1.7 using comparative quantitation analysis. Each miRNA was normalized by the internal reference RNU6B.

The overall survival was evaluated from the date of the diagnosis till the date of the death from the malignancy. The overall survival was analyzed using the Log-rank (Mantel-Cox) test. The level of the statistical significance was set at the 95%. The median follow-up period of the breast cancer patients was 46 months. Mann–Whitney test was used to calculate miRNA expression differences between two groups. Clinical and pathological characteristics between the *BRCA1* gene mutation carriers and non-carriers were compared by T-test and Fisher's exact test.

3. RESULTS

3.1. Patient characteristics and clinical data comparison between hereditary and sporadic breast cancer patients

A clinical data analysis for the hereditary and sporadic breast cancer patients was performed. The median age (range) of hereditary breast cancer patients at the time of the diagnosis was 46 (27–76) years, while the median age (range) at the time of the diagnosis of sporadic breast cancer patients was 58 (28–78) years. Hereditary breast cancer patients at the time of the diagnosis was significantly younger than sporadic breast cancer patients ($p = 0.0004$, t-test). Hereditary breast cancer patients with c.5266dupC mutation oh the *BRCA1* gene were 69.57%, with c.4034delA mutation were 26.09%, and with c.181T> G mutation was 4.35%. Comparing the T stage between the hereditary and sporadic groups, no statistically significant differences

($p = 0.4726$, Fisher's exact test) were found. Comparing N and M stages between hereditary and sporadic groups, no statistically significant differences were observed ($p = 0.4432$, Fisher's exact test) and ($p = 0.4577$, Fisher's exact test), respectively. There were no statistically significant differences in the clinical stage between both groups ($p = 0.0952$, Fisher's exact test). No statistically significant difference in regard of molecular subtypes between both groups were observed ($p = 0.6713$, Fisher's exact test). Hereditary breast cancer patients showed statistically significantly higher median Ki67 proliferation index than sporadic breast cancer patients ($p = 0.0175$, Fisher's exact test). Hereditary breast cancer patients more frequently had tumors with lower differentiation than sporadic breast cancer patients, but observation did not reach statistical significance ($p = 0.0555$, Fisher's exact test). Clinical characteristics are shown in the table 3.1.1.

Table 3.1.1.

Clinical data comparison between hereditary and sporadic breast cancer patients

Characteristics	Hereditary N=24 N (%)	Sporadic N=48 N (%)	P value
Medium age (range)	46 (27–76)	58 (28–78)	0.0004
BRCA1 gene mutations c.5266dupC c.4034delA c.181T>G *BRCA2* gene mutations 886delTG	16 (69.57) 6 (26.09) 1 (4.35) 1 (100)	-	-
T stage T_1 T_2 T_3 T_4 No data	 10 (41.67) 8 (33.33) 2 (8.33) 3 (12.50) 1 (4.17)	 12 (25.00) 20 (41.67) 8 (16.67) 8 (16.67) -	0.4726
N stage N_0 N_1 N_2 N_3 No data	 9 (37.50) 2 (8.33) 6 (25.00) 1 (4.17) 6 (25.00)	 20 (41.67) 12 (25.00) 9 (18.75) 6 (12.50) 1 (2.08)	0.4432
M stage M_0 M_1 No data	 16 (66.67) 1 (4.17) 7(29.17)	 47 (97.92) 1 (2.08) -	0.4577
Stage I II III IV	 9 (37.50) 7 (29.16) 6 (25.00) 1 (4.17)	 7 (14.58) 21 (43.75) 19 (39.58) 1 (2.08)	0.0952

Table 3.1.1. Continuation

Characteristics	Hereditary N=24 N (%)	Sporadic N=48 N (%)	P value
Histological type D L M P D+M D+P No data	 14 (58.33) 1 (4.17) 4 (16.67) 1 (4.17) 1 (4.17) 1 (4.17) 2 (8.33)	 42 (87.50) 1 (2.08) 2 (4.17) - 1 (2.08) 1 (2.08) 1 (2.08)	0.0670
Differentiation G2 G3 No data	 6 (25.00) 13 (54.17) 5 (20.83)	 3 (6.25) 31 (64.58) 14 (29.17)	0.0555
Tumor size ≤ 50 mm > 50 mm No data	 18 (75.00) 5 (20.83) 1 (4.17)	 32 (66.67) 16 (33.33) -	0.2709
Ki67 (%) Median (range)	 70 (5–97)	 52 (2–98)	0.0175
Molecular subtype LA LB HER2 TN	 4 (16.67) 1 (4.17) 1 (4.17) 18 (75.00)	 9 (18.75) 6 (12.50) 1 (2.08) 32 (66.67)	0.6713
Relapse Yes No No data	 4 (16.67) 18 (75.00) 2 (8.33)	 3 (6.25) 45 (93.75) -	0.1949
Death Yes No No data	 1 (4.17) 21 (87.50) 2 (8.33)	 8 (16.67) 40 (83.33) -	0.1276

D – Ductal; L-lobular; M – medullary, P – papillary

3.2. MikroRNS expression differences in breast cancer tissues and normal control tissues

3.2.1. MicroRNA expression in tumor and healthy control tissues

Relative expression of five different microRNAs (miR-10b, miR-21, miR-29a, miR-31 and miR-214) in breast cancer tissue and normal control tissues were compared. The median ± interquartile range (Q1, Q3) expression in breast cancer tissue of miR-10b, miR-21, miR-29a, miR-31 and miR-214 was 0.315 ± 0.320 (0.170, 0.490), 5.920 ± 7.688 (2.587; 10.275), 1,415 ± 1,300 (0,710, 2,010), 0.260 ± 520 (0.105, 0.625) and 1.110 ± 1.195 (0.595, 1.790), respectively. The median ± interquartile range (Q1, Q3) expression in healthy control tissue of miR-10b, miR-21, miR-29a, miR-31 and miR-214 were 0.215 ± 0.233 (0.142, 0.375), 0.720 ± 0.820 (0.467; 1.287), 0.558 ± 0.493 (0.432, 0.925), 0.034 ± 0.100 (0.012, 0.112) and 0.803 ± 0.633 (0.487, 1.120), respectively. Expression of miR-21, miR-29a, miR-31 and miR-214 was significantly higher in tumor than in healthy control tissues ($p<0.0001$, $p<0.0001$, $p<0.0001$, and $p = 0.002$, respectively; Mann–Whitney test) (from figures 3.2.1.1. to 3.2.1.4.).

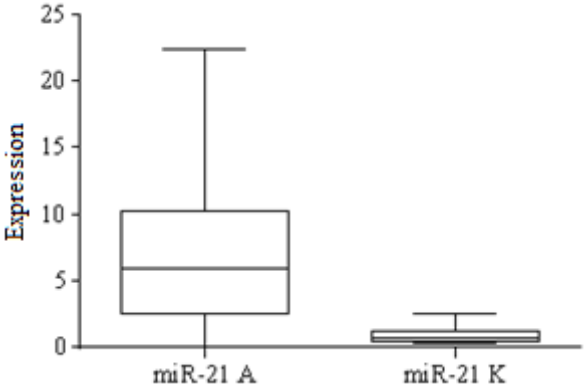


Fig. 3.2.1.1. **Relative expression of miR-21 in tumor (A) and healthy control (K) tissues; p<0.0001**

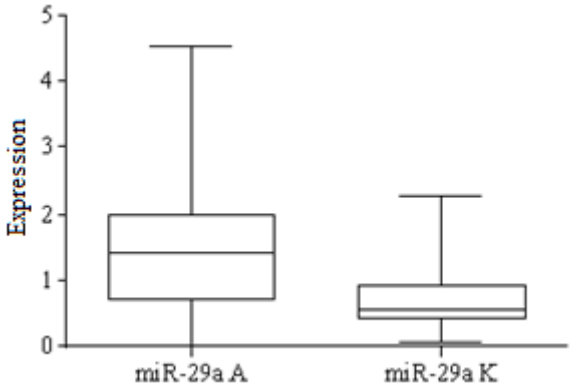
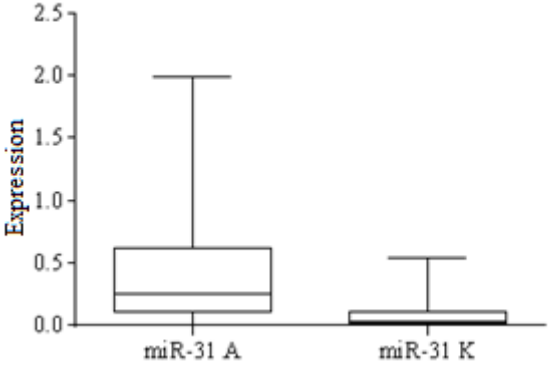


Fig. 3.2.1.2. **Relative expression of miR-29a in tumor (A) and healthy control (K) tissues; p<0.0001**



3.2.1.3. Fig. **Relative expression of miR-31 in tumor (A) and healthy control (K) tissues; p<0.0001**

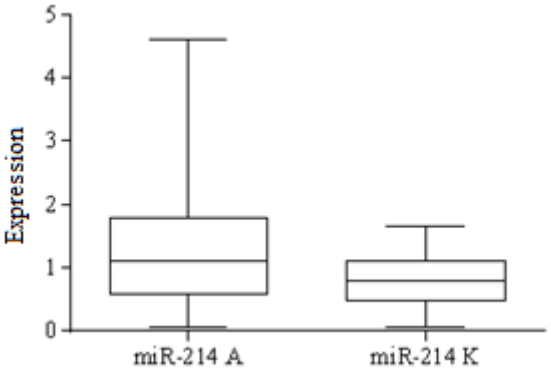


Fig. 3.2.1.4. **Relative expression of miR-214 in tumor (A) and healthy control (K) tissues; p = 0.002**

In the case of miR-10b, no statistically significant differences between tumor and healthy control tissues were observed (p = 0.081; Mann–Whitney test) (figure 3.2.1.5.).

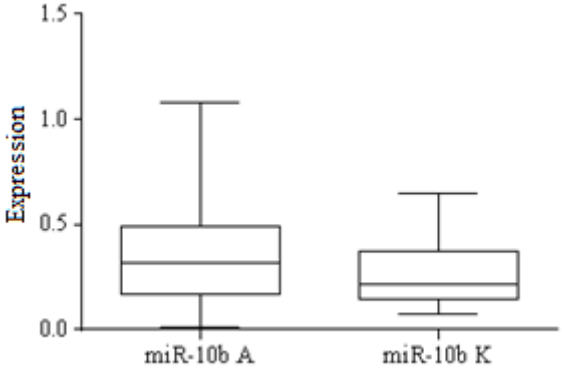


Fig. 3.2.1.5. **Relative expression of miR-10b in tumor (A) and healthy control (K) tissue; p = 0.081**

3.2.2. Overall survival of breast cancer patients in the regard of high and low microRNA expression

High and low expression levels of four different microRNAs: miR-21, miR-29a, miR-31, and miR-214 in tumor tissues in relation with overall

survival of the breast cancer patients was analyzed. High relative expression was defined value that was higher than the median expression value and low expression was defined the value that was lower than the median expression. MicroRNA: miR-21, miR-29a, miR-31 and miR-214 median expression value in breast cancer tissues was 5.960, 1.400, 0.280, and 1.120, respectively. Breast cancer patients with a high level of miR-31 and miR-214 in breast cancer tissues showed a trend of worse overall survival than patients with low expression (HR = 0.283, 95% CI: 0.076 to 1.052, p = 0.0596) and (HR = 0.413, 95% CI: 0.111 to 1.542, p=0.188), respectively (results shown in figures 3.2.2.1. and 3.2.2.2.).

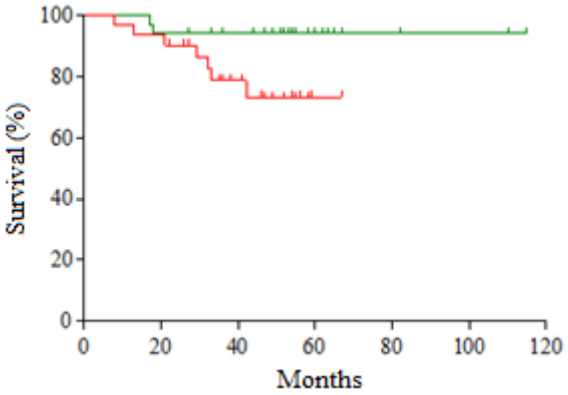


Fig. 3.2.2.1. **Overall survival of breast cancer patients with high and low miR-31 expression; p = 0.0596**
Orange line – high expression; Green line – low expression

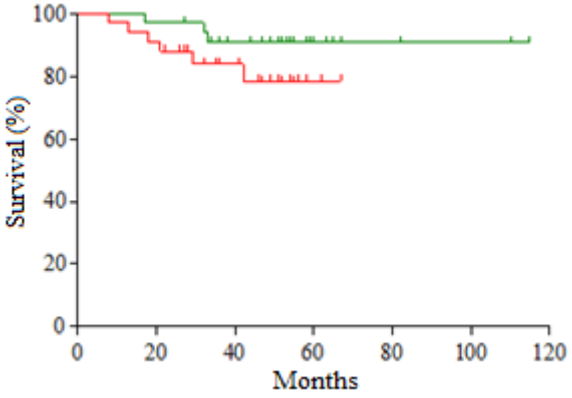


Fig. 3.2.2.2. **Overall survival of breast cancer patients with high and low miR-214 expression; p = 0.188**
Orange line – high expression; Green line – low expression

No statistically significant differences with high and low miR-21 and miR-29a expression in regard of overall survival was observed (HR = 0.744, 95% CI: 0.201–0.754, p = 0.658) and (HR = 0.397, 95% CI: 0.090 to 1.752, p = 0.222) (results shown in figures 3.2.2.3. and 3.2.2.4.).

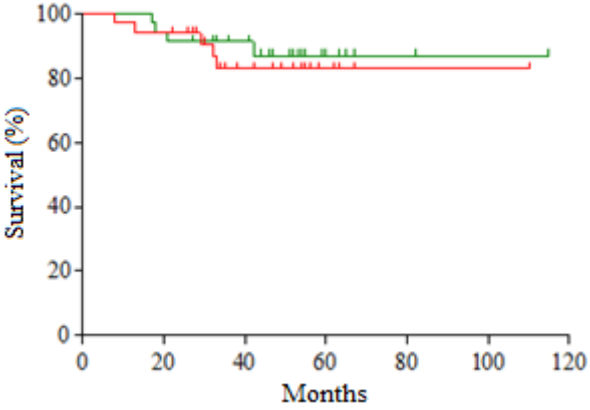


Fig. 3.2.2.3. **Overall survival of breast cancer patients with high and low miR-21 expression; p = 0.658**
Orange line – high expression; Green line – low expression

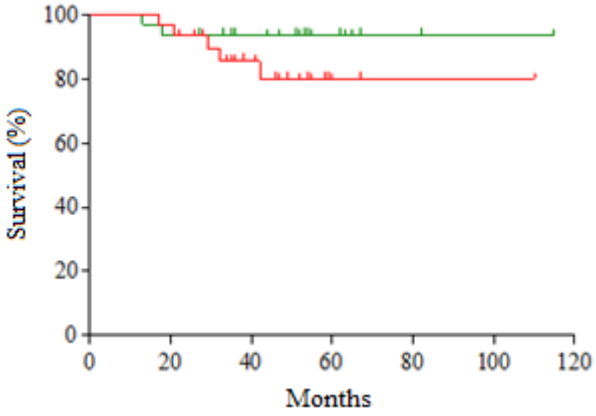


Fig. 3.2.2.4. **Overall survival of breast cancer patients with high and low miR-29a expression; p = 0,222**
Orange line – high expression; Green line – low expression

3.2.3. MicroRNA expression in hereditary and sporadic breast cancer tissues

Expression of five different microRNAs (miR-10b, miR-21, miR-29a, miR-31 and miR-214) in hereditary and sporadic breast cancer tissues were compared. The median ± interquartile range (Q1, Q3) expression of miR-10b, miR-21, miR-29a, miR-31 and miR-214 in hereditary breast cancer tissues was 0.320 ± 0.290 (0.135, 0.425), 5.640 ± 4.540 (2.520, 7.060), 1.330 ± 1.500 (0.530, 2.030), 0.220 ± 0.455 (0.042, 0.497) and 0.755 ± 0.910 (0.417, 1.327). The median ± interquartile range (Q1, Q3) expression of miR-10b, miR-21, miR-29a, miR-31 and miR-214 in sporadic breast cancer tissues was 0.305 ± 0.370 (0.200, 0.570), 6.550 ± 8.940 (2.610, 11.550), 1.430 ± 1.255 (0.800, 2.055), 0.300 ± 0.510 (0.125, 0.635) and 1.325 ± 1.362 (0.778, 2.140), respectively. Expression of miR-214 was significantly higher in sporadic breast cancer tissues than in hereditary breast cancer tissue (p = 0.003) (results shown in 3.2.3.1. figure).

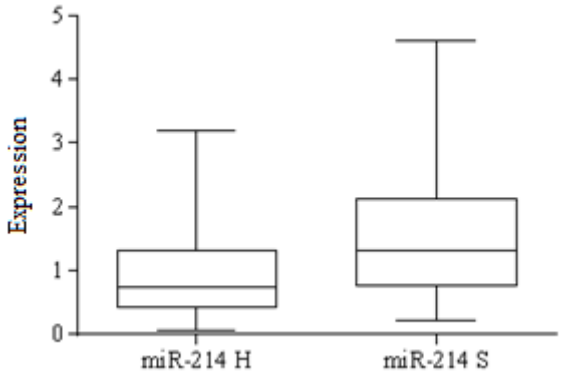


Fig. 3.2.3.1. **Relative expression of miR-214 in hereditary (H) and sporadic (S) breast cancer tissues; p = 0.003**

No statistically significant differences between hereditary and sporadic breast cancer tissues in the case of miR-10b, miR-21, miR-29a and miR-31 was observed (p = 0.431, p = 0.332, p = 0.909 and p = 0.188, respectively) (results shown from figures 3.2.3.2. to 3.2.3.5.).

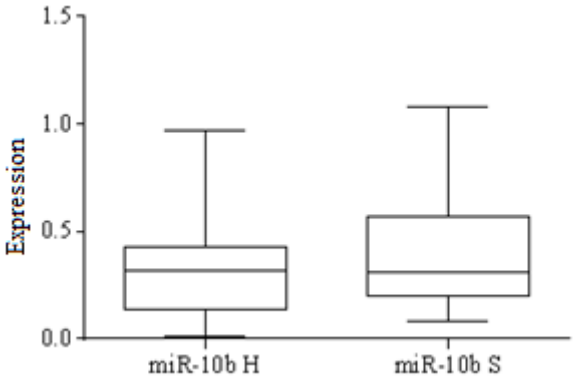


Fig. 3.2.3.2. **Relative expression of miR-10b in hereditary (H) and sporadic (S) breast cancer tissues; p = 0.431**

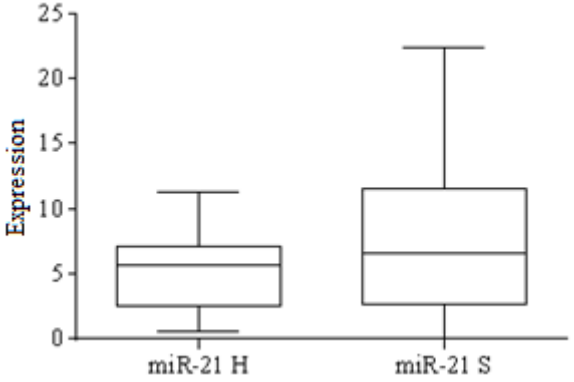


Fig. 3.2.3.3. **Relative expression of miR-21 in hereditary (H) and sporadic (S) breast cancer tissues; p = 0.332**

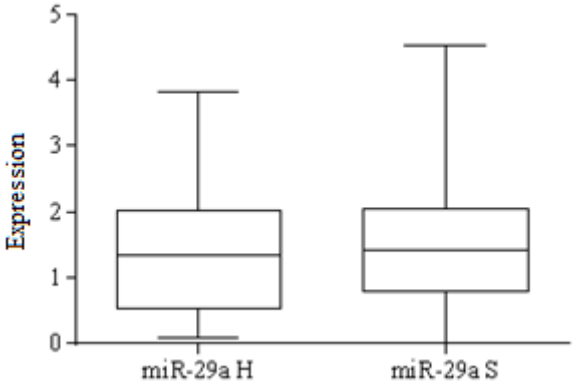


Fig. 3.2.3.4. **Relative expression of miR-29a in hereditary (H) and sporadic (S) breast cancer tissues; p = 0.909**

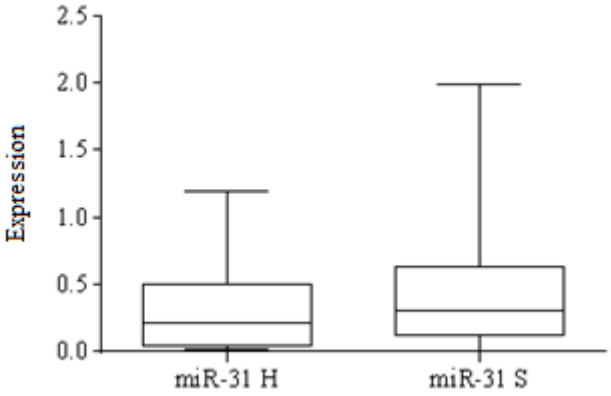


Fig. 3.2.3.5. **Relative expression of miR-31 in hereditary (H) and sporadic (S) breast cancer tissues; p = 0.188**

3.2.4. Overall survival in regard of high and low miR-214 expression in sporadic breast cancer tissues

The overall survival in regard of high and low miR-214 expression in sporadic breast cancer tissues was evaluated. High relative expression was defined value that was higher than median expression of miR-214 and low was defined value that was below median expression. The median expression value of miR-214 in sporadic breast cancer tissues was 1.370. Sporadic breast cancer patients with high expression of miR-214 had a non-significant trend of having worse overall survival compared to those with low expression of miR-214 (HR = 0.421 95% CI: 0.102 to 1.734, p = 0.231) (results shown in figure 3.2.4.1.).

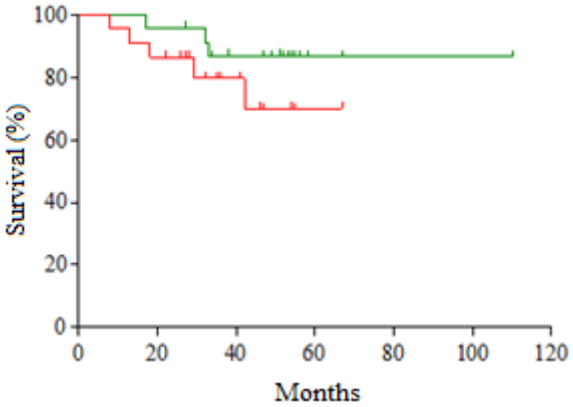


Fig. 3.2.4.1. **Overall survival in regard of high and low expression of miR-214 in sporadic tissues; p = 0.231**
Orange line – high expression; Green line – low expression

3.2.5. MicroRNA expression in TN and LA, LB and HER2 breast cancer tissues

Expression of five different microRNAs (miR-10b, miR-21, miR-29a, miR-31, and miR-214) in TN versus LA, LB, and HER2 tissues was analyzed. The median ± interquartile range (Q1, Q3) expression of miR-10b, miR-21, miR-29a, miR-31, and miR-214 in TN breast cancer tissues was 0.325 ± 0.363 (0.192, 0.555), 6.990 ± 9.595 (2.855, 12.450), 1,485 ± 1,330 (0.832, 2.162), 0,490 ± 0,610 (0.210, 0.820) and 1.455 ± 1.645 (0.607, 2.252), respectively. The median ± interquartile range (Q1, Q3) expression of miR-10b, miR-21, miR-29a, miR-31, and miR-214 in LA, LB and HER2 breast cancer tissues was 0.240 ± 0.275 (0.120, 0.395), 3.090 ± 4.215 (1.877, 6.092), 0.975 ± 1.138 (0.510, 1.648), 0.155 ± 0.155 (0.087, 0.242) and 0.945 ± 0.585 (0.575, 1.160), respectively. Expression of miR-21, miR-31, and miR-214 was significantly higher in TN tissues than in LA, LB and HER2 breast cancer tissues ($p = 0.002$, $p < 0.0001$ and $p = 0.012$, respectively; Mann–Whitney test) (results shown from figures 3.2.5.1. to 3.2.5.3.).

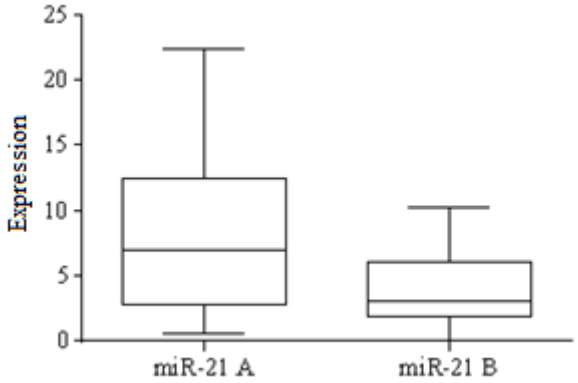


Fig. 3.2.5.1. **Relative expression of miR-21 in TN (A) and LA, LB and HER2 (B) breast cancers; p=0.002**

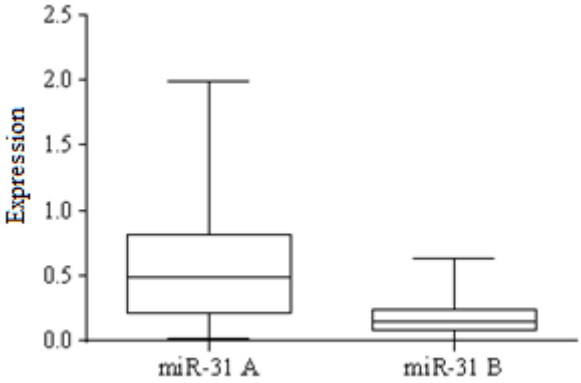


Fig. 3.2.5.2. **Relative expression of miR-31 in TN (A) and LA, LB and HER2 (B) breast cancers; p<0.0001**

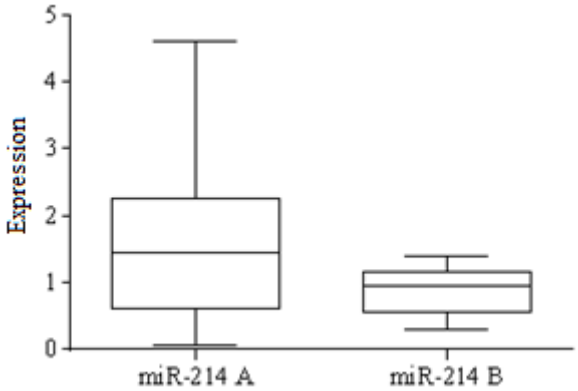


Fig. 3.2.5.3. **Relative expression of miR-214 in TN (A) and LA, LB and HER2 (B) breast cancers; p = 0.012**

No statistically significant differences between TN and LA, LB, and HER2 group in the case of miR-10b and miR-29a was observed (p = 0.190 and p = 0.171, respectively) (results are shown in figures 3.2.5.4. and 3.2.5.5).

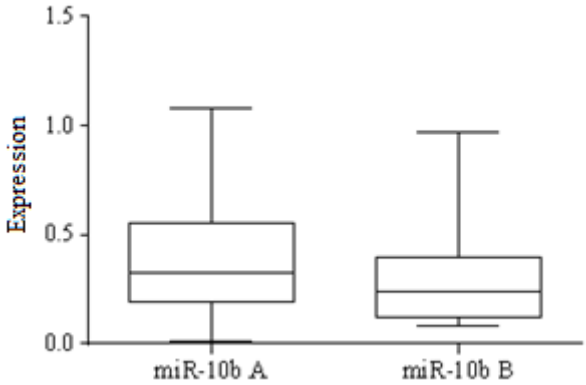


Fig. 3.2.5.4. **Relative expression of miR-10b in TN (A) and LA, LB and HER2 (B) breast cancers; p = 0.190**

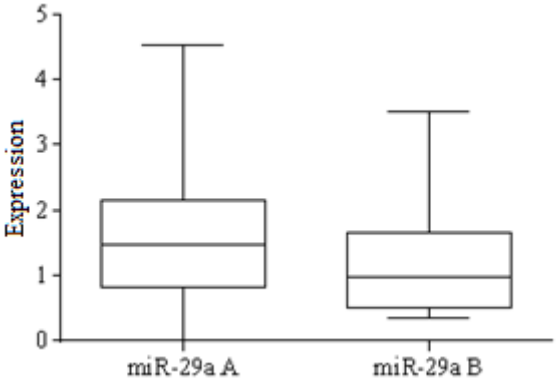


Fig. 3.2.5.5. **Relative expression of miR-29a in TN (A) and LA, LB and HER2 (B) breast cancers; p = 0.171**

3.2.6. Overall survival of TN breast cancer patients in regard of high and low microRNA expression

Overall survival of TN breast cancer patients in regard of high and low microRNA expression in cancer tissues was evaluated. High expression was defined value above median expression in TN tissues whereas low expression was defined value below median expression. The median expression of miR-21, miR-31 and miR-214 in TN breast cancer tissues was 6.99, 0.48 and 1.45, respectively. TN breast cancer patients with a high level of mir-214 expression was significantly worse overall survival than patients with a low miR-214 expression (HR = 5.152, 95% CI: 1.158 to 22.930, p = 0.0314) (Figure 3.2.6.1.).

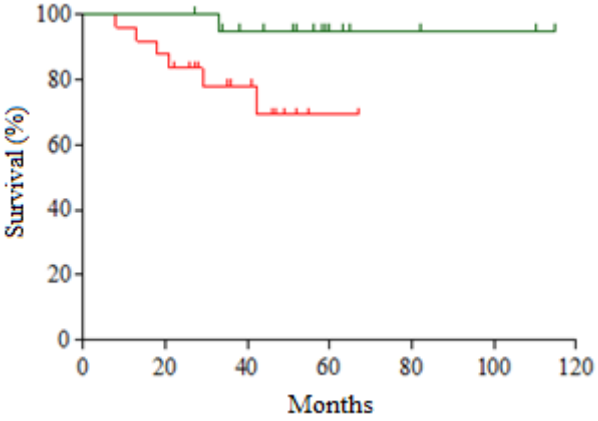


Fig. 3.2.6.1. **Overall survival of TN breast cancer patients in regard of high and low miR-214 expression; p = 0.0314**
Orange line – high expression; Green line – low expression

TN breast cancer patients with high and low expression of miR-21 and miR-31 do not have a statistically significant differences in overall survival (HR = 1.443, 95 % CI: 0.328 to 6.367, p = 0.628) and (HR = 2.622, 95% CI: 0.591 to 11.630, p = 0.205), respectively (results are shown in figures 3.2.6.2. and 3.2.6.3.).

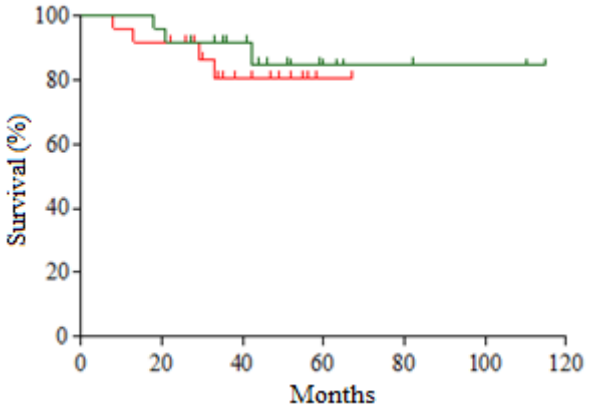


Fig. 3.2.6.2. **Overall survival of TN breast cancer patients in regard of high and low miR-21 expression; p = 0.628**
Orange line – high expression; Green line – low expression

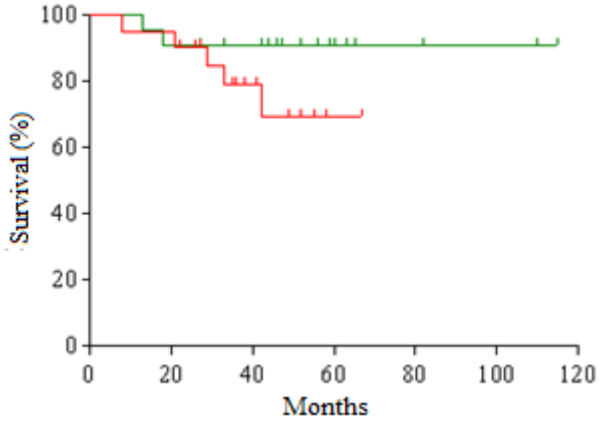


Fig. 3.2.6.3. **Overall survival of TN breast cancer patients in regard of high and low miR-21 expression; p = 0.205**
Orange line – high expression; Green line – low expression

3.2.7. MicroRNA expression in TN hereditary and sporadic breast cancer tissues

Expression of five different microRNAs (miR-10b, miR-21, miR-29a, miR-31, and miR-214) in TN hereditary (TN-H) and TN sporadic (TN-S) breast

cancer tissues was analyzed. The median ± interquartile range (Q1, Q3) expression of miR-10b, miR-21, miR-29a, miR-31, and miR-214 in TN-H breast cancer tissues was 0.275 ± 0.287 s (0.122, 0.408), 5.725 ± 4.250 (2.408, 6.658), 1.330 ± 1.552 (0.478, 2.030), 0.254 ± 0.642 (0.041, 0.684) and 0.489 ± 1.027 (0.350, 1.378), respectively. The median ± interquartile range (Q1, Q3) expression of miR-10b, miR-21, miR-29a, miR-31, and miR-214 in TN-S breast cancer tissues was 0.330 ± 0.381 (0.249, 0.631), 9.580 ± 7.545 (5,405, 12,950), 1,490 ± 0,990 (1,180, 2,170), 0.592 ± 0.487 (0.332, 0.819) and 1.800 ± 1.250 (1.170, 2.420), respectively. Expression of miR-214 was significantly higher in TN-S than in TN-H breast cancer tissues (p = 0.0005, Mann–Whitney test) (results are shown in figure 3.2.7.1.).

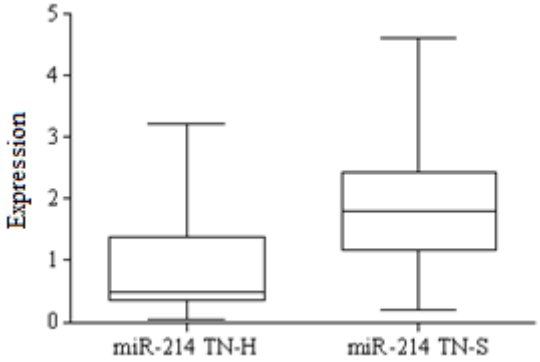


Fig. 3.2.7.1. **Expression of miR-214 in TN-H and TN-S breast cancer tissues; p = 0.0005**

MicroRNAs: miR-10b, miR-21, and miR-31 expression was observed higher in TN-S than TN-H breast cancer tissues, however, observation was considered not quit statistically significant (p = 0.0516, p = 0.0501 and p = 0.0597, respectively; Mann–Whitney test). Results are shown in figure 3.2.7.2. to 3.2.7.4.

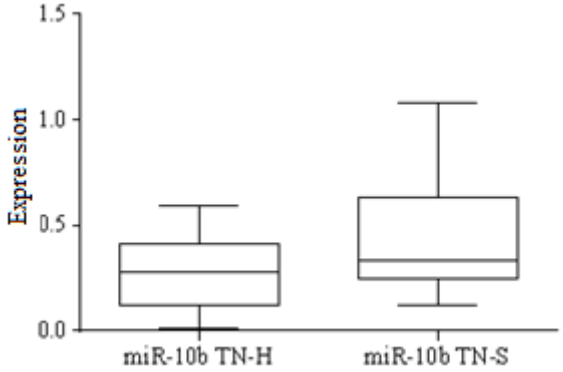


Fig. 3.2.7.2. **Expression of miR-10b in TN-H and TN-S breast cancer tissues; p = 0.0516**

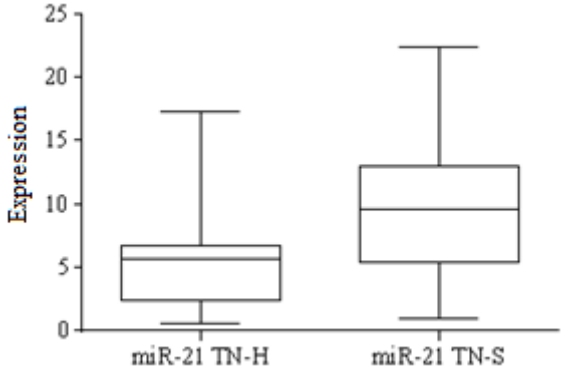


Fig. 3.2.7.3. **Expression of miR-21 in TN-H and TN-S breast cancer tissues; p = 0.0501**

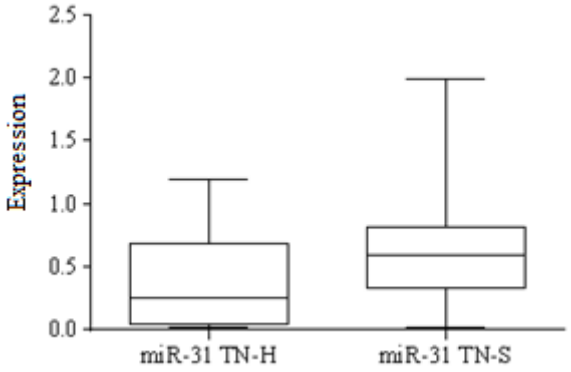


Fig. 3.2.7.4. **Expression of miR-31 in TN-H and TN-S breast cancer tissues; p = 0.4574**

No statistically significant differences between TN-H and TN-S breast cancer tissues in regard of miR-29a was observed (p = 0.4574) (results are shown in figure 3.2.7.5.).

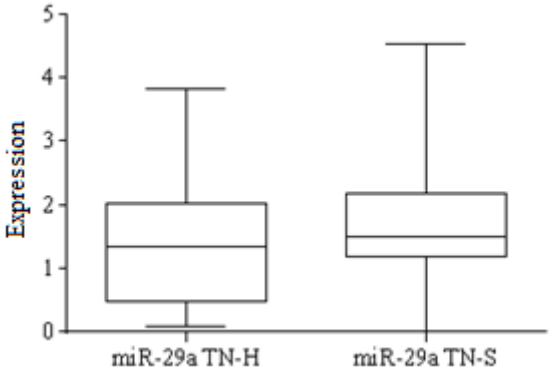


Fig. 3.2.7.5. **Expression of miR-29a in TN-H and TN-S breast cancer tissues; p = 0.0597**

3.3. Clinical data analysis of TN-H and TN-S breast cancer patients

Clinical data between TN-H and TN-S breast cancer patients were compared. TN-H breast cancer patients at the time of the diagnosis were significantly younger than TN-S breast cancer patients (p = 0.0300, t-test). The

median age (range) of the TN-H breast cancer patients at the time of the diagnosis was 46 (27–72) years, while the median age (range) at the time of the diagnosis of TN-S breast cancer patients was 55 (28–78) years. TNM stages between

TN-H and TN-S breast cancer patients at the time of the diagnosis were compared. No statistically significant differences between TN-H and TN-S breast cancer groups in regard of T stages were observed (p = 0.3021, Fisher's exact test). No statistically significant differences in N and M stages between both groups were observed as well (p = 0.3324, Fisher's exact test) and (p = 0.5412, Fisher's exact test), respectively. Clinical stages between TN-H and TN-S breast cancer groups were compared. No statistically significant differences were observed (p = 0.1438, Fisher's exact test). TN-S breast cancer were observed to have more frequently ductal type of cancer than TN-H breast cancer patients (p = 0.0370, Fisher's exact test). Clinical data is shown in table 3.3.1.

Table 3.3.1.

TN-H and TN-S breast cancer patient's clinical data

Characteristics	TN-H N=18 N (%)	TN-S N=32 N (%)	P value
Median age (range)	46 (27–72)	55 (28–78)	0.0300
T stage T_1 T_2 T_3 T_4	 7 (38.89) 6 (33.33) 2 (11.11) 3(16.67)	 7 (21.88) 16 (50.00) 7 (21.88) 2 (6.25)	0.3021
N stage N_0 N_1 N_2 N_3 No data	 8 (44.44) - 5 (27.78) 1 (5.56) 4 (22.22)	 15 (46.88) 6 (18.75) 8 (25,00) 2 (6.25) 1 (3.13)	0.3324
M stage M_0 M_1 No data	 14 (77.78) 1 (5.56) 3(16.67)	 31 (96.87) 1 (3.13) -	0.5412

Table 3.3.1. Continuation

Characteristics	TN-H N=18 N (%)	TN-S N=32 N (%)	P value
Stage I II III IV	 7 (38.89) 5 (27.78) 5 (27.78) 1 (5.56)	 4 (12.50) 15 (46.88) 12 (37.50) 1 (3.13)	 0.1438
Histological type D L M P D+M D+P No data	 9 (50.00) - 4 (22.22) 1 (5.56) 1 (5.56) 1 (5.56) 2 (11.11)	 27 (84.38) 1 (3.13) 2 (6.25) - - 1 (3.13) 1 (3.13)	 0.0370
Differentiation G2 G3 No data	 2 (11,11) 12 (66,67) 4 (22.22)	 1 (3,13) 25 (78,13) 6 (18.75)	 0.2763
Tumor size ≤ 50 mm > 50 mm	 13 (72.22) 5 (27.78)	 23 (71.88) 9 (28.13)	 0.7438
Median Ki67 (%) (range)	 75 (33–97)	 70 (27–98)	 0.4269
Relapse Yes No	 3 (16.67) 15 (83.33)	 3 (9.37) 29 (90.63)	 0.6538
Death Yes No	 1 (5.56) 17 (94.44)	 6 (18.75) 26 (81.25)	 0.3978

D – Ductal; L – lobular; M – medullary, P – papillary

3.4. TN breast cancer patient's gene expression data analysis

3.4.1. Gene expression data analysis with CLC Workbench

Gene expression data was analyzed with CLC Workbench 7 software according to the manufacturer's instructions.

For 18 TN-H and 30 TN-S breast cancer tissues gene expression analysis was performed. Genetic analyzer, due to the poor quality of the samples, read 43 spots on the BeadChip. In analysis 43 (15 TN-H and 28 TN-S) breast cancer

patient gene expression data were included. Gene expression quality control data were evaluated by quartiles. Since the distribution of the quality control plots between samples were too high and a large proportion of the data did not meet quality control criteria, large proportion of the samples were excluded from the further analysis (Figure 3.4.1.1.). In the further analysis 21 (10 TN-H and 11 TN-S) breast cancer patient's gene expression data were included. Data were logarithmically transformed and quality control plots were inspected (Figure 3.4.1.2.) Logarithmically transformed data were normalized and quality control

plots were inspected. All samples met quality control criteria and were included to evaluate gene expression (Figure 3.4.1.3.).

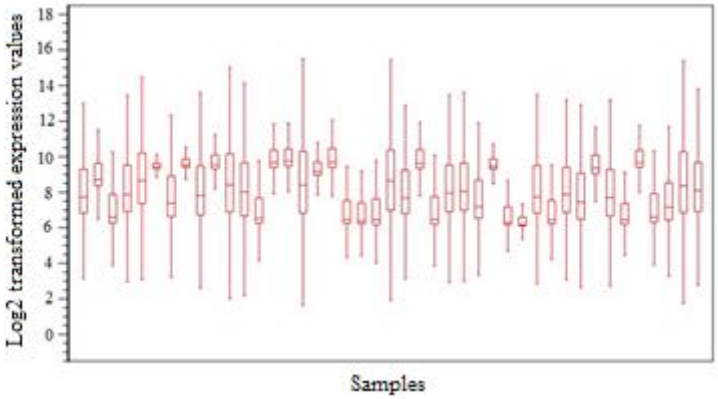


Fig. 3.4.1.1. **Distribution of the Log_2 transformed gene expression data**

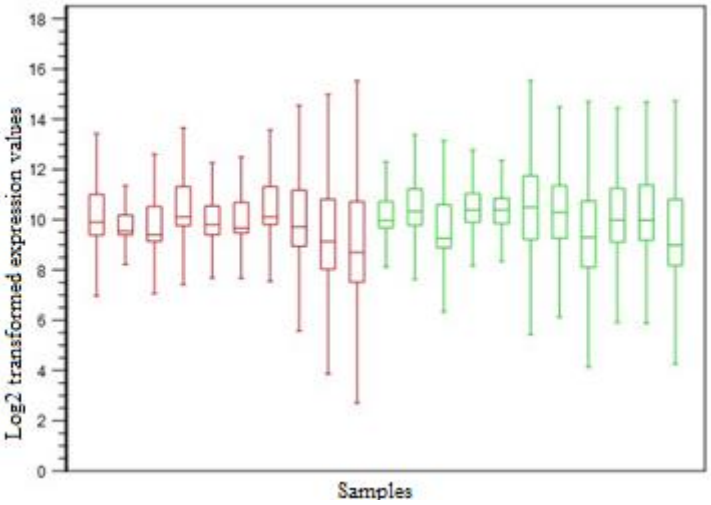


Fig. 3.4.1.2. **Quality control plots for Log_2 transformed gene expression data**

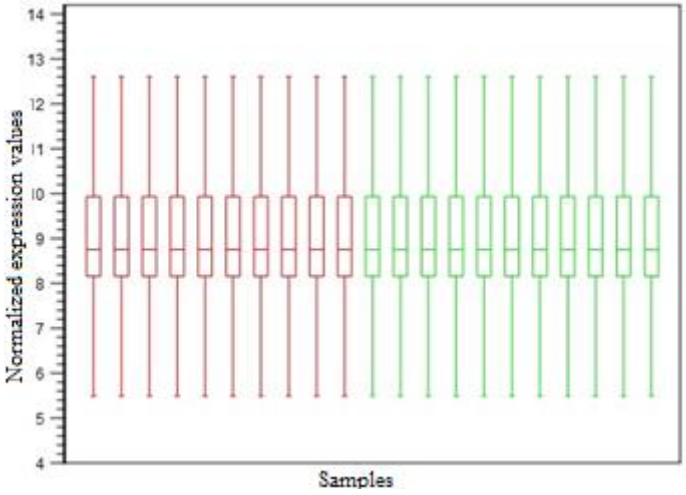


Fig. 3.4.1.3. **Quality control plots for normalized gene expression data**

3.4.2. TN-H versus TN-S gene expression profile

For 10 TN-H and 11 TN-S breast cancer samples gene expression analysis was performed. Gene expression profile for TN-H versus TN-S breast cancer tissues before data filtering is shown in Figure 3.4.2.1.

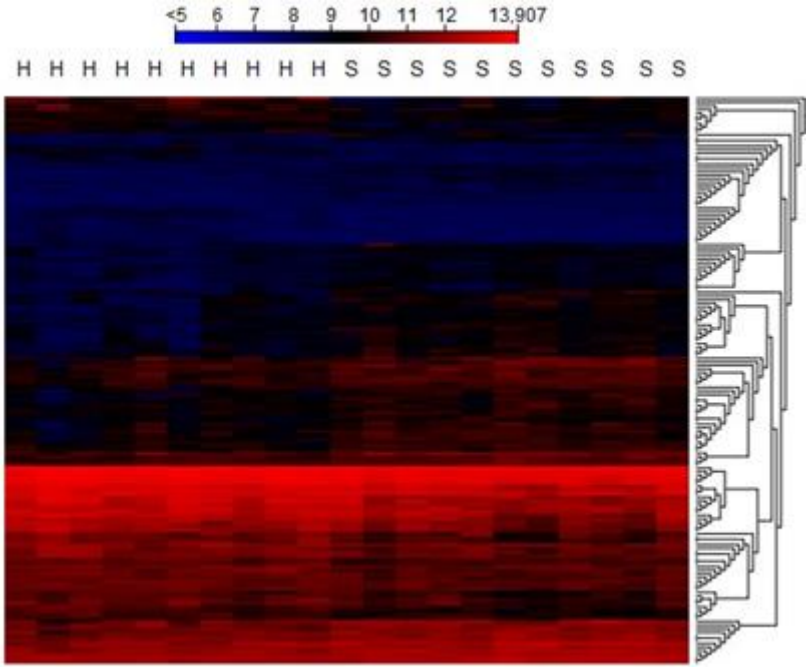


Fig. 3.4.2.1. **Gene expression profile between TN-H versus TN-S before data filtration**

To identify genes that are expressed differently between TN-H and TN-S breast cancer tissues t-test was performed. To identify most significantly different genes, threshold was set at $p < 0.0005$. Between TN-H and TN-S breast cancer tissues were found 22 differently expressed genes (Figure 3.4.2.2.).

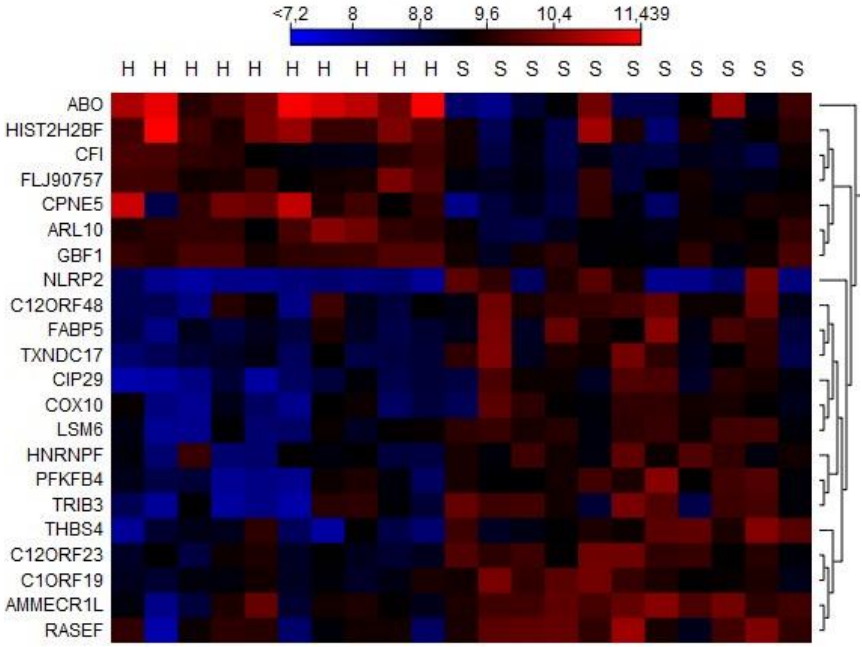


Fig. 3.4.2.2. **Differently expressed genes between TN-H and TN-S breast cancer tissues; p<0.0005**

In the TN-H breast cancer tissues 7 gene: *ABO*, *HIST2H2BF*, *CFI*, *FLJ90757*, *CPNEP5*, *ARL10* and *GBF1* were up-regulated whereas in the TH-S breast cancer tissues these genes were down-regulated. In TN-H breast cancer tissues 15 genes: *NLRP2*, *C12ORF48*, *FABP5*, *TXNDC17*, *CIP29*, *COX10*, *LSM6*, *HNRNPF*, *PFKFB4*, *TRIB3*, *THBS4*, *C12ORF23*, *C1ORF19*, *AMMERCR1L* and *RASEF* were down-regulated whereas in the TN-S breast cancer tissues these genes were up-regulated. The description of the genes is shown in the table 3.4.2.1.

Table 3.4.2.1.

Differently expressed genes between TN-H and TN-S breast cancer tissues

Gene	Gene name*
ABO	Transferase A, Alpha 1-3-N-Acetylgalactosaminyltransferase; Transferase B, Alpha 1-3-Galactosyltransferase
HIST2H2BF	Histone cluster 2, H2bf
CFI	Complement factor I
FLJ90757	BAIAP2 antisense RNA 1 (head to head)
CPNE5	Copine V
ARL10	ADP-ribosylation factor-like 10
GBF1	Golgi brefeldin A resistant guanine nucleotide exchange factor 1
NLRP2	NLR family, pyrin domain containing 2
C12ORF48	Chromosome 12 open reading frame (known as well as PARP1 binding protein)
FABP5	Fatty acid binding protein 5 (psoriasis-associated)
TXNDC17	Thioredoxin domain containing 17
CIP29	SAP domain containing ribonucleoprotein
COX10	Cytochrome c oxidase assembly homolog 10
LSM6	LSM6 homolog, U6 small nuclear RNA associated
HNRNPF	Heterogeneous nuclear ribonucleoprotein F
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
TRIB3	Tribbles pseudokinase 3
THBS4	Thrombospondin 4
C12ORF23	Transmembrane protein 263
C1ORF19	TSEN15 tRNA splicing endonuclease subunit
AMMECR1L	AMMECR1-like
RASEF	RAS and EF-hand domain containing

*NCBI- National Center for Biotechnology Information

3.4.3. Differently expressed gene analysis in relation with microRNA expression

Gene expression analysis in relation with microRNA regulation revealed that three genes: *C12ORF23*, *C1ORF19* and *AMMECR1L* are regulated by miR-214 which previously has been found up-regulated in TN-S breast cancer tissues in comparison to TN-H ones. According to the mentioned above, relation between *C12ORF23*, *C1ORF19* and *AMMECR1L* gene expression and high and low miR-214 expression in tumor tissues was assessed. High expression was defined as value above median expression whereas low expression was defined as value below median expression of a particular microRNA. Analyzing expression of the *C12ORF23* gene in relation to the miR-214 expression was determined that in the 7 cases of the TN-H tissues expression of the *C12ORF23* gene and expression of miR-214 match and can be defined as low in the both cases. Contrary, in the 6 cases of the TN-H tumors, expression of the *C12ORF23* gene and expression of the miR-214 was observed as high and in this case the expression pattern between miR-214 and the *C12ORF23* gene overlapped as well (Fig. 3.4.3.1.). In the case of the *C1ORF19* gene 8 TN-H breast cancer patients had low expression of miR-214 and low *C1ORF19* gene expression, while 5 TN-S breast cancer patients with high *C1ORF19* gene expression was observed high expression of miR-214 (Fig. 3.4.3.1.). Low *AMMECR1L* gene expression and low-miR-214 expression was observed to have 6 TN-H breast cancer patients, while high gene and microRNA expression was observed in 5 TN-S tissues (Fig. 3.4.3.1.).

Analyzing expression of the *C12ORF23* gene and expression of the miR-21 was determined that 9 TN-H breast cancer patients had low gene and microRNA expression. Equally, in the 9 cases of the TN-S breast cancer tissues high expression of the *C12ORF23* gene and miR-214 was observed (Fig. 3.4.3.2.).

Expression of the *TRIB3* gene was analyzed in regard to high and low expression of the miR-31. In the 6 TN-H tumor tissues low expression of the *TRIB3* gene and low expression of the miR-31 was observed. High expression of the *TRIB3* gene and miR-31 was in 4 TN-S tumor tissues (Fig. 3.4.3.3.).

Low *AMMECR1L* gene expression and low expression of the miR-31 was found in 3 TN-H tumor tissues, while high *AMMECR1L* gene expression and high expression of the miR-31 was found in 4 TN-S tissues (Fig. 3.4.3.3.). Expression of the *AMMECR1L* gene was analyzed in regard to high and low expression of the miR-29a. Low expression of the *AMMECR1L* gene and low expression of the miR-29a was found in 2 TN-H tumor tissues whereas high expression of the *AMMECR1L* gene and high expression of the miR-29a was found in 2 TN-S tumor tissues only (Fig. 3.4.3.4.).

Table 3.4.3.1.

Differently expressed genes and microRNAs regulated by them

Gene	microRNA*
ABO	-
HIST2H2BF	miR-623
CFI	miR-1253, miR-186
FLJ90757	miR-1226, miR-1233, miR-1300, miR-143, miR-665 etc.
CPNE5	miR-1265, miR-220b, miR-223, miR-452, miR-486, miR-661 etc.

Table 3.4.3.1. Continuation

Gene	microRNA*
ARL10	let-7a, miR-16, miR-204, miR-211, miR-224, miR-24 etc.
GBF1	let-7a, miR-1205, miR-15b, miR-17, miR-1182, miR-20a, miR-194 etc.
NLRP2	miR-580
C12ORF48	miR-10a, miR-134, miR-34a, miR-212 etc.
FABP5	miR-144, miR-198, miR-203, miR-603, miR620 etc.
TXNDC17	miR-641
CIP29	miR-1265, miR-1827, miR-320b, miR-335, miR-940 etc.
COX10	miR-210
LSM6	miR-488, miR-518c
HNRNPF	miR-141, miR-144, miR-19a, miR-19b, miR-27a, miR-27b etc.
PFKFB4	miR-122, miR-128, miR-188, miR-24, miR-27a, miR-34a etc.
TRIB3	miR-31, miR-24, miR-204, miR-205, miR-211, miR-212, miR-1237 etc.
THBS4	miR-190, miR-190b, miR-296-3-p, miR-299-3-p etc.
C12ORF23	miR-21, miR-214, miR-15b, miR-30a, miR-106a etc.
C1ORF19	miR-214, miR-29b, miR-34a, miR143 etc.
AMMECR1L	miR-29a, miR-31, miR-214, miR-101, miR-103 etc.
RASEF	miR-34b, miR-143, miR-224, miR-492, miR-610 miR-630 etc.

*G2SBC – Gene-to System Breast Cancer Database

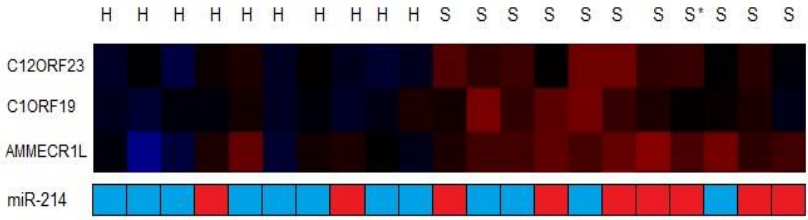


Fig. 3.4.3.1. ***C12ORF23*, *C1ORF19* and *AMMECR1L* gene expression in regard of miR-214**

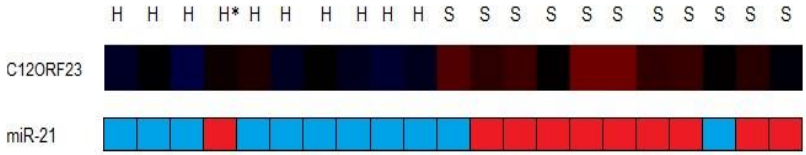


Fig. 3.4.3.2. ***C12ORF23* gene expression in regard of miR-21**

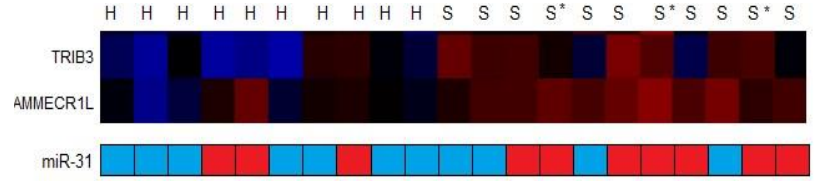


Fig. 3.4.3.3. ***TRIB3* and *AMMECR1L* gene expression in regard of miR-31**

4. DISCUSSION

TN sporadic breast cancer tissues showed significantly higher expression level of miR-214 than TN hereditary individuals that are consistent with the finding in other study; miR-214 is expressed differentially in ovary cancer patients with and without the *BRCA1* gene mutations [*Lee*, 2009]. High grade serous carcinoma patients with any loss within the *BRCA1* gene show lower expression of miR-214 than patients with no change [*Lee*, 2009]. The disease-specific survival in respect of high and low expression level of miR-214 was analyzed. TN breast cancer patients with high expression level of miR-214 have significantly worse overall survival than patients with low expression of miR-214. According to the results of this study, in the breast cancer, miR-214 may act similarly as oncogene that is consistent with the finding in other study. MiR-214 is up-regulated in preoperative serum samples of breast cancer patients; whereas, in post-operative serum samples, it is decreased and increased miR-214 correlates with positive lymph node status [*Schwarzenbach*, 2012]. MiR-214 plays an important role not only in the ovary cancer but as well in the breast cancer development. It is not clear how BRCA1 dysfunction can influence the level of miR-214 in ovarian and breast tumors as yet. It is known that miR-214 targets the *PTEN* gene; by targeting *PTEN* Akt pathway is activated thus resulting in the cell survival [*Yang*, 2008]. In many different types of cancers, in about 40% of ovarian and breast cancers, Akt kinase activity has been detected increased [*Ma*, 2007]. In addition, reported that the group with high mir-214, most patients had sporadic breast cancer patients. The group of low-mir-214 expression was 13 (54%) H and TN-11 (46%) TN-S breast cancer patients, while the group with high MIR-214 had five (21%) H and TN-19 (79%) TN-S breast cancer patients. It is known that TN-S breast cancer patients is worse survival than TN-H breast cancer patients [*Maksimenko*, 2013].

In advanced (metastatic) breast cancers, expression of miR-10b is up-regulated as compared to the primary ones [*Ma*, 2007]. MiR-10b is directly involved in the suppression of the *HOXD10* that in turn activates expression of the pro-metastatic gene *RHOC* [*Ma*, 2007]. MiR-10b correlation between tumor size, histological grade, clinical stage, positive lymph node status, and HER2 expression is positive [*Liu*, 2012]. While the correlation between high expression of miR-10b and HER2 expression is positive; the correlation between miR-10b expression and PR and ER status is negative [*Liu*, 2012]. Over-expression of miR-10b* is associated with reduced disease-free, relapse-free, and metastasis-free survivals, compared to those with low expression level [*Biagioni*, 2012].

Another miRNA that in this study was up-regulated in TN-S tissues as compared to TN-H ones was miR-21. As well as in this case, difference between groups was not quite statistically significant. MiR-21 is up-regulated in TN primary breast cancers as compared to healthy breast tissues [*Radojicic*, 2009]. Expression of miR-21 is significantly higher in ERα positive, ErbB2 negative, and PR positive than in ERα negative, ErbB2 positive, and PR negative breast cancers [*Mattie*, 2006]. MiR-21 is regulated by both ER (ERα and ERβ) receptors. Interaction between estradiol (E2) and one of the two ER receptors leads to the inhibition of miR-21 expression thus resulting in a loss of suppression of PDCD4, PTEN and BCL2 protein expression [*Wickramasinghe*, 2009]. In addition, interaction between E2 and ERα directly increases transcription of BCL2 [*Wickramasinghe*, 2009]. The mRNA profiling analysis revealed that in the adjacent normal breast tissues compared to TN ones, oncogenic BCL2 is down-regulated whereas miR-21 in TN breast cancer tissues is over-expressed [*Cascione*, 2013]. Breast cancer patients with ER negative and PR negative receptor status have significantly higher expression of miR-21 than breast cancer patients with ER positive and PR positive receptor status [*Hafez*, 2012]. TN breast cancer patients with high expression

level of miR-21 have a non-significant tendency of worse overall and disease-free survival than to those with low expression of miR-21 [*Radojicic*, 2011].

In this study higher expression level of miR-31 was in TN-S tumor tissues than in TN-H ones; however, as well in this case the difference was not quite statistically significant. Up-regulation of miR-31 is associated with less aggressive breast cancer subtypes, like luminal ones; whereas down-regulation is associated with more aggressive breast cancer subtypes, like triple-negative ones. In the MDA-MB-231 (triple-negative breast cancer subtype) cell lines miR-31 is found down-regulated whereas in the MCF7 (luminal breast cancer subtype) cell lines up-regulated [*Atchley*, 2008; *Augoff*, 2012].

Genes that were differently expressed between TN-H and TN-S groups in regard to their involvement in different signaling pathways and interaction with other genes were examined by PathCard, Reactome, G2SBC and KEGG databases. *TRIB3* gene that was expression more in sporadic breast cancer tissues is involved in the PI-3K signaling pathway and participates in the following processes: in the PI3K/AKT activation, PIP3 activation of AKT signaling, PI-3K cascade, PI3K events in ERBB2 signaling etc. pathways and signaling [*PathCard*; *Reactome*; *KEGG*; *G2SBC*]. In the PI-3K cascade *TRIB3* gene interacts with: *STAT1*, *PTEN*, *PIK3CA*, *KRAS*, *AKT1*, *RHOA*, *CHEK1* etc. genes. *THBS4* and *TRIB3* genes are involved in the FGFR signaling pathway where they interact with the following genes: *EGFR*, *KRAS*, *PIK3CA*, *PTEN*, *mTOR*, *SPRY2* etc. genes [PathCard; Reactome; KEGG; G2SBC]. *THBS4* gene as well is involved in the PI3-AKT signaling pathway where it interacts with *BRCA1*, *TP53*, *PIK3CA*, *BCL2*, *MYC*, etc. genes [PathCard; Reactome; KEGG; G2SBC].

PFKFB4 gene expression was observed lower in the TN-H breast cancer tissue and this gene is involved in the AKT signaling pathway and interacts with the following genes: *TP53*, *FGFR4*, *HRAS*, *NFKB1*, *PAK3*, *ErbB2*, *BCL2*,

ITGA3, *CHE1* etc. genes [PathCard; Reactome; KEGG; G2SBC]. The *C12ORF48* gene encodes a protein that interacts with PARP-1 [*Piao*, 2011].

MicroRNAs are promising biomarkers that can be used not only for the retrospective analysis but to monitor the efficacy of the chemotherapy and side the effects during the treatment as well. One of such option is to analyze free circulating nucleic acids (DNAs, mRNAs and microRNAs) in plasma or serum samples by the collected before each chemotherapy course and by evaluating correlation between the change of the free circulating nucleic acids during the treatment and response to certain chemotherapeutic drug. MicroRNA which potentially can be used for this purpose is miR-214. By analyzing the expression of miR-214 in plasma samples of breast cancer patients was found that the expression of miR-214 was significantly higher in the pre-operative serum samples samples than to post-operative serum samples [*Schwarzenbach*, 2012]. In addition, it was observed that increased miR-214 expression correlates with positive lymph node status [*Schwarzenbach*, 2012]. Thus it would be very valuable to continue this research by exploring changes in the expression of miR-214 in the neoadjuvant and/or metastatic breast cancer patients by collecting serum or plasma samples before each chemotherapy course. In the case of the ovarian cancer it has been found that high expression of the miR-214 in tumor tissue correlates with the resistance to cisplatin-based chemotherapy [*Yang*, 2008]. Based on the above, it would be interesting and very important to assess whether there is such a connection in the case of the breast cancer when to platinum-based chemotherapy is applied. Especially it would be very interesting to evaluate it in the case of the TN breast cancer when the *BRCA1* gene mutation is present. The platinum-based chemotherapy efficacy in the case of the TN breast cancer is unclear. The study in which the progression-free survival of the patients who received the platinum-based therapy and patients who did not receive platinum-based therapy were examined was found that the first group had 10 months of events-free survival in compared with the second group where it was only 5 months [*Hong*, 2014].

5. CONCLUSIONS

1. MicroRNAs: miR-21, miR-29a, miR-31, and miR-214 relative expression is significantly higher in breast cancer tissues than in normal breast epithelial tissues ($p<0.05$).
2. MiR-21, miR-31, and miR-214 expression is significantly higher in TN than in LA, LB and HER2 breast cancers ($p<0.05$).
3. MiR-214 expression is significantly higher in TN-S than in TN-H breast cancer tissues ($p = 0.0005$).
4. High expression of miR-214 in TN breast cancer tissues correlates with worse overall survival ($p = 0.0314$).
5. Between TN-H and TN-S breast cancer tissues 22 differently expressed genes are found ($p = 0.0005$).
6. There is a certain association between *C12ORF23*, *C1ORF19* and *AMMECR1L* gene and miR-21 and miR-214 expression.

6. LIST OF PUBLICATIONS AND REPORTS

6.1. Scientific publications

1. Dagnija Kalniete, Miki Nakazawa-Miklaševiča, Ilze Štrumfa, Arnis Āboliņš, Arvīds Irmejs, Jānis Gardovskis, Edvīns Miklaševičs. High expression of miR-214 is associated with a worse disease-specific survival of the triple-negative breast cancer patients. Hereditary Cancer in Clinical Practice 2015, 13(1):7.
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6.2. Reports

1. Kalniete, D., Nakazawa-Miklasevica, M., Strumfa, I., Abolins, A., Irmejs, A., Gardovskis, J., Miklasevics, E. MicroRNA expression in triple-negative versus other subtypes of breast cancer. European Journal of Cancer. Proceedings Book. 2014. S67.
2. Kalniete, D., Nakazawa-Miklaševiča, M., Štrumfa, I., Āboliņš, A., Irmejs, A., Gardovskis, J., Miklaševičs, E. TN krūts vēža pacientēm ar augstu miR-214 ir sliktāka kopējā dzīvildze nekā pacientēm ar zemu miR-214 ekspresiju. 2014. gada Zinātniskās konferences tēzes, RSU 2014., 314.
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