

MICRO RNA-21 EXPRESSION LEVELS IN INVASIVE BREAST CARCINOMA WITH A NON-INVASIVE COMPONENT

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Abstract: Invasive ductal carcinomas with a non-invasive component (IDC-DCIS) are classified as a group of invasive breast carcinomas, together with pure invasive ductal carcinomas of the breast (IDC). MicroRNA-21 (miR-21) has been characterized as a factor of breast cancer invasiveness, however the difference in miR-21 expression levels between IDC-DCIS and pure IDC tumors and the correlations with standard diagnostic and prognostic parameters inside the IDC-DCIS group are unknown. Our aim was to determine if miR-21 had the ability to distinguish these two invasive breast cancer groups. Levels of miR-21 expression were measured by a stem-loop quantitative Real-Time PCR (RT-qPCR) method. Expression levels of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her-2) and proliferative index Ki-67 were evaluated by immunohistochemistry. IDC-DCIS tumors had significantly lower levels of miR-21 expression in grade 2 ($P=0.003$, Mann-Whitney U test), ER positive ($P=0.025$, Mann-Whitney U test) and PR positive tumors ($P=0.024$, Mann-Whitney U test) than pure IDCs. miR-21 levels showed a different pattern of expression in IDC-DCIS compared to IDC tumors, which is based on the difference in miR-21 expression between Her-2 negative and Her-2 positive IDC-DCIS tumors ($P=0.030$, Mann-Whitney U test) and high negative correlation of miR-21 levels with PR levels ($\rho=-0.886$, $P=0.006$, Spearman correlation). According to our results, IDC-DCIS breast carcinomas act in a different manner in pure IDC tumors with regard to the relations between miR-21 expression levels and the standard diagnostic and prognostic parameters, such as Her-2 status, ER and PR status and protein levels.

Key words: Invasive ductal carcinoma with non-invasive component (IDC-DCIS); miR-21 expression levels; the difference between IDC-DCIS and IDC

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INTRODUCTION

Ductal carcinoma in situ (DCIS) is a breast cancer disease characterized by the cells that proliferate inside the basement membrane comprised of myoepithelial cells in the breast ducts (Virnig et al., 2010). In some cases, they accompany invasive forms of the disease. About 25.4% cases of invasive breast carcinomas are associated with a non-invasive component (Soliman et al., 2012), and 30-60% patients with IDC contain DCIS form (Dieterich et al., 2014).

Solitary DCIS forms are very difficult to detect in Serbia because of the insufficient sensitivity of mammography to DCIS histology (Brem et al., 2007). Only one in 10-20 cases of diagnosed breast cancer contains DCIS form (Verkooijen et al., 2003; Wong et al., 2010).

IDC-DCIS are classified according to the current American Joint Committee on Cancer staging system in the group of invasive breast carcinomas, based on the presence of invasive form (Singletary, 2002). Some

studies have demonstrated that DCIS associated with invasive forms share similar genomic profiles to pure invasive forms (Iakovlev et al., 2008). Although patients with IDC-DCIS were classified together with pure IDC according to its invasiveness (Chagpar et al., 2009), most researchers analyze IDC-DCIS and pure IDC samples together. However, IDC-DCIS tumors on the molecular level differ considerably from pure IDC tumors, as Castro et al. (2008) have pointed out.

Several researchers expect tumors containing both-IDC and DCIS components to develop a less aggressive phenotype (Wong et al., 2010). Others suggest that the presence of an *in situ* part does not influence patient prognosis at all (Castro et al., 2008). However, other researchers developed an opposite hypothesis. Because of the diffuse nature of DCIS, after the breast-conserving surgeries, IDC-DCIS patients might have a greater chance for local recurrence than pure IDC patients, and also might have a worse prognosis (Chagpar et al., 2009).

As the invasiveness of a tumor is a preliminary process to metastasis, it is now in the focus of many studies. MicroRNA-21 has been shown to be a very powerful factor in breast cancer invasion (Yan et al., 2008; Huang et al., 2009; Song et al., 2010; Tang et al., 2012; Petrović et al., 2014a; Petrović et al., 2014b), but the expressional levels of miR-21 and its potential correlations with the clinical and pathological features in IDC-DCIS tumors have not been characterized yet. miR-21 is a 22-nucleotide-long epigenetic factor that silences gene expression by binding to the mRNAs of its target genes (Qi et al., 2009). MicroRNA-21 is an oncogenic regulatory element that blocks or delays transcription of tumor suppressor genes (Kim et al., 2009; Pan et al., 2010). This small silencer of translation is engaged with six out of ten key moments in breast cancer development and progression: growth suppressor evasion, proliferative signaling maintenance, resistance to cell death, tumor-promoting inflammation, induction of angiogenesis, and induction of invasion and metastasis (Hanahan and Weinberg, 2011), by trapping five target-gene-mRNAs: *TIMP3*, *PDCD4*, *PTEN*, *TPM1* and *RECK* (Yang et al., 2009; Buscaglia, 2011).

The IDC-DCIS form of a breast cancer disease might be a transitional state between *in situ* and invasive breast carcinomas (Pinder and Ellis, 2003). An IDC-DCIS tumor might occur *de novo* (Farabegoli et al., 2002; Patla et al., 2002), or it can arise as a result of genetic and epigenetic changes in normal and/or DCIS tissue (Velds et al., 2006; Wong et al., 2010). The tumor could also be the result of changes in the signaling pathways in neighboring cells of the tumor or normal tissue that will be transformed to malignant (by paracrine regulation) (Polyak and Hu, 2005; Daniel et al., 2011). The association of miR-21 expression levels with standard diagnostic and prognostic parameters in these entities is still unknown. It is also unknown whether they act in a similar manner to their potential “pure invasive relatives” or the changes occur as the consequences of actions in different molecular pathways.

In our previous study, we have shown that invasive with non-invasive ER positive tumors have lower miR-21 expression than pure invasive tumors (ILC, IDC and mixed-ILC-IDC tumors) and higher than non-invasive ER+ tumors (Petrović et al., 2014a). We also found that invasive associated with non-invasive tumors had lower expression of miR-21 in the PR-positive subgroup compared to both pure invasive and even non-invasive tumors. Therefore, further research into and characterization of these entities was necessary. We performed an additional study to examine their unusual behavior.

Based on our previous research (Petrović et al., 2014b) and according to miR-21 expression levels that were distributed between non-invasive and pure invasive breast cancers, we suggest that IDC-DCIS might represent transitional forms during breast cancer invasion and progression. We have continued investigating only IDC-DCIS cases to characterize their effect on miR-21 expression levels. In this study, we have compared i) the miR-21 expression levels of 12 invasive associated with non-invasive breast carcinomas with 11 pure IDC tumors from the same groups based on standard diagnostic and prognostic breast cancer parameters; ii) the miR-21 expression levels within the group of IDC-DCIS tumors divided into

subgroups formed according to standard diagnostic and prognostic parameters of breast tumors, and iii) the miR-21 expression levels with DCIS contribution in IDC-DCIS breast carcinomas.

Our basic assumption was that IDC-DCIS tumors differ from pure IDC breast carcinomas (although both groups are classified as invasive breast carcinomas) in miR-21 expression levels in groups formed according to standard diagnostic and prognostic parameters such as age at diagnosis, menstrual status, size of a tumor, tumor grade, lymph node status, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (Her-2) and proliferation index Ki-67. Our second assumption (based on the experimentally confirmed role of miR-21 in the process of breast cancer invasion) was that miR-21 expression levels negatively correlate with DCIS contribution.

MATERIALS AND METHODS

Study subjects

During this research, we have analyzed 12 invasive breast cancer samples that contained non-invasive components in different percent from patients that had undergone surgery at the Institute for Oncology and Radiology of Serbia between June 2012 and May 2013. We compared their miR-21 expression levels with 11 samples of pure invasive ductal carcinomas (selected according to similar diagnostic and prognostic parameters to IDC-DCIS samples).

Tumor samples were divided into two sections, and characterized immediately after surgery. Parts of the tissues with at least 75% of malignant cells were used for further molecular analysis and parts were fixed with formalin and embedded in paraffin for routine pathological and histological characterization, analysis, and archiving.

This study was approved by an Institutional Review Board approval according to the National Health Regulation 5002/1-01, and informed consent from all the examined patients was obtained. Histologi-

cal type, histological and nuclear grade, lymph-node status were determined by two pathologists. ER, PR, Her-2 status and Ki-67 were evaluated by a standard scoring system to determine adequate post-operative therapy. IDC and DCIS ratios in IDC-DCIS entities were presented in percentage determined by the two pathologists.

Breast cancer tissue and molecular analysis Immunohistochemistry

For estrogen receptor (ER) and progesterone receptor (PR) protein level evaluation, rabbit and mouse monoclonal antibodies (RM-9101-S1, Thermo Fisher Scientific, Cheshire, UK, and M3569, Dako Copenhagen, Denmark, respectively) were used on 4-5- μ m sections of formalin-fixed paraffin-embedded blocks. The evaluation of human epidermal growth factor receptor 2 (Her-2) status was performed with antibody rabbit-antihuman A0485, (Dako, Copenhagen, Denmark). Ki-67 proliferation index was characterized with RM9106-S1 (Thermo Fisher Scientific, Cheshire, UK). For ER and PR status, Her-2 expression was considered positive at 3+ by immunohistochemistry (IHC) and at >2 by IHC and positive by chromogenic *in situ* hybridization (CISH) if = 2 (2+) (Di Palma et al., 2006). Ki-67 levels were evaluated as the percentage of positively stained cells. After deparaffinization, rehydration and treatment with 3% H₂O₂ for 10 min, tissue slides were immersed in 10 mM of citrate buffer (pH 6) in a microwave oven for 25 min. Samples were cooled and washed in TBS, pH 7.4. Sections were incubated with the antibodies at 1:50 dilutions with Ab Diluent Buffer (Code No.S0809 Dako, Copenhagen, Denmark). Then, samples were treated with the TP-125-HL (Thermo Fisher Scientific, Cheshire, UK) ready-to-use polymer system. For the visualization, we used diaminobenzidine. Brown nuclear staining in cells was scored according standards from Leake (2000).

RNA extraction and purification

All tissue samples were stored at -80°C immediately after surgery. Fresh-frozen tissues of breast cancer samples were homogenized in liquid nitrogen for fur-

ther RNA isolation. Total RNA was extracted from powdered tissues with TRI Reagent (Ambion, Foster City). After a 10-min incubation in TRI Reagent, 0.2 ml of chloroform was added to each sample. The samples were centrifuged for 10 min at 14000 RPM (4°C) and RNA samples were precipitated with isopropanol. Then, the samples were centrifuged (14000 RPM, 4°C) and the pellet was rinsed with 1 ml of 70% ice-cold ethanol. The pellets were dissolved in 100 µL DEPC-DW and incubated at 65°C for 10 min. Quantification of RNA samples was performed by Biospec-Nano (Shimadzu Corporation, Japan). The quality of RNA samples has been also confirmed by denaturing 1% agarose gel electrophoresis.

miR-21 reverse transcription and stem-loop qRT-PCR

The expression of mature hsa-miR-21 5p and RNU6B as endogenous control was measured with TaqMan® Assays (ID: 000397 and ID: 001093, respectively). For reverse transcription we used in TaqMan Micro RNA Reverse Transcription Kit components and the following thermal conditions: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min. Following the steps from the TaqMan® Small RNA Assays Protocol (Applied Biosystems, Foster City, CA), we performed reverse transcription. In the second step, we quantified RT-qPCR amplicons with Applied Biosystems (Foster City, CA) specific TaqMan® miR-21 and RNU6B Assays with the following thermal conditions: 95°C 10 min and 95°C 15 s; 60°C 60 s for 40 cycles.

MicroRNA-21 expression levels were presented in relative units, and the expression level values were normalized to RNU6B (small nuclear endogenous control), and the samples were calibrated to the sample with the lowest relative expression (set as referent-1x sample). Relative quantity values were analyzed with 7500 System SDS software. We used the relative-quantity- $\Delta\Delta Ct$ method, with the equation: $RQ_{\text{sample}} = 2^{-(\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}})}$ (where RQ_{sample} represents relative quantity of sample, while $\Delta Ct = Ct_{\text{miR-21}} - Ct_{\text{RNU6B}}$).

Statistical analysis

DCIS involvement was presented by percents and miR-21 relative expression levels were characterized by their medians. We used the Mann-Whitney U non-parametric test to compare 2 independent groups of samples. For correlation analysis, we used Spearman's. P values ≤ 0.050 were statistically significant, while those between 0.1 and 0.05 were considered a statistical trend. For the calculations of P values, we used GraphPad Prism 5 software (GraphPad Software, Inc. CA).

RESULTS

miR-21 expression levels in IDC-DCIS and IDC tumor groups

We detected a statistical trend in the difference in miR-21 expression levels between the group of IDC-DCIS and pure IDC tumor samples ($P=0.053$, Mann-Whitney U test). A statistically significant difference in miR-21 expression levels between IDC-DCIS and pure IDC samples did not appear in patients younger than 60 years, older than 60 years, postmenopausal patients. Ten IDC-DCIS tumors differed with high significance in miR-21 expression level from pure IDC grade 2 samples with $P = 0.003$ (Mann-Whitney U test, Table 1, Fig. 1A). In lymph node-positive and lymph node-negative tumors there was no statistically significant difference in miR-21 expression level between IDC-DCIS and IDC carcinomas according to the Mann-Whitney U test. All IDC-DCIS patients were postmenopausal with no difference in miR-21 expression compared with postmenopausal patients with pure IDC tumors. In tumor samples with ER+ status, IDC-DCIS significantly differed from pure IDC tumor samples in miR-21 expression level with $P=0.025$ (Mann-Whitney U test, Table 1, Fig. 1B). In tumor samples with PR+ status, there was also a statistically significant difference between IDC-DCIS and pure IDC tumor samples in miRNA-21 expression levels ($P=0.024$, Mann-Whitney U test, Table 1, Fig. 1C). In Her-2-negative tumors, we detected

Table 1. The difference in miR-21 relative expression levels between size-matched IDC-DCIS and IDC tumor samples

Diagnostic and prognostic parameters of breast tumors	^a Relative miR-21 expression levels				^{c,d} P value
	IDC-DCIS	^b N	Pure IDC	N	
Age					
≤ 60	50.817 (35.137-179.896)	4	239.382 (89.001-333.937)	8	P = 0.461
>60	50.895 (35.867-64.087)	6	90.459 (23.365-97.989)	3	P = 0.548
Menstrual status					
Postmenopausal	99.775 (86.448-113.363)	8	88.802 (39.053-164.623)	4	P = 0.683
Grade					
2	50.817 (35.867-64.087)	10	141.412 (97.989-270.900)	6	P = 0.003
Lymph node status					
Positive	61.777 (35.867-64.811)	6	95.479 (38.874-150.484)	6	P = 0.310
Negative	50.817 (42.323-122.854)	6	141.412 (58.097-231.405)	5	P = 0.537
ER status					
Positive	52.278 (35.867-64.268)	8	141.412 (87.121-231.405)	9	P = 0.025
PR status					
Positive	50.895 (34.779-64.449)	8	141.412 (87.121-231.405)	9	P = 0.024
Her-2 status					
Negative	39.095 (33.691-45.088)	4	95.479 (56.956-145.948)	8	P = 1
Positive	64.087 (58.737-314.074)	6	239.382 (89.001-333.937)	3	P = 0.059
Ki-67 index (%)					
≤ 20	64.449 (59.467-122.854)	7	150.484 (85.093-262.923)	5	P = 0.429
> 20	42.323 (37.481-44.397)	3	83.782 (36.807-141.412)	6	P = 0.381

^aRelative miR-21 expression with 25th-75th percentile in parentheses. ^bN-number of samples. ^cP values equal or less than 0.05 were considered significant according to the Mann-Whitney U test (between 2 groups). ^dP values less than 0.1 were considered a statistical trend.

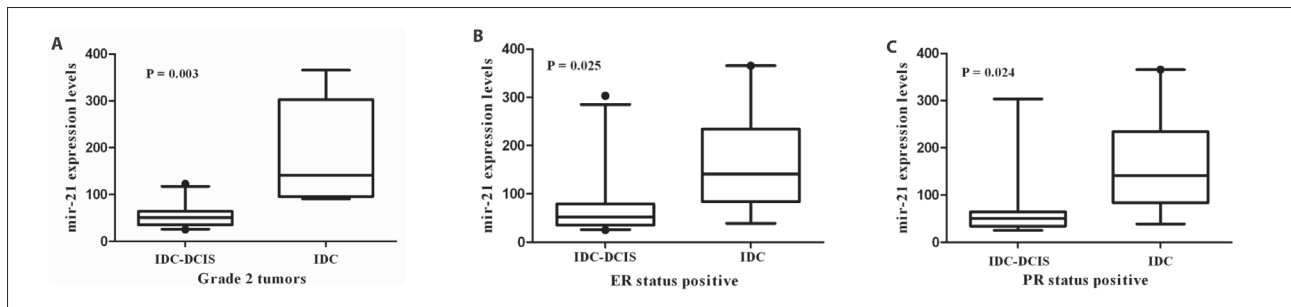


Fig. 1. The difference in miR-21 expression levels between IDC-DCIS and pure IDC tumor samples in (A) grade 2 tumors; (B) ER positive subgroup; (C) PR positive subgroup. The values of miR-21 expression levels are shown in relative units, normalized to RNU6B and calibrated to the sample with lowest miR-21 expression. The plot shows interquartile range (boxes) contoured with 25-75% of median values. Middle line indicates the median value and the whiskers extended from the boxes represent the highest and the lowest values, i.e. non-outlier ranges. Statistically significant differences were considered for $P < 0.05$ values.

a statistical trend towards increased levels of miR-21 expression in pure IDC tumors compared with IDC-DCIS tumor samples ($P = 0.059$, Mann-Whitney U test, Table 1). In Her-2-positive breast carcinoma samples, a statistically significant difference between

IDC-DCIS and pure IDC tumor samples was not found. In addition, there were no significant differences in tumors with Ki-67 $\leq 20\%$ and tumors with Ki-67 $> 20\%$ between IDC-DCIS and pure IDC tumors.

miR-21 expression levels in the IDC-DCIS tumor group

In the group of patients younger than 60 years, significantly different levels of miR-21 were not detected when compared with the group of patients older than 60 years. Statistically significantly higher miR-21 expression was found in Her-2-positive compared to Her-2-negative tumors with $P=0.030$ in the IDC-DCIS samples (Mann-Whitney U test, Table 2, Fig. 2A). Tumors with proliferative index Ki-67 $\leq 20\%$ compared with Ki-67 $> 20\%$ tumors had a statistical trend towards higher miR-21 expression levels in the IDC-DCIS samples ($P=0.024$, Mann-Whitney U test

Table 2. The difference in miR-21 relative expression levels between groups of IDC-DCIS tumor samples formed according to standard diagnostic and prognostic parameters of breast tumors.

Diagnostic and prognostic parameters of tumors	^a Relative miR-21 expression in IDC-DCIS tumors	^b N	^c P value
Age			
≤ 60	50.817 (35.137-179.896)	4	P = 1
>60	50.895 (35.867-64.087)	6	
^d Size			
≤ 2cm	42.323 (31.565 -167.952)	5	P = 0.530
> 2cm	59.467 (42.783-64.268)	7	
Lymph node status			
Negative	50.817 (42.323-122.854)	6	P = 0.937
Positive	61.777 (35.867-64.811)	6	
ER status			
Negative	201.554 (56.547-346.561)	2	P = 0.334
Positive	52.278 (35.867-64.811)	8	
PR status			
Negative	89.701 (50.870-234.707)	5	P = 0.283
Positive	39.095 (33.691-59.467)	7	
Her-2 status			
Negative	39.095 (33.691-45.088)	6	P = 0.030
Positive	64.087 (58.737-314.074)	4	
Ki-67 index (%)			
≤ 20	64.449 (59.467-122.854)	7	P = 0.024
> 20	42.323 (37.481-44.397)	3	
DCIS contribution (%)			
≤ 30	64.449 (50.895-213.049)	4	P = 0.109
> 30	50.817 (34.779-40.477)	8	

^aRelative miR-21 expression with 25th-75th percentile in parentheses.
^bN-number of samples. ^cP Values equal or less than 0.05 were considered significant according to the Mann-Whitney U test (between 2 groups).
^dMaximal tumor diameter.

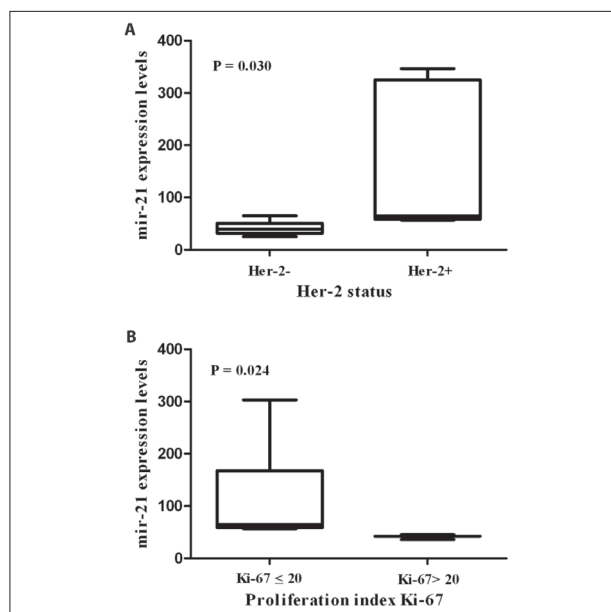


Fig. 2. The difference in miR-21 expression inside the IDC-DCIS tumor group divided according to (A) Her-2 status and (B) the values of Ki-67 proliferation index. The values of miR-21 expression levels are shown in relative units, normalized to RNU6B and calibrated to the sample with lowest miR-21 expression. The plot shows interquartile range (boxes) contoured with 25-75% of median values. Middle line indicates the median value and the whiskers extended from the boxes represent the highest and lowest values, i.e. non-outlier ranges. Statistically significant differences were considered for $P < 0.05$ values.

Table 2, Fig. 2B). Significant correlation between miR-21 expression level and the age of patients and tumor size was not found. MicroRNA-21 expression levels highly negatively correlated with PR status ($\rho = -0.886$, $P = 0.006$, Spearman's correlation), but showed no correlation with ER, proliferation index expression levels (Ki-67%) (Table 3), nor with DCIS contribution in percentage (Table 4).

DCIS contribution in IDC-DCIS tumors

Interestingly, there were no statistically significant differences in miR-21 expression between IDC-DCIS tumors with $\leq 30\%$ of DCIS component compared with IDC-DCIS that contained $> 30\%$ DCIS. The distribution of DCIS in percentage positively correlated with tumor size, with a high correlation coefficient value, $\rho = 0.718$ ($P = 0.015$, Spearman's correlation) (Table 3).

Table 3. Correlation of miR-21 expression levels with standard diagnostic and prognostic parameters of tumors and DCIS contribution.

Variables	Number of samples	Coefficient of correlation	^a P value
Age	10	-0.353	0.387
Size	12	0	0.968
ER	10	-0.206	0.578
PR	10	-0.886	0.006
Ki-67	10	0.359	0.353
^b DCIS %	12	-0.418	0.297

ER, estrogen receptor; PR, progesterone receptor; Ki-67, proliferation index. ^aP-values less than 0.05 were considered statistically significant.

^bContribution of DCIS component.

Table 4. Correlation of DCIS contribution in percentage with standard diagnostic and prognostic parameters of breast tumors.

Variables	Number of samples	Coefficient of correlation	^a P value
Age	10	-0.089	0.785
Size	10	0.718	0.015
ER	10	0.344	0.283
PR	10	-0.082	0.797
Ki-67	10	-0.0289	0.924

ER, estrogen receptor; PR, progesterone receptor; Ki-67, proliferation index.

^aP-values less than 0.05 were considered statistically significant.

^bContribution of DCIS component.

Table 4. Correlation of DCIS contribution in percentage with standard diagnostic and prognostic parameters of breast tumors.

Variables	Number of samples	Coefficient of correlation	^a P value
Age	10	-0.089	0.785
Size	10	0.718	0.015
ER	10	0.344	0.283
PR	10	-0.082	0.797
Ki-67	10	-0.0289	0.924

ER, estrogen receptor; PR, progesterone receptor; Ki-67, proliferation index.

^aP-values less than 0.05 were considered statistically significant. ^bContribution of DCIS component.

In our experiment, DCIS contribution in IDC-DCIS entities correlated neither with patient age, ER and PR status, nor with the percentage of proliferation index Ki-67 expression levels (Table 4).

DISCUSSION

IDC-DCIS are classified according to the American Joint Committee in Cancer staging system as pure invasive forms (Singletary, 2002). Some studies have demonstrated that DCIS associated with invasive forms share similar genomic profiles with pure DCIS (Iakovlev et al., 2008), but Catro et al. (2008) showed that IDC-DCIS tumors significantly differ from IDC breast cancers at the molecular level.

The concept and the idea of our approach derived from our previous research where we noticed an interesting behavior of invasive breast carcinomas with non-invasive component in that they significantly differed from pure invasive forms according to miR-21 expression in the groups formed according to ER, PR, grade 2, and K-67 $\leq 20\%$. However, in the first study, we analyzed all types of pure invasive carcinomas together (invasive lobular, ductal and mixed). In order to highlight the significance of these unique entities and to emphasize the potential influence of certain prognostic and diagnostic factors, via changes in miR-21 expression levels, we excluded all types of breast carcinoma (BC) that had originated from other than breast ducts. Additionally, we included only IDCs with similar sizes to IDC-DCIS. In this study, we increased the number of IDC-DCIS samples. The novelty of this work lies in the fact that no research to date, to the best of our knowledge, has analyzed the association of miR-21 expression levels with standard diagnostic and prognostic parameters inside the IDC-DCIS group. In addition, no one has yet compared DCIS contribution (as a potential factor that might be related to BC invasion) with the factor of BC invasiveness such as miR-21.

We observed a statistical trend towards increased levels of miR-21 expression in 11 pure IDC samples compared with 12 IDC-DCIS tumors. In grade 2-IDC

and IDC-DCIS tumors, the statistically significant difference appeared to be very high (with low P value). When we compared high miR-21 IDC-DCIS with high miR-21 IDC (according to their median ranges), we observed that the IDC tumors have significantly higher miR-21 levels, whereas samples with low miR-21 levels did not differentiate between IDC-DCIS and IDC samples. Additionally, this might be a special target group: high miR-21-IDC-DCIS or IDC-grade 2 group for future stratification and classification of breast carcinoma patients.

MicroR-21 expression levels found in IDC-DCIS tumors did not correlate with ER status, which is unusual, because in the group of pure invasive tumors, levels of miR-21 expression correlated highly positively with the levels of ER expression, which was also shown by Petrović et al. (2014a). In addition, ER+ and PR+ IDC-DCIS significantly differed from IDC ER+ and PR+ tumors in miR-21 expression levels. Although it has been shown that miR-21 expression might be a process dependant on ER expression (Mattié et al., 2006; Wickramasinghe et al., 2009; Petrović et al., 2014a), in IDC-DCIS tumors, this might not be the case.

As we expected, a difference in miR-21 expression levels in PR+ patients appeared between the IDC-DCIS and IDC tumor groups, which indicates that higher miR-21 expression also might not be induced by increased levels of progesterone receptor. Surprisingly, the miR-21 levels of IDC-DCIS tumors highly negatively correlated with PR status, while pure invasive BCs from our previous study (Petrović et al., 2014a) highly positively correlated with PR levels. The fact that miR-21 levels do not correlate with age, while in pure invasive they correlate negatively with the age of patients (Petrović et al., 2014b), could be due to the absence of ER/PR regulation of miR-21 expression in IDC-DCIS tumor specimens because of the differences in hormonal status related to patient age (Ma et al., 2006). In IDC-DCIS tumors, Her-2 positive tumors had significantly higher levels of miR-21 expression, while pure invasive (IDC and ILC) tumors acted independently from Her-2 status in the previous analysis (Petrović et al., 2014a). Huang et

al. (2009) described Her-2-dependent miR-21 expression. We have also found that IDC-DCIS tumors do not have ER- and/or PR-dependant miR-21 expression, so these cases of multi-component tumors might have Her-2-dependant expression of miR-21, which supports our finding that there was significance in the difference of miR-21 expression levels between Her-2-negative and Her-2-positive tumors. We detected significant difference in miR-21 expression levels between IDC-DCIS and IDC Her-2-positive tumors. This means that the Her-2 receptor is another factor that separates IDC-DCIS and IDC tumors with regard to miR-21 expression (Huang et al., 2009). Ki-67 is a marker of a tumor activity. Higher Ki-67 levels might indicate potential progression from non-invasive towards aggressively invasive phenotype (Gerdes et al., 1991). Elevated Ki-67 levels in DCIS tumors might anticipate recurrence of the in situ carcinoma after breast-conserving surgery or progression to IDC. Wong et al. (2010) showed that the frequency of IDC-DCIS was associated with Ki-67 levels in a low Ki-67 group. We did not find statistically significant differences between miR-21 levels in IDC-DCIS and IDC groups when divided into tumors with $Ki-67 \leq 20\%$ and $Ki-67 > 20\%$. In the IDC-DCIS tumor group, we observed that tumors with $Ki-67 \leq 20$ had significantly higher miR-21 expression levels than $Ki-67 > 20$ tumors, similar to pure invasive tumors from our previous study, (Petrović et al., 2014a). Our findings could be explained by the dual form of the tumor, i.e. the influences of both-DCIS and IDC components that are individual. Wong et al. (2010) implied in their research similar findings. We assumed that the presence of DCIS might not be silent, and might have some impact based on the changes in genetic and epigenetic levels that might have some repercussion on the behavior of the entire tumor (Chagpar et al., 2009). However, our findings showed that there were no relations between miR-21 expression levels and DCIS contribution. Furthermore, we compared IDC-DCIS tumors with less than or equal to 30% of DCIS with tumors containing more than 30% of DCIS, and there was a lack of significant difference. DCIS % highly positively correlated with tumor size, but correlations with age, ER, PR or Ki-67 were not found. Also,

miR-21 expression does not depend on the histological type of a tumor (whether we compare IDC-DCIS with IDC, with ILC, or ILC and IDC together) according to the results of previous and present studies (Petrović et al., 2014a; Petrović et al., 2014b).

The question whether these are transitional forms between *in situ* and invasive tumors is impossible to answer, because each person is unique and breast cancer is a very heterogeneous disease, influenced by many genetic, epigenetic and microenvironmental factors (Allinen et al., 2004; Polyak and Hu, 2005; Polyak, 2007).

In our previous research (Petrović et al., 2014a) we implied that it was necessary to add new non-standard diagnostic and prognostic markers and that it was also necessary to form additional systems of classification and therapeutic approach. New therapy approaches should be used especially in ER+/PR+ and ER+/PR- groups of patients with higher miR-21 expression levels, in order to prevent or halt further invasion and metastases in patients that do not respond or are resistant to tamoxifen. It has already been shown that miR-21 expression was related to ER expression (Wickramasinghe et al., 2009; Yan et al., 2011). Also, miR-21 expression levels in ER+/PR+, ER+PR- or ER-/PR- subgroups of patients with invasive BCs associated with non-invasive should be considered to act differently. These findings confirm our assumption that invasive cancers with non-invasive breast cancer forms act in a different manner when compared to pure invasive tumors according to miR-21 expression levels. In addition, based on our findings related to the different behavior of miR-21/ER/PR/Her-2 factors, high- miR-21/ER+/PR+/PR-/grade 2 and/or Her-2+ IDC-DCIS tumors might have therapeutically challenging phenotype for future anti-miR therapies.

MicroRNA-21 expression in IDC-DCIS tumors that were investigated might have been Her-2-dependent; this could have been responsible for the difference in miR-21 expression between Her-2- and Her-2+ subgroups (that was not detected in pure invasive tumors) (Huang et al., 2009; Petrović et al., 2014a; Petrović et al., 2014b). Thus, Her-2+ groups with

higher miR-21 expression levels in patients that do not respond to conventional therapies with herceptin should be considered for future anti-miR-21 therapy (in contrast to pure invasive breast carcinomas, according to our study).

In summary, in the previous research we showed that miR-21 expression in groups formed according to ER, PR, grade 2 and K-67 \leq 20% significantly differed from those in pure invasive forms, while in our novel research (with fewer factors included and with a slightly larger IDC-DCIS group), these two groups differed with higher significance in our present research, and differed in Her-2-positive tumors, unlike in our previous research (Petrović et al., 2014b). Also in this study, the difference in K-67 \leq 20% group did appear, unlike previous results (Petrović et al., 2014b).

CONCLUSIONS

This study shows the complexity and heterogeneity of breast cancer and the need for additional systems of classification as well as the need to identify new factors/biomarkers. In order to improve access to treatment and therapy, multi-component tumors should be considered as special entities. It is necessary to seek new ways of treatment and to move towards a personalized and away from a generalized approach to patients with breast carcinoma.

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