Am J Cancer Res 2023;13(6):2471-2487
www.ajcr.us /ISSN:2156-6976/ajcr0149287

Original Article

Association of high miR-27a, miR-206, and miR-214 expression with poor patient prognosis and increased chemoresistance in triple-negative breast cancer

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Received January 23, 2023; Accepted April 9, 2023; Epub June 15, 2023; Published June 30, 2023

Abstract: Triple-negative breast cancer (TNBC) represents the most aggressive breast cancer subtype, associated with early metastasis and recurrence as well as poor patient outcome. TNBC does not or weakly respond to hormonal or HER2-targeted therapies. Therefore, there is a strong need to identify other potential molecular targets for TNBC therapy. Micro-RNAs play important roles in the post-transcriptional regulation of gene expression. Thus, micro-RNAs, displaying an association between elevated expression and poor patient prognosis, may represent candidates for such novel tumor targets. In the present study, we evaluated the prognostic impact of miR-27a, miR-206, and miR-214 in TNBC via qPCR in tumor tissue (n=146). In univariate Cox regression analysis, elevated expression of all three analyzed micro-RNAs was significantly associated with shortened disease-free survival (hazard ratio [HR] for miR-27a: 1.85, P=0.038; miR-206: 1.83, P=0.041; miR-214: 2.06, P=0.012). In multivariable analysis, the micro-RNAs remained independent biomarkers for disease-free survival (HR for miR-27a: 1.99, P=0.033; miR-206: 2.14, P=0.018; miR-214: 2.01, P=0.026). Furthermore, our results suggest that elevated levels of these micro-RNAs are linked to enhanced resistance to chemotherapy. Based on the association of high expression levels with shortened patient survival and increased chemoresistance, miR-27a, miR-206, and miR-214 may represent novel molecular targets for TNBC.

Keywords: miR-27a, miR-206, miR-214, miRNA, triple-negative breast cancer, biomarker, chemoresistance

Introduction

Breast cancer is the world's most prevalent cancer accounting for 1 in 8 cancers diagnosed worldwide. It affects women at any age after puberty and corresponds to 16% of cancer related deaths in women [1]. However, breast cancer treatment can be highly effective, especially, if the disease is detected at an early stage. Most of the patients are eligible for surgery and radiation therapy and many of them benefit from available efficient targeted therapies such as anti-hormonal therapy or HER2-directed antibody treatment. However, the triple-negative breast cancer (TNBC) subtype is characterized by the lack of estrogen receptor (ER), progesterone receptor (PR) expression, as well as HER2 receptor overexpression. This phenotype leaves patients with a very aggressive disease with limited therapeutic options such as systemic chemotherapies (platinum-, taxane-, or anthracycline-containing regimens

or post-adjuvant capecitabine therapy). TNBC patients with mutations of the BRCA1/2 gene are susceptible to PARP inhibition, however, only 20% of all TNBC cases show the relevant genetic aberrations. The therapeutic options might be expanded by additional antibody-based therapies such as antibody-drug conjugates (trastuzumab duocarmazine or sacituzumab govitecan) or immune-checkpoint blockade (pembrolizumab). The persistent poor prognosis of TNBC patients together with the lack of efficient therapy options fuels the need for the finding of new biomarkers potentially enabling the development of new therapeutic strategies for TNBC [2] or enabling the sustainability of existing therapies together with the prevention of therapy resistance.

Micro-RNAs are non-coding, short (22-24 nucleotides) RNAs, which mediate degradation or inhibition of the targeted mRNA [3]. The implication of micro-RNAs in the progression of breast cancer has been extensively investigated in different studies evaluating several potential interaction nodes for cancer progression: (a) modulation of tumor aggressiveness by targeting interaction partners involved in proliferation, migration, cell cycle arrest, apoptosis, and angiogenesis [4]; (b) involvement in immune escape by providing immune cell impairment [5, 6]; and (c) influence on metastasis by interacting with epithelial-to-mesenchymal-transition (EMT)-associated proteins [4, 7]. Besides their functional implication, the prognostic potential of miRNAs has been thoroughly described [8] and the accuracy of miRNA expression profiles is superior to protein-coding mRNA-based approaches [9].

Mostly half of the known human miRNA-encoding genes are associated with chromosomal instabilities in cancer-linked regions [10]. Breast cancer-related micro-RNAs can function both as tumor suppressors and oncogenes [11]. The roles of individual micro-RNAs can even change depending on the tumor type, progression, or stage of the disease. The improvement of diagnostic and therapeutic tools for breast cancer treatment has significantly reduced cancer-related death rates. However, the therapy options for TNBC remain limited and advances in treatment and diagnosis could further improve patients' outcomes and survival. Three micro-RNAs that are upregulated in breast cancer and have been particularly associated to TNBC, are miR-27a [12-15], miR-214 [16-21], and miR-206 [22-25]. Moreover, all of these micro-RNAs have been linked to cancer-related micro-RNA clusters, which further strengthens their functional involvement in TNBC progression.

miR-27a has been observed to be upregulated in breast tumor tissue, compared to adjacent healthy tissue [12, 13]. TNBC subtype-associated expression levels have not been determined yet, albeit *in vitro* studies described the increased expression of miR-27a in several TNBC cell lines [14, 15]. The expression pattern of miR-214 is so far variously discussed in breast cancer. Several studies observed downregulation of miR-214 in breast cancer tissue compared to healthy controls [16-18], however, contrary results, particularly in TNBC, have been reported as well, with lower miR-214 levels in normal tissue compared to breast cancer tissue [19-21]. Similar conflicting results were found for miR-206. Some studies reported downregulation of miR-206 in tumor tissue [22, 23], whereas others described an upregulation of miR-206 [24, 25].

To further extend the knowledge concerning the clinical relevance of the three breast cancer-related micro-RNAs miR-27a, miR-214, and miR-206 in TNBC, in the present study, we assessed the expression levels of these micro-RNAs in a cohort of 146 triple-negative breast cancer patients and correlated them with clinical parameters and patients' disease-free survival by univariate and multivariable regression analysis. Moreover, subgroup analyses related to the nodal status of the patients (nodal-negative versus nodal-positive) as well as chemotherapy treatment (untreated versus treated) were performed to evaluate whether the micro-RNA expression levels are subgroup-specifically associated with patient prognosis.

Materials and methods

Patients

One hundred and forty-six patients with TNBC were enrolled in the study. The patients were diagnosed between 1988 and 2012 at the Department of Obstetrics and Gynecology, Klinikum rechts der Isar, Technical University of Munich (TUM). There was no administration of

chemotherapy before surgery. Tumor tissues were collected after inspection by pathologists and immediately stored in liquid nitrogen. Tumors were routinely tested for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) expression and categorized as TNBC if there was a lack or low expression of ER and PR (immunohistochemistry staining below 1% for ER and 5% for PR) and absence of or low HER2 expression (immunohistochemically determined score 0, 1+; or score 2+ with no signs of HER2 gene amplification by fluorescence *in situ* hybridizations [FISH] test). In 2013, the TNBC status was collectively re-evaluated by pathologists.

All patients primarily underwent standard surgical procedures including mastectomy or breast-conserving surgery including axillary sentinel lymph node biopsy or dissection. None of them had distant metastasis at the time of surgery. Adjuvant therapy (anthracycline- or cyclophosphamide-based) was administered based on consensus recommendations at that time. Chemotherapy was administered in 64% (89/146) of cases. Patients did not receive chemotherapy due to clinical contraindication, early-stage or small tumors, a negative lymph node status, or declined therapy by the patients. The predominant breast cancer subtype in this cohort was classified as invasive ductal, grade 2 (n=26) and grade 3 (n=121). The remaining cases consisted of less frequent subtypes including medullary and lobular cancers. The age of the patients ranged from 30 to 96 years (median 59 years) with a median follow-up time of 68 months (2-296 months). The tumor size ranged from 2 to 140 mm (median 26 mm).

The biomarker study in breast tumor tissues was approved by the local Ethics Committee, based on the declaration of Helsinki, and written informed consent for the study was available for all patients. Subgroup analyses were performed for the lymph node status (N0 vs. N+) and therapy (untreated vs. treated).

Real-time polymerase chain reaction

Isolation of total RNA from fresh frozen tissue including both mRNA and micro-RNA was performed with the automated QIAcube sample preparation machine (Qiagen) applying the All Prep DNA/RNA/miRNA Universal Kit (Qiagen). RNA quality and concentration were spectrophotometrically quantified and 1 µg was used for reverse transcription using the miScript II RT Kit (Qiagen).

The analysis of the micro-RNAs was performed with specific primers of the miScript Primer Assay (Qiagen) together with miScript SYBR Green PCR Kit 218076 (Qiagen). The following assays were used: miR-27a-3p (MI0000085), miScript assay number: ms00003241 mature miRNA sequence: UUCACAGUGGCUAAGUUCCGC; miR-206-3p (MI0000490): miScript Primer Assay ms00003787 mature miRNA sequence: UGGAAUGUAAGGAAGUGUGUGG; miR-214-3p (MI0000290): miScript Primer Assay ms00031605 mature miRNA sequence: ACAGCAGGCACAGACAGGCAGU; miR-16-3p (MI0000070) MS00031493 mature micro-RNA sequence: UAGCAGCACGUAAAUAUUGGCG.

Standard dilution series were employed to determine the reaction efficiency and sensitivity. Threshold cycles (Ct) were utilized to calculate the relative micro-RNA expression levels of the analyzed miRs normalized to the housekeeping gene miR-16 using the $2^{-\Delta\Delta Ct}$ method. The quality of the acquired q-PCR data was controlled, whereby one or more of the following criteria led to the exclusion of individual samples: a Ct value for miR-16 > 35 as an indicator for non-sufficient RNA quality; an error of $2^{-\Delta\Delta CT}$ between sample and reference above 30%; a standard deviation higher than 47.1% after two repeated analysis.

In silico analysis

Target prediction of miRNAs of interest (miR-27a, miR-206, miR-214) was performed by miRDB (http://mirdb.org/) [26] and by miRWalk (http://mirwalk.umm.uni-heidelberg.de/) [27]. Thereafter, common predicted target genes were subjected to Gene Ontology (GO) enrichment analyses of biological processes, cellular components, and molecular functions through the functional annotation tool of DAVID v6.8 (https://david.ncifcrf.gov/summary.jsp) [28].

Statistics

The association of the micro-RNA expression levels with clinicopathological parameters of the patients was assessed by the Chi-square test. Disease-free survival (DFS) was defined

Table 1. Association of relative micro-RNA expression levels and clinicopathological parameters in patients with triple-negative breast cancer

Clinical parameter	miR-27a low/high[a]	miR-206 low/high[a]	miR-214 low/high[a]
Age	P=0.874	P=0.509	P=*0.077*
< 60 years	39/37	50/21	54/21
≥ 60 years	35/35	43/23	40/29
Lymph node status	P=0.428	P=0.231	P=0.812
N0	40/43	56/22	54/28
N+	34/28	36/22	39/22
Tumor size	P=0.649	P=0.601	P=0.943
≤ 20 mm	16/17	20/11	22/11
> 20 mm	52/46	64/28	64/33
Histological grade	P=0.709	P=0.785	P=*0.066*
Grade II	12/13	15/8	10/14
Grade III	62/57	77/36	82/36
Chemotherapy	P=0.954	P=0.169	P=*0.073*
No	25/24	29/19	26/22
Yes	49/48	64/25	68/28

[a]Chi-square test (cut-off point: miR-27a = median, miR-206 = 66th percentile, miR-214 = 66th percentile). Trends towards significance are indicated in italics. Due to missing values, samples do not add up to 146.

as the time from the primary surgery to the first documented DFS event (disease recurrence or death of any cause). Survival analyses were performed by constructing Kaplan-Meier curves. The log-rank test was applied to evaluate group differences in survival functions. Associations of micro-RNAs and clinical parameters with patients' survival were additionally determined by univariate and multivariable Cox regression analysis and expressed as hazard ratio (HR) as well as its 95% confidence interval (95% CI). All calculations were performed with the SPSS statistical analysis software (version 20.0; SPSS Inc., Chicago, IL, USA). *p*-values of less than 0.05 were considered statistically significant.

Results

Expression of miR-27a, miR-206, and miR-214 in tumor tissues of triple-negative breast cancer patients and their correlation with clinical parameters

Expression levels of micro-RNAs were quantified by qPCR assays in 146 cases of TNBC tissues. miR-27a levels ranged from 0.06 to 5.07 (median: 0.62), miR-206 mRNA expression from undetectable to 873.10 (median: 0.62), and miR-214 mRNA expression from 0.16 to 1335.66 (median: 44.43) arbitrary units (au). Expression of miR-27a was categorized by the median into a low versus a high expression group, in the case of miR-206 as well as miR-214 the tertials were used (tertial 1 + 2 versus tertial 3).

The associations between relative micro-RNA expression levels and the established clinical variables including age, lymph node status, tumor size, chemotherapy, and histological grade were examined (**Table 1**). Neither miR-27a nor miR-206 nor miR-214 mRNA expression levels were significantly associated with any clinicopathological parameters of TNBC.

Association of miR-27a, miR-206, and miR-214 expression levels with DFS in univariate Cox regression analysis

Univariate Cox regression analysis was performed to evaluate the prognostic relevance of the micro-RNAs and the clinicopathological parameters regarding the patient outcome in the TNBC cohort (**Table 2**). Among the clinical variables, a positive lymph node status indicated a significantly shorter DFS, whereas the administration of chemotherapy was an indicator of prolonged survival or onset of recurrence.

Elevated expression levels of all three analyzed micro-RNAs were found to be a significant predictive factor for worse DFS with an about two-fold increased recurrence probability in the high-expressing groups (miR-27a: HR=1.85, P=0.038; miR-206: HR=1.83, P=0.041; miR-214: HR=2.06, P=0.012).

The impact of these factors on patient survival was also confirmed by Kaplan-Meier estimation. As shown by the respective survival curves, again high miR-27a (P=0.034, **Figure 1A**), high miR-206 (P=0.038, **Figure 1B**), and high miR-214 expression levels (P=0.010, **Figure 1C**) were significantly associated with shortened DFS. The obtained data were compared and in part validated by an *in silico* analysis of publicly available micro-RNA expression data of a genome-wide study focusing on TNBC [29]. Here, expression of miR-27a displayed a

Table 2. Clinical outcome in triple-negative breast cancer patients: univariate and multivariable COX regression analysis including clinicopathological parameters and micro-RNA expression

Clinical parameter	univariate analysis			multivariable analysis		
	Disease-free survival (DFS)					
	No.[a]	HR (95% CI)[b]	p	No.	HR (95% CI)[b]	p
Age			*0.065*			0.672
< 60 years	68	1		55	1	
≥ 60 years	63	1.69 (0.97-2.94)		54	1.16 (0.57-2.37)	
Lymph node status			**0.012**			**0.003**
N0	74	1		62	1	
N+	56	2.06 (1.18-3.62)		47	2.70 (1.39-5.33)	
Tumor size			0.314			0.667
≤ 20 mm	34	1		29	1	
> 20 mm	89	1.43 (0.71-2.88)		80	1.19 (0.55-2.58)	
Chemotherapy			**0.005**			**0.003**
no	39	1		31	1	
yes	62	0.45 (0.26-0.78)		78	0.33 (0.16-0.69)	
miR-27a[c]			**0.038**			**0.033**
low	64	1		57	1	
high	63	1.85 (1.04-3.30)		52	1.99 (1.06-3.74)	
miR-206[c]			**0.041**			**0.018**
low	80	1		73	1	
high	40	1.83 (1.02-3.26)		36	2.14 (1.14-4.02)	
miR-214[c]			**0.012**			**0.026**
low	84	1		73	1	
high	42	2.06 (1.17-3.64)		36	2.01 (1.08-3.72)	

[a]Due to missing follow-up or values, samples do not add up to 146. [b]HR: hazard ratio, CI: confidence interval. [c]Dichotomization (cut-off point: miR-27a = median, miR-206 = 66th percentile, miR-214 = 66th percentile). Significant *p* values (P < 0.05) are indicated in bold, trends towards significance are indicated in italics.

trend towards significance concerning association with overall survival (P=0.112, **Figure 2A**), and high miR-214 expression was significantly associated with shorter overall survival (P=0.030, **Figure 2B**). In the dataset from de Rinaldi et al. [29], there were no expression data available for miR-206.

Association of miR-27a, miR-206, and miR-214 expression levels with DFS in multivariable Cox regression analysis

The independence of the micro-RNAs as prognostic factors in TNBC was studied in multivariable Cox regression analysis (**Table 2**). We established a base model encompassing age, lymph node status, tumor size, and chemotherapy. Here, a positive lymph node status was significantly associated with an increased risk for disease recurrence or death (HR=2.89, P=0.002) and the administration of chemotherapy is strongly linked to a more favorable outcome (HR=0.30, P=0.001). Among the tumor biological factors (added separately to the base model), all three miR-27a (HR=1.99, P=0.033), miR-206 (HR=2.14, P=0.018) and miR-214 (HR=2.01, P=0.026) expression, significantly contributed to the base model.

Because all three micro-RNAs were independent factors in the multivariable analysis, we also analyzed whether a combined factor of two micro-RNAs may be able to further discriminate between a high- and low-risk patient group. Indeed, the high/high group of the combined factors displayed an almost 3-fold increased risk for recurrence or death as compared to the low and/or low group (Table S1).

Differences in the prognostic value of micro-RNA expression levels in treated and non-treated TNBC patients

The study cohort encompassed patients which had received surgery between 1988 and 2012,

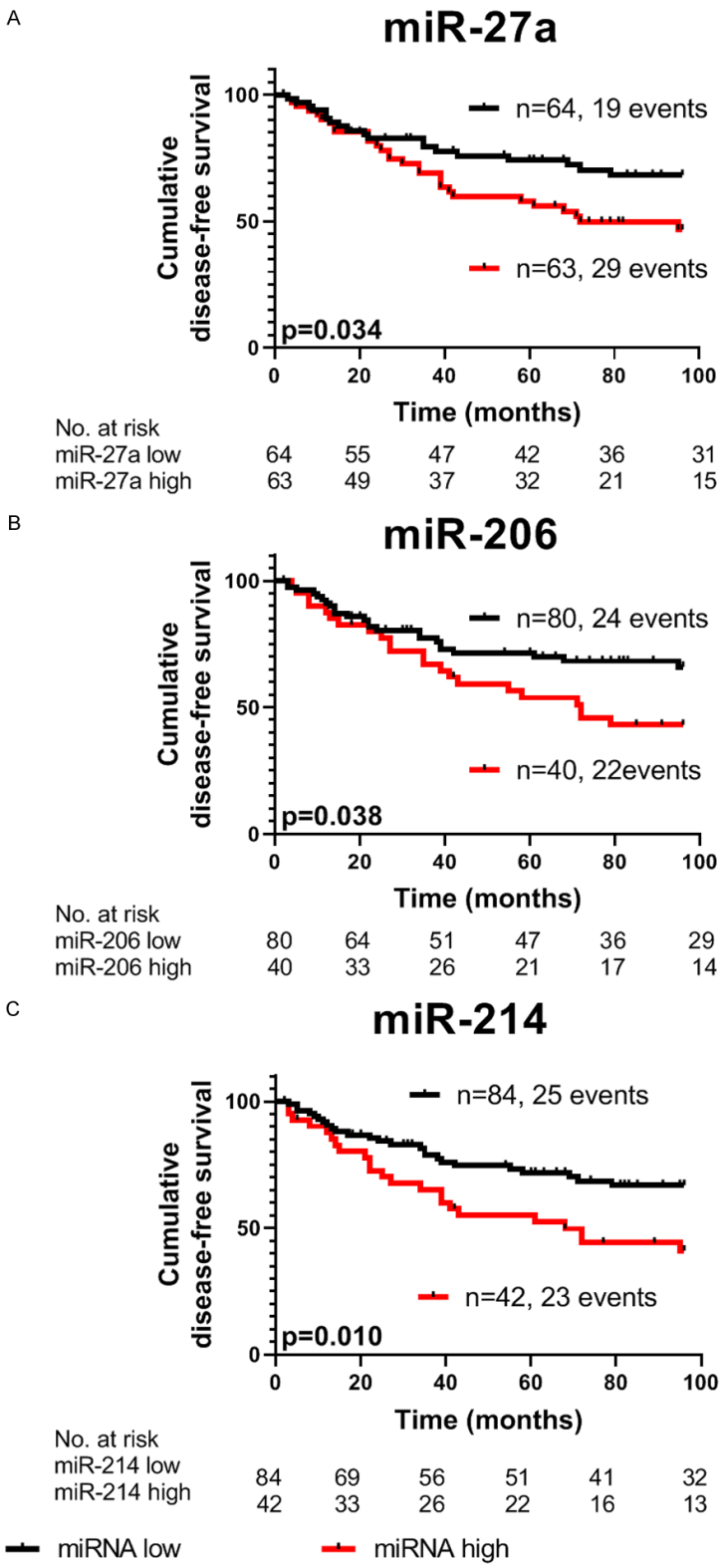

Figure 1. Kaplan-Meier survival analysis showing the impact of increased micro-RNA expression on disease-free survival of patients afflicted with triple-negative breast cancer. (A) miR-27a, (B) miR-206, and (C) miR-214 expression in primary tumor tissue. Micro-RNA expression was dichotomized into low (black) and high (red) expression by the median for miR-27a and by the 66th percentile for miR-206 and miR-214, respectively.

whereby about one-third of the patients had not received any chemotherapy. Subgroup analysis (treated versus non-treated patients) revealed that in the miR low-expressing groups, there was a significant association of treatment with chemotherapy (anthracycline and cyclophosphamide-based) with better survival. This positive predictive value was true for all three micro-RNAs (miR-27a: P=0.004; miR-206: P=0.043; miR-214: P=0.003). However, such statistically significant beneficial effects of chemotherapy administration were not observed in the miR high-expressing groups for all three factors (**Figure 3**). These results indicate that an elevated tumor-associated expression of either of the analyzed micro-RNAs is related to weak or no response to chemotherapy. In line with these results, an additional subgroup analysis with a retrospective stratification for treatment (yes vs. no) instead of micro-RNA expression, reveals a subgroup of patients with high miR-expression, that does not sufficiently benefit from the applied chemotherapy treatment (Figure S1).

Differences in the prognostic value of micro-RNA expression levels in lymph node-negative (N0) versus lymph node-positive (N+) TNBC patients

When the N0 and N+ patients were analyzed separately (**Figure 4**), we observed that both low expression levels of miR-27a and to a lesser extent miR-206 were able to define a favorable prognosis group in the N+, but not N0, subgroup (miR-27a: P=0.03; miR-206: P=0.07). By contrast, low expression of miR-214 was significantly associated with a favorable patient prognosis in N0, but not N+, patients (P=0.003).

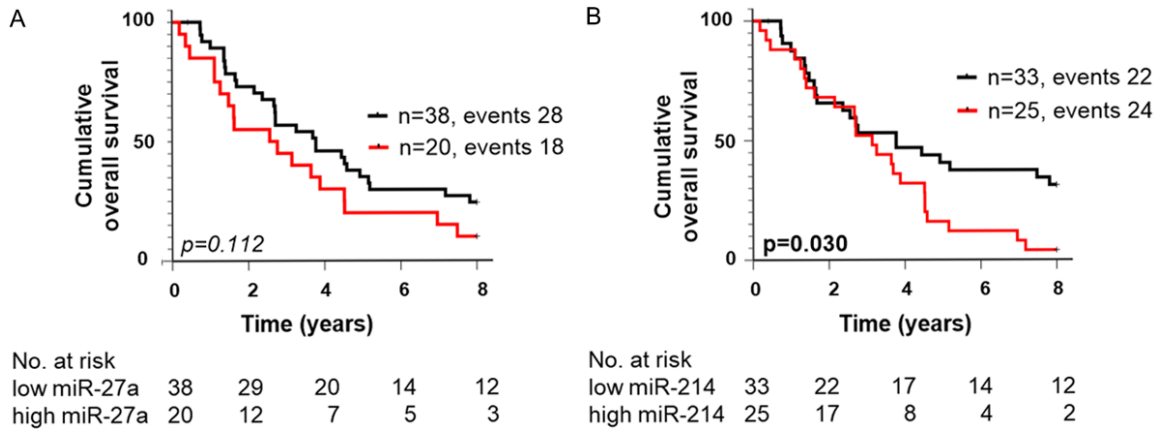

Figure 2. Kaplan-Meier survival curves illustrating the relation of micro-RNA expression levels with overall survival in a publicly available dataset. The data for *in silico* analysis were taken from [29]. A. miR-27a; B. miR-214. *p*-values were calculated by log-rank test. A trend towards significance is indicated in italics. Black, low expression; red, high expression.

Target prediction and gene enrichment analyses of miR-27a, miR-206 and miR-214

The identification of potential target genes for the three micro-RNAs was performed via target prediction analysis by miRDB and miRWalk. The overlap of potential target genes of both databases was used for further interpretation. After this adjustment, 415 target genes were identified for miR-27a, 277 for miR-206 and 617 for miR-214 (**Figure 5A-C**). A gene enrichment analysis was performed with the positively selected genes using the GO enrichment by the DAVID functional annotation tool. For each micro-RNA, the twenty most enriched terms for biological processes, cellular components, and molecular functions, with a p-value ≤ 0.05, are illustrated in **Figure 5A-C**. Among the most enriched genes were those involved in regulation of RNA metabolic processes, cell junction, and intracellular organelles.

Discussion

Clinical relevance of micro-RNAs in TNBC

Quantification of miR-27a, miR-214, and miR-206 in tumor tissue of a cohort of 146 triple-negative breast cancer patients revealed an association of elevated expression of these micro-RNAs with shortened DFS in univariate analysis. This holds also true in multivariate COX regression analyses, where all three micro-RNAs significantly contribute to the base model encompassing age and the clinical factors lymph node status, tumor size and chemotherapy. Thus, miR-27a, miR-206 and miR-214 can be considered independent negative prognostic biomarkers for TNBC.

miR-27a

Although its tumor biological role in breast cancer - evaluated *in vitro* - is controversially discussed, miR-27a seems to represent a biomarker for cancer progression and therapy sensitivity for all breast cancer subtypes [30]. Tang et al. [31] have proposed high miR-27a expression as an unfavorable prognostic marker for breast cancer due to its correlation with shortened disease-free and overall survival in 124 patients with mixed breast cancer subtypes. Based on these studies, miR-27a has been included in a 4-miR marker panel that allowed for the identification of patients with poor prognosis and therapy response. The negative prognostic impact is in line with an *in silico* analysis using a TCGA data set [14] showing also a shorter overall survival of patients with high miR-27a expression. Furthermore, in the present study, an analysis performed with another publicly available data set [29] revealed a trend towards significance concerning poor outcome of TNBC patients with high miR-27a expression in terms of overall survival as well.

The association of elevated miR-27a levels with poor prognosis is in line with its tumor-promoting properties that have been described *in*

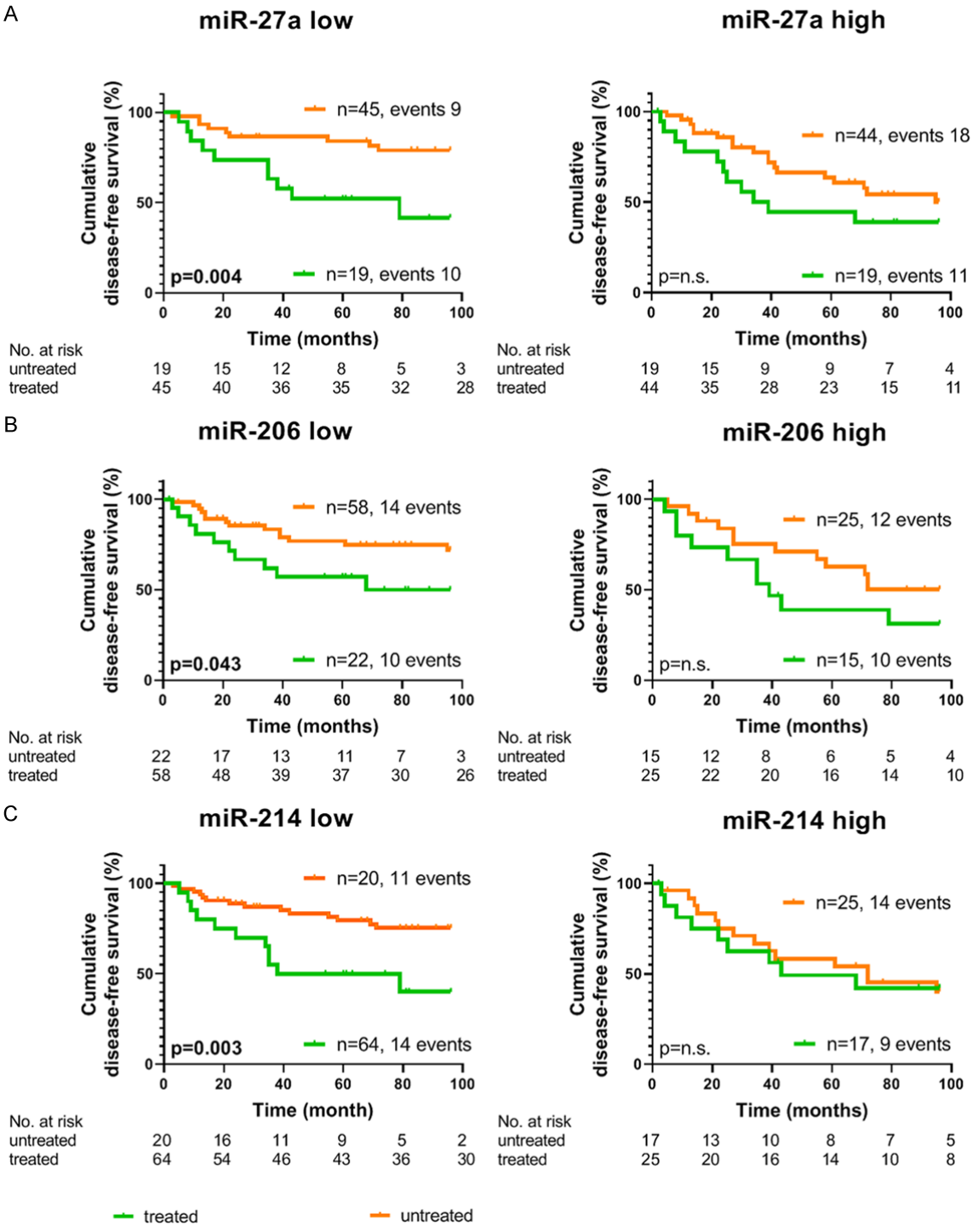

Figure 3. Prognostic value of micro-RNA expression levels in the subgroups of non-treated versus treated TNBC patients. miR-27a (A), miR-206 (B) and miR-214 (C) expression levels, respectively, can discriminate between a low- and high-risk group in treated, but not untreated, patients. *p*-values were calculated by log-rank test. Green, untreated; orange, treated.

vitro. Upregulation of miR-27a fosters invasion, migration, proliferation, and reduced sensitivity to chemotherapy in different cell culture systems [12, 14, 32]. Some of these effects, *e.g.* on migration and EMT, were shown to be reversed upon miR-27a inhibition in TNBC cell

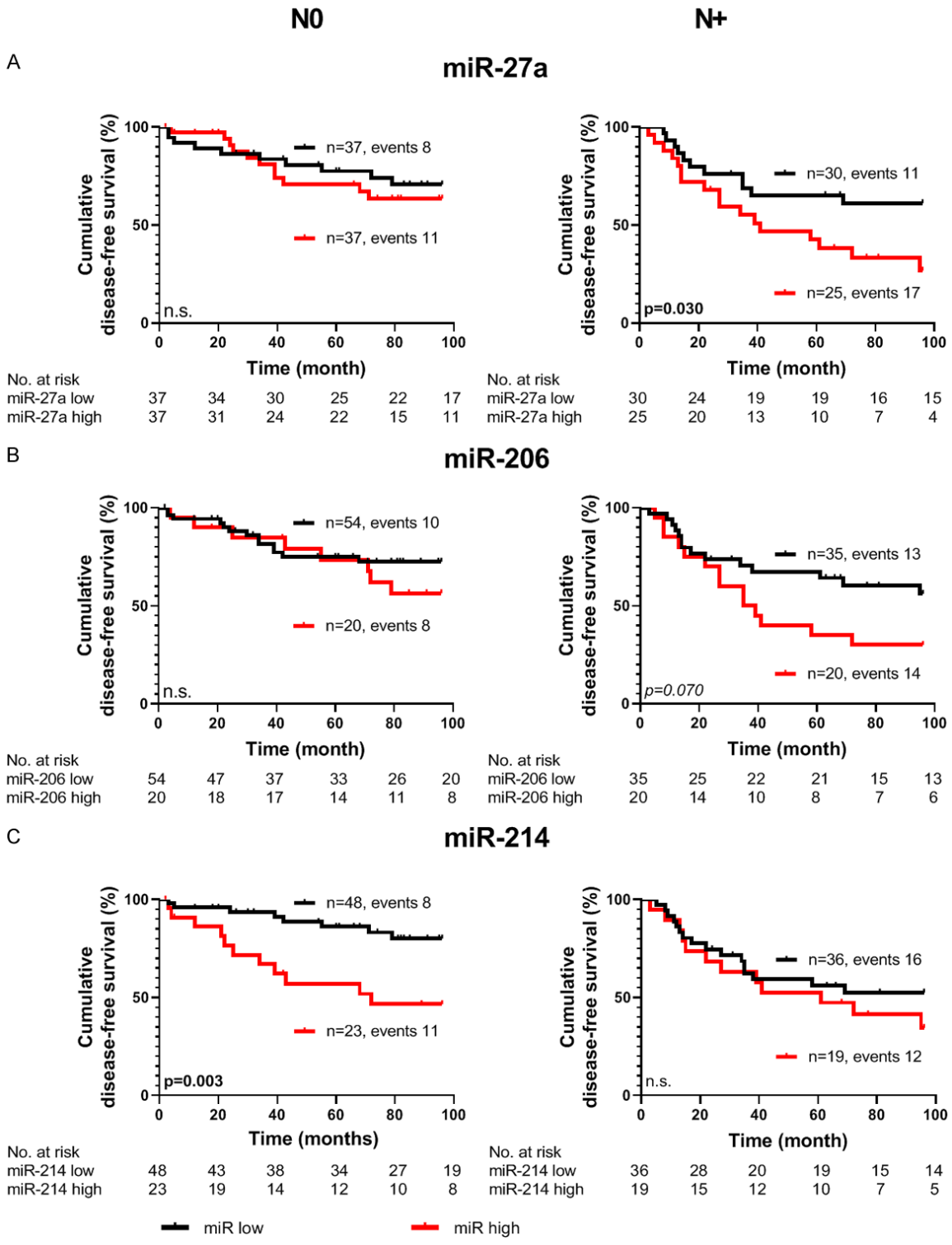

Figure 4. Prognostic value of micro-RNA expression levels in the subgroups of lymph node-negative (N0) versus lymph node-positive (N+) TNBC patients. miR-27a expression levels (A) are able to discriminate between a low- and high-risk group in N+, but not N0, patients. miR-206 (B) displays a similar trend (indicated in italics). Conversely, miR-214 expression levels (C) significantly discriminate between a low- and high-risk group in N0, but not N+, patients. *p*-values were calculated by log-rank test. Black, low expression; red, high expression.

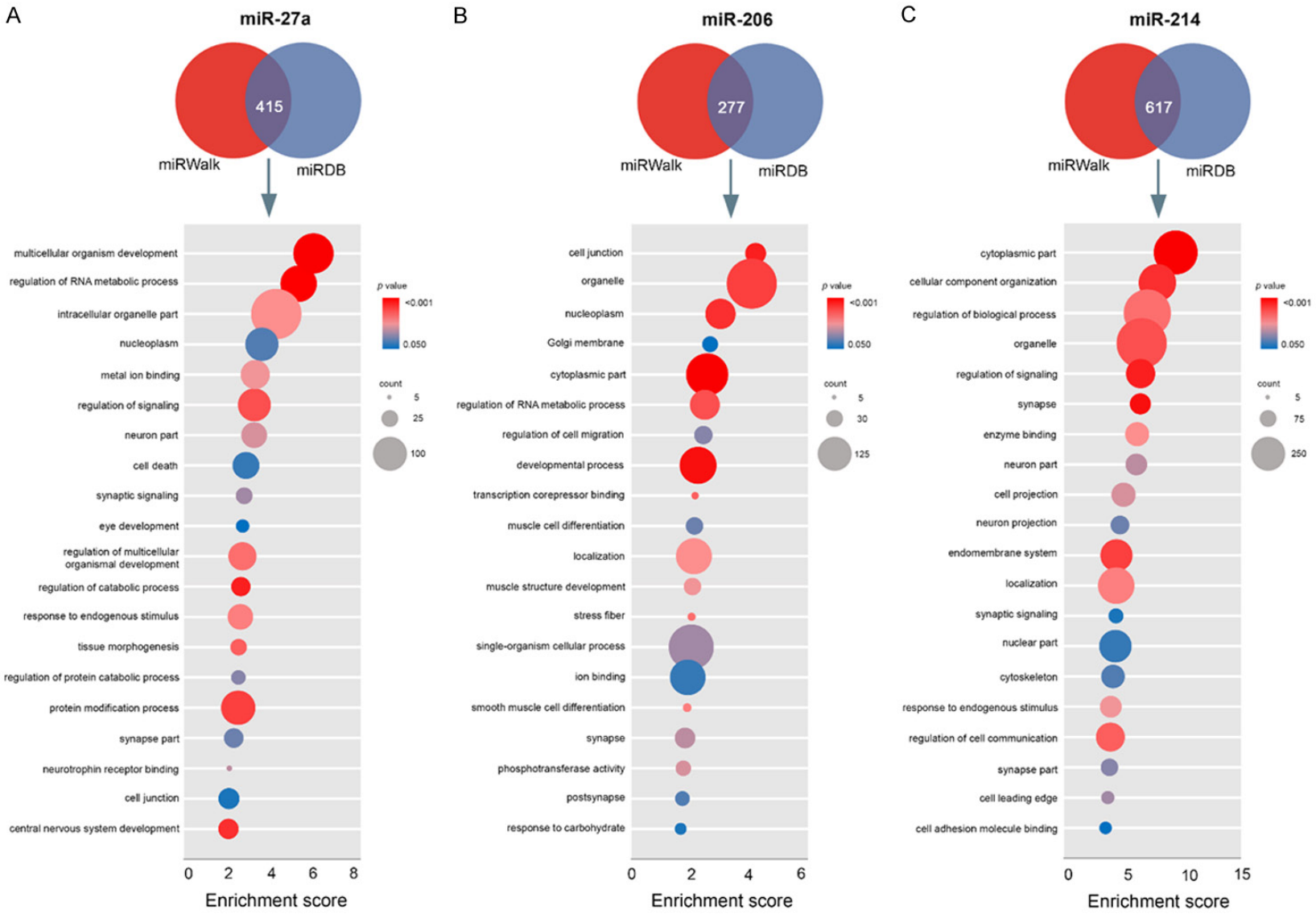

Figure 5. Target prediction and gene ontology (GO) analysis of miR-27a, miR-206 and miR-214. Overlapping circles of Venn diagrams indicate the common predicted target genes as obtained by miRDB and miRWalk. GO enrichment analysis of biological processes, cellular components, and molecular functions of the predicted (A) miR-27a, (B) miR-206 and (C) miR-214 targets illustrated as a bubble plot.

lines [13]. These findings are consistent with the miR-27a-promoted tumorigenesis *in vivo* resulting in increased tumor growth in an MDA-MB-231 xenograft mouse model [33].

The performed target prediction analysis points to an involvement of miR-27a in the regulation of RNA metabolic processes, which is in line with the general dysregulation of RNA and miRNA profiles during cancer progression [10]. Furthermore, additional modes of action, that have previously been associated with miR-27a expression and tumor progression, such as stem cell differentiation [34] or mitochondrial dysfunction [35], may contribute to the observed data in our study.

Clinically relevant biomarkers display a major advantage, if they are non-invasively accessible for routine testing and quantification, for example during regularly performed blood sampling. The potential application of miR-27a as a cancer biomarker in plasma samples of breast cancer patients has been assessed. Here, one study revealed the downregulation of miR-27a in the plasma of breast cancer patients but failed to resolve between different gradings or receptor statuses [36]. Of note, however, a more recent study showed increased serum levels of miR-27a in a cohort of 40 breast cancer patients compared to healthy controls, benign cases, and high-risk groups determined by family history for breast cancer [37].

Our data strongly suggest that miR-27a is an unfavorable prognostic factor in the TNBC subgroup. The adverse effect of miR-27a expression in breast cancer is in concordance with the described tumor-promoting characteristics *in vitro* and the suggested overexpression in breast cancer tissue. Quantification of miR-27a in TNBC tissues may thereby provide additional predictive information for further risk stratification and may represent a novel target for interventive strategies such as micro-RNA inhibition.

miR-214

Research on the clinical impact of miR-214 expression in breast cancer patients has been published earlier indicating an association of high miR-214 expression with worse overall survival in a group of 50 triple-negative breast cancer composed of sporadic and BRCA1-driven carcinogenesis [38] and an association of low miR-214 expression with prolonged disease-free survival in 31 breast cancer patients [39]. In addition to the results of the present work revealing a negative prognostic impact of miR-214 in our TNBC cohort, we also performed an *in silico* analysis using publicly available data [29]. Here, the results in our TNBC cohort were validated. Our data as well as the *in silico* analysis demonstrate an association of miR-214 expression with clinical outcome, thus, confirming the previous results obtained by small-scale studies of TNBC patients and breast cancer patients of mixed subtypes, respectively, revealing that elevated miR-214 expression in TNBC tissue of patients is a prognostic factor for worse DFS.

The functional significance of miR-214 in breast cancer progression is just as opaque as the expression profiles in breast cancer tissue. On the one hand, miR-214 overexpression in breast cancer cell lines was reported to decrease proliferation and invasion [40], increase the sensitivity towards cisplatin treatment [17], increase apoptosis [41], and decrease migration together with reduced expression of EMT-related proteins indicating a mesenchymal phenotype [42].

On the other hand, miR-214 has been associated with several tumor-promoting mechanisms. Wang et al. [43] reported increased invasion, viability and reduced apoptosis of MCF7 cells upon artificial miR-214 overexpression. Moreover, suppression of miR-214 reduced migration and invasion of MDA-MB-231 cells [19]. In a xenograft mouse model, inhibition of miR-214 reduced tumor dissemination, formation of lung metastasis, as well as the number of circulating tumor cells in the blood [44].

These findings are also reflected by the genes identified in our target prediction. Potential interaction sites include cellular component organization, encompassing cell junction and migration. Furthermore, several tumor-driving parameters have been identified as potential interaction sites. Among the most enriched genes are those involved in the regulation of signaling or biological processes. This particularly includes TGF beta activation, a hallmark driver of EMT, and hormone secretion. Moreover, interaction sites linked to altered func-

tionality of organelles have been identified, such as damaged mitochondria [45].

Concerning their value as liquid biomarkers, Schwarzenbach et al. [46] analyzed the prognostic potential of miR-214 as a liquid biopsy biomarker in the serum of breast cancer patients and observed that miR-214 is upregulated in breast cancer-derived serum compared to healthy controls or benign neoplasia. Moreover, miR-214 levels dropped after successful surgery of breast cancer patients indicating the potential of miR-214 also as a cancer progression marker.

miR-206

In *in vitro* analyses, miR-206 has been proposed as a tumor suppressor in breast cancer. Upregulation of miR-206 reduced cell proliferation, migration, and viability in several breast cancer cell lines [24, 47-50]. These findings are in line with the reduced total tumor burden in TNBC patient-derived xenograft mouse models [51], as well as the decreased accumulation of brain and bone metastasis [52]. A study focussing on the clinical potential of miR-206 reported an association of low miR-206 expression with unfavorable prognosis for overall survival in a cohort of 128 breast cancer patients. Moreover, lower expression levels were significantly associated with advanced clinical staging and lymph node metastasis [53].

Still, overexpression of this micro-RNA in breast cancer has also been associated with poor prognosis in a cohort of 372 breast cancer patients [25]. Consistent with this finding, in the present study, we observed that elevated miR-206 levels are associated with a shorter DFS in TNBC patients. *In silico* analysis of the prognostic impact of miR-206 in the TCGA data set [54] did not reveal any association with survival.

One reason for the, in part, controversial findings may be that in our study we used a highly curated cohort encompassing TNBC patients only and in this highly aggressive breast cancer subtype miR-206 may act differently as compared to other subtypes. In addition to its tumor-suppressive functions, miR-206 has also been discussed as a cancer stem cell marker and it is, therefore, tempting to speculate that in TNBC the stem cell function might overcome an initial protective role by faster recurrence [55]. Moreover, miR-206 has been reported to be associated with increased therapy resistance despite its inherent tumor-suppressive effects. This impact on therapy may result in a worse outcome for those patients afflicted with the highly chemotherapy-intensive TNBC [23, 56].

The impact of predicted target genes of miR-206 reflects the previously proposed role in cell junction and migration [24]. Moreover, the impact on RNA metabolic processes might also foster the dualistic outcome of miR-206 expression in terms of enhanced RNA dysregulation [10].

Taken together, there is major evidence in the literature that all three micro-RNAs have a substantial influence on the progression of cancers and breast cancer in particular. The classification as acting either tumor-suppressive or tumor-promotive can, however, be hard to perform, since many more factors are involved in cancerogenesis. For breast cancer, also the different aspects of the - distinctly differing - molecular subtypes can influence the biology behind the disease. Since we have analyzed miR-27a, miR-206, and miR-214 expression in a cohort encompassing only patients afflicted with TNBC, our results suggest that all three micro-RNAs represent unfavorable prognostic markers in this subtype and may be more valid as compared to the use of heterogeneous breast cancer patient cohorts.

Micro-RNAs for risk stratification in lymph node-negative and lymph node-positive TNBC

The evaluation of tumor-affected lymph nodes is still one of the most powerful prognostic markers for breast cancer patients after surgery [57-59]. However, the application of the lymph node staging system is constantly criticized and suggested to be modified and adjusted to increase the accuracy and prognostic power [60-62]. The lymph node status is taken into consideration for therapy decisions, but always in concordance with the tumor staging, which, especially for TNBC, is the overall superior predictive marker [63]. There are recent approaches to improve the lymph node-derived readout via the application of gene expression-based tests. But in most cases, these tests are not appropriate or not validated for TNBC

patients [64]. In our study, we identified two micro-RNAs, miR-27a and miR-206, that were able to identify a group of TNBC patients with one or more tumor-affected lymph nodes that show a lower risk of recurrence or death and, strikingly, have an outcome comparable to lymph node-negative patients. Contrariwise, miR-214 was shown to be a biomarker capable of determining a high-risk group among lymph node-negative TNBC patients whose survival is similar to that of lymph node-positive patients. It might be useful to include the expression profiles of the upper-mentioned micro-RNAs in gene expression-based tests in order to utilize this technology also for TNBC patients.

The implication of micro-RNAs in estrogen receptor modulation and therapy resistance

Besides the complex molecular features which potentially modulate micro-RNA function in cancer progression, several additional factors may have an impact in an *in vivo* setting only and also may implement after a certain time. One of these possible mechanisms is a regulatory interaction between the expression of hormone receptors with those micro-RNAs that might foster the TNBC phenotype. Another mechanism may be the induction of chemotherapy resistance which may certainly, at least in part explain the poor prognosis of those patients.

In the case of miR-27a, a direct link between micro-RNA overexpression and increased ER-alpha concentration has been established, however, this interrelation seems to lead to regulatory feedback also resulting in miR-27a downregulation after ER expression [65]. Further analyses revealed a ZBTB10-mediated, miR27a-dependent ER-alpha regulation [66]. The interaction between miR-27a and the ER was also discussed as a mediator of tamoxifen resistance in ER-positive cell lines [67]. An inverse interaction was described for miR-206, demonstrating that ER-downregulation induces the expression of miR-206 [68]. Moreover, the blockade of miR-206 *vice versa* inhibited the expression of ER response genes and promoted a luminal A and basal-like breast cancer phenotype [69]. Furthermore, post-transcriptional regulation of the ER by miR-206 was postulated [70]. For miR-214, direct interaction with the ER has not been shown so far, however, miR-214 can help to overcome endocrine resistance in breast cancer cell lines, also indicating a cross-regulation of the hormone receptors [21].

Another persistent problem in the therapy of TNBC is the accumulation of resistance to the limited choices of standard chemotherapy. All of the analyzed micro-RNAs in this study have been linked to therapy resistance before and, in fact, in the present study, we were able to identify subgroups of patients that seem to not benefit from chemotherapy treatment. There is no statistically significant association between micro-RNA expression and the status of treatment (chemotherapy yes vs. no). miR-27a has been shown to increase the resistance to platinum-based chemotherapy in ovarian [71] as well as colon cancer [72] and can induce multidrug resistance in esophageal [73] and gastric cancer [74]. Similar results have been described for miR-214, whereupon elevated micro-RNA expression results in increased resistance to platinum-based chemotherapy in ovarian cancer, osteosarcoma, and esophageal squamous cell carcinoma were reported [75-77]. Moreover, the efficacy of erlotinib, a tyrosine kinase inhibitor that is generally administered after the failure of first-line chemotherapy in non-small cell lung cancer, was impaired by miR-214 overexpression [78]. It has, however, been reported that miR-206 reduces the 5-FU resistance in colon cancer [79] as well as the chemosensitivity towards platinum-based chemotherapy in lung adenocarcinoma [80] and gastric cancer [81]. In TNBC, an increase in chemosensitivity upon miR-206 overexpression has been described as well [23]. The latter was suggested to be achieved via miR-206-mediated downregulation of the ABCB1 transporter [56]. In the present study, we did not find a substantial benefit in terms of therapy success for patients with high miR-206 expression, but the absence of a significant difference in DFS between treated and untreated patients rather strongly indicates a higher therapy resistance in the miR-206 high expression group.

All in all, both mechanisms, the interrelation between hormone receptors and micro-RNAs as well as modulation of therapy resistance, may contribute to the negative impact of the three analyzed micro-RNAs. A hallmark of TNBC is the lack of hormone receptor expression,

which could result in an altered expression pattern of selected micro-RNAs and thereby favor their accumulation. Otherwise, dysregulation of micro-RNA expression via cancer-derived mutations could induce the lack of hormone receptor expression and thereby foster the manifestation of the TNBC phenotype. Lack of hormone receptor expression together with the normal (or lower) HER2 expression excludes those patients from the available, efficient targeted therapies, and treatment options are more or less limited to conventional anthracycline or taxane-based chemotherapy. Unfortunately, these agents are particularly susceptible to acquired cellular resistance which could be induced via micro-RNA regulation including those analyzed in this study.

Conclusions

We have analyzed the expression pattern of three different micro-RNAs in the tumor tissue of TNBC patients. Based on the results of the present study, all of them represent biomarker potential for poor prognosis of patients afflicted with TNBC, if present in elevated levels. Given the relation between miR-27a, miR-206 and miR-214 expression and prognosis, a therapeutic intervention to reduce the expression of these micro-RNAs may be rational.

Acknowledgements

We would like to thank Jenny Probst for excellent technical assistance.

Disclosure of conflict of interest

None.

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Table S1. Clinical outcome in triple-negative breast cancer patients: multivariate COX regression analysis including clinicopathological parameters age, lymph node status, histological grade, and combinations of micro-RNA expression

Clinical parameter	Disease-free survival (DFS)		
	No.	HR (95% CI)[a]	p
Age			0.681
< 60 years	55	1	
≥ 60 years	54	1.16 (0.57-2.37)	
Lymph node status			**0.004**
N0	62	1	
N+	47	2.70 (1.38-5.29)	
Tumor size			0.658
≤ 20 mm	29	1	
>20 mm	80	1.19 (0.55-2.58)	
Chemotherapy			**0.003**
no	31	1	
yes	78	0.33 (0.16-0.69)	
miR-206 + miR-214[b]			**0.004**
low	92	1	
high	17	2.70 (1.37-5.30)	
miR-206 + miR-27a[b]			**0.002**
low	95	1	
high	14	3.07 (1.50-6.27)	
miR-27a + miR-214[b]			**0.003**
low	79	1	
high	30	2.54 (1.37-4.71)	

[a]HR: hazard ratio, CI: confidence interval. [b]Dichotomization for the combined analysis was low and/or low vs. high and high based on the defined cut-offs (cut-off point: miR-27a = median, miR-206 = 66th percentile, miR-214 = 66th percentile). Significant *p* values (P < 0.05) are indicated in bold.

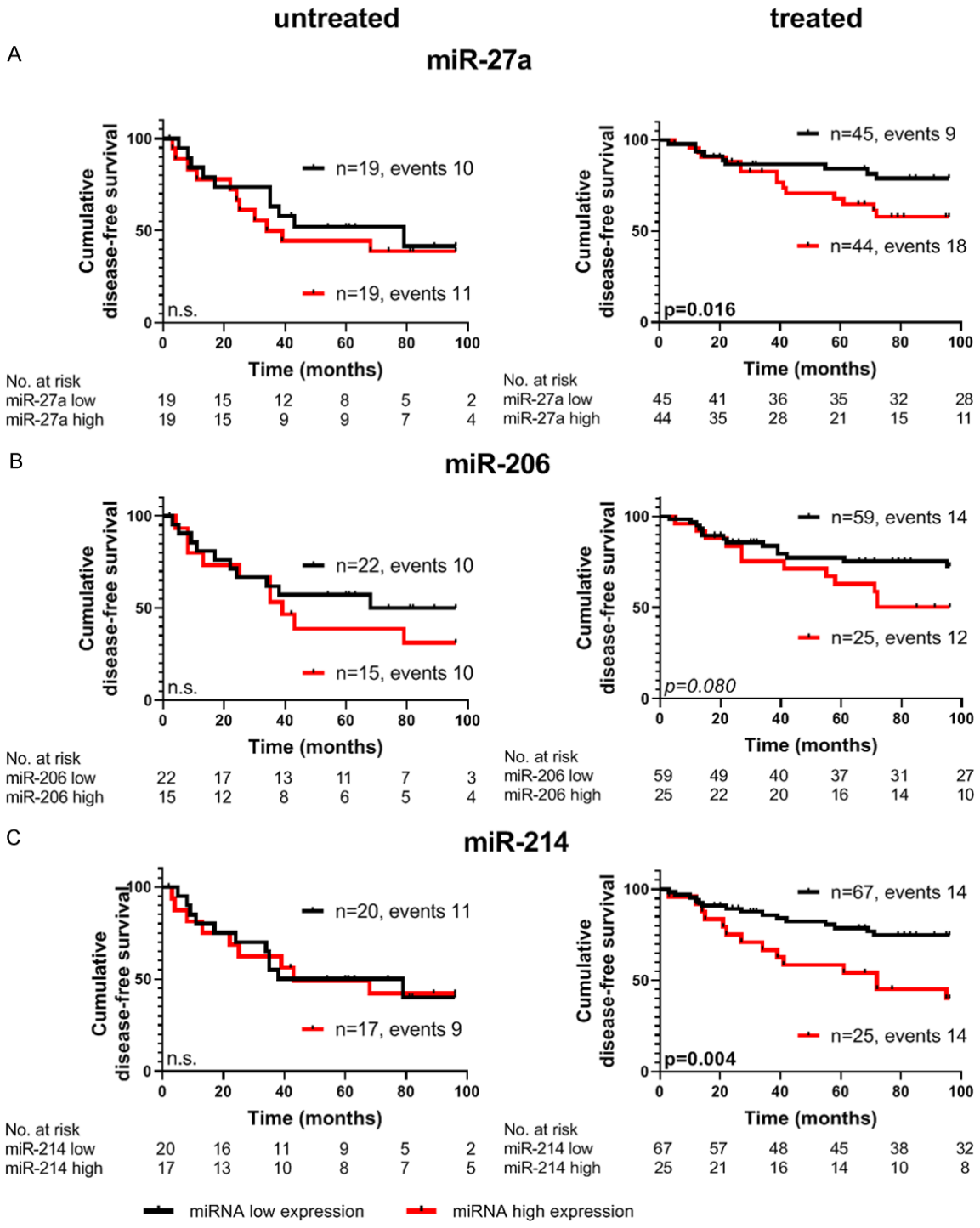

Figure S1. Prognostic value of micro-RNA expression levels in the subgroups of non-treated versus treated TNBC patients. miR-27a (A) and miR-214 (C) expression levels, respectively, are able to discriminate between a low- and high-risk group in treated, but not untreated, patients. miR-206 (B) displays a similar trend (indicated in italics). *p*-values were calculated by log-rank test. Black, low expression; red, high expression.