miR-205-5p Downregulation and ZEB1 Upregulation Characterize the Disseminated Tumor Cells in Patients with Invasive Ductal Breast Cancer

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Abstract: Background: Dissemination of breast cancer (BC) cells through the hematogenous or lymphogenous vessels leads to metastatic disease in one-third of BC patients. Therefore, we investigated the new prognostic features for invasion and metastasis. Methods: We evaluated the expression of miRNAs and epithelial-to-mesenchymal transition (EMT) genes in relation to CDH1/E-cadherin changes in samples from 31 patients with invasive ductal BC including tumor centrum (TU-C), tumor invasive front (TU-IF), lymph node metastasis (LNM), and CD45-depleted blood (CD45-DB). Expression of miRNA and mRNA was quantified by RT-PCR arrays and associations with clinicopathological characteristics were statistically evaluated by univariate and multivariate analysis. Results: We did not verify CDH1 regulating associations previously described in cell lines. However, we did detect extremely high ZEB1 expression in LNMs from patients with distant metastasis, but without regulation by miR-205-5p. Considering the ZEB1 functions, this overexpression indicates enhancement of metastatic potential of lymphogenously disseminated BC cells. In CD45-DB samples, downregulated miR-205-5p was found in those expressing epithelial and/or mesenchymal markers (CTC+) that could contribute to insusceptibility and survival of hematogenously disseminated BC cells mediated by increased expression of several targets including ZEB1. Conclusions: miR-205-5p and potentially ZEB1 gene are promising candidates for markers of metastatic potential in ductal BC.

Keywords: invasive ductal breast cancer; CDH1 gene; EMT genes; miRNA and mRNA expression; E-cadherin

1. Introduction

The International Agency for Research on Cancer’s GLOBOCAN 2018 reported the global burden of cancer across 20 world regions and revealed almost 2.1 million new breast cancer (BC) patients and 0.63 million associated deaths. That means a 24.2% incidence and 15% mortality rate in female cancer patients [1]. Among BC patients, metastatic disease has been reported in the range from 7% to 35% [2–4]. Metastatic breast cancer is generally considered incurable and regardless of some improvement in overall survival only 13% of BC patients with primary stage IV survive 10 years after diagnosis [5]. Cancer cells spread from the tumor mass either via blood or via lymphatic circulation after the intensive
neo-vascularization and neo-lymphangiogenesis, respectively [6]. Detached cancer cells enter blood vessels directly, but those traveling through the lymphatic vasculature either form metastases in lymph nodes or they pass into the blood circulation through the thoracic duct [7]. However, the factors determining the method of cancer cell dissemination depend mainly on the cancer type and features of the tumor microenvironment [8].

In BC, lymphovascular invasion inside and around the tumor tissue indicate the mechanisms of cancer spread and metastases in lymph nodes are considered as the key prognostic marker of tumor spread and aggressiveness [9–11]. On the other hand, many studies performed in the last decade have investigated the role of circulating tumor cells (CTCs) in haematogenous dissemination of cancer and their clinical utility in prognosis is under examination in ongoing clinical trials. Detection and count of CTCs are used as independent prognostic factors for primary and metastatic BC contributing to the monitoring and treatment stratification of BC patients [12–14]. Various technologies for CTC detection and isolation have been developed (including the validated CellSearch® system) and most of them have utilized the epithelial characters for CTC enrichment [15,16]. In BC, heterogeneous CTC subpopulations were found, counting cells with epithelial characters co-existing with those with epithelial-to-mesenchymal transition (EMT) features; however, EMT CTCs have been identified more frequently in metastatic BC patients and are associated with poor prognosis [17–20].

Mesenchymal cell phenotype is associated with an increased migratory capacity, invasiveness, apoptosis, and resistance; therefore, EMT is considered an essential event in BC haematogenous dissemination [20,21]. On the other hand, TGF-1-induced EMT was recently described to promote the chemotaxis-mediated migration of BC cells through the lymphatic vessels [22]. The main feature of EMT induction is the loss of cell–cell adherent junctions via inhibition of E-cadherin encoded by the CDH1 gene. Key EMT inducers, which act as direct CDH1 repressors, belong to three distinct families; the Snail family (SNAIL1, SNAIL2/SLUG, and SNAIL3/SMUC), the Zeb family (ZEB1/2), and the b-HLH family (TWIST1/2). Their encoding genes inhibit E-cadherin expression via binding to the E-box elements in the promoter region of the CDH1 gene [23,24].

Genetic and epigenetic mechanisms of E-cadherin downregulation were previously described. Several CDH1 somatic or germline mutations and loss of heterozygosity were found almost exclusively in invasive lobular BC [25,26]. In addition, epigenetic modulation by CDH1 promoter methylation [27,28] or by miRNA post-transcription regulation have been documented [29].

In BC cells, MYC/MYCN-activated miR-9 was found to be a direct regulator of the CDH1 gene, and increased miR-9 levels were associated with metastatic status and local recurrence in BC patients [30,31]. The miR-221 a member of the miR-221/222 cluster directly targeted the open reading frame of CDH1 and a higher miR-221 expression, significantly upregulated by SNAIL2/SLUG, was observed in metastatic BC cells and BC patients with LNMs and distant metastases [32–34]. Moreover, the miR-221/222 cluster decreased expression of E-cadherin indirectly via targeting of transcription repressor TRPS1, the direct regulator of ZEB2 transcription, resulting in abundance of ZEB2 and promotion of EMT [35].

Several CDH1 repressors and therefore EMT inducers were found to be regulated by members of a well-investigated miR-200 cluster including miR-200b/200a/429 and miR-200c/141. Decreased expression of all members of the miR-200 cluster followed by upregulation of SNAIL1, SNAIL2, ZEB1, and ZEB2 genes was observed in EMT in vitro models of breast basal cell lines and BC patients, more markedly in those with metaplastic tumors. Moreover, the inactivation of miR-200c/141 expression could be caused by hypermethylation of its promoter [36]. Among them, miR-200c targeted ZEB1 and the other two TKS5 and MYLK genes acting in invadopodia formation. Co-expression of these three genes and low expression of miR-200c in several BC lines as well as in BC patients are associated with EMT activation, higher invasion and invadopodia creation [37,38]. In a recent study, miR-200c targeted ZEB2 and the role of this repressor in metastasis was found
in triple-negative BC (TNBC) cells and tissues [39]. Furthermore, both \textit{ZEB1} and \textit{ZEB2} were directly regulated by miR-205 and decreased levels of miR-205 initiated EMT and were associated with a metastatic phenotype of BC patients [40,41].

Other BC in vitro studies showed that expression levels of miR-203 and miR-200 cluster were decreasing in a time-dependent manner during \textit{SNAI1}-induced EMT. The miR-203-reduced \textit{SNAI1} endogenous levels generated a double-negative miR-203/\textit{SNAI1} feedback loop and together with the miR200/\textit{ZEB1} feedback loop, the plasticity of the epithelial cell during differentiation and tumorigenesis was controlled [42]. The miR-203 also directly regulated \textit{SNAI2} gene and this miRNA was upregulated and downregulated in non-metastatic and metastatic cell lines, respectively, compared to non-tumorigenic cells. Moreover, decreasing levels of miR-203 in metastatic cells were associated with promoter hypermethylation [43]. Downregulation of miR-124 was found in TNBC cell lines and patient tissues. It was documented that miR-124 directly targeted the \textit{ZEB2} gene and contributed to EMT and metastasis suppression [44]. Other authors showed that miR-124 also directly targeted the \textit{SNAI2}/\textit{SLUG} gene, which allowed E-cadherin expression and inhibition of cell invasion and metastasis. In BCs, the significantly reduced miR-124 levels negatively correlated with histological grade [45].

Compared to the above-mentioned EMT-associated transcription factors, the miRNA regulation of \textit{TWIST1} expression in BC has been less frequently investigated. The \textit{TWIST1} gene was directly targeted by miR-720 and significant downregulation of miR-720 followed by increasing \textit{TWIST1} levels were observed predominantly in metastatic BC [46].

Generally, in BCs and many other epithelial cancers, the attenuation of E-cadherin adhesion is considered the main event in invasion and metastasis. Regarding the prognostic value, high levels of E-cadherin were found to be a good prognostic marker in most cancers. In a previous study, the variable trend of decreasing E-cadherin expression was observed from ductal BCs in situ, from ductal BCs without LNM to those with LNM, but increased E-cadherin levels were found in LNM tissues compared to primary tumors [47]. However, a more recent study showed that high E-cadherin correlated with shorter survival in invasive ductal BCs in contrast to the lobular subtype of BC, and reduced or lost E-cadherin expression was inversely associated with tumor stage, indicating more complex and possible divergent functions of this protein in BCs [48]. This hypothesis is supported by results from mouse experimental models, where the loss of E-cadherin improved cancer cell invasion, but reduced cell proliferation and survival, the number of CTCs in systemic circulation, and dissemination of cancer cells in distant organs. The authors indicated that E-cadherin could contribute to metastasis through apoptosis inhibition [49].

In this study, we investigated epigenetic changes associated with regulation of the E-cadherin encoding \textit{CDH1} gene to contribute to a better understanding of the specific functions of E-cadherin and associated miRNAs and genes in invasive ductal BCs. We analyzed the expression of \textit{CDH1}, five well-known EMT genes, and seven regulating miRNAs (Figure 1) in tumor centrum (TU-C), tumor invasive front (TU-IF), and in lymph node metastasis (LNM) and CTC-enriched blood fraction samples (by CD45 depletion) to identify aberrantly expressed miRNAs and genes, and understand their associations with clinical parameters of invasive and metastatic processes including LNM, CTC, lymphovascular invasion (LVI), and distant metastasis (MTS). Our results did not confirm \textit{CDH1} regulating associations previously described in cell line models. However, we detected extremely high \textit{ZEB1} expression in LNM samples from patients with MTS, which was not regulated by miR-205-5p. Due to \textit{ZEB1} functions, its overexpression indicates enhancement of metastatic potential of disseminated BC cells spread through the lymphatic vessels. In CD45-depleted blood (CD45-DB) fractions, the downregulated miR-205-5p was found in samples expressing epithelial and/or mesenchymal markers (CTC+) that could contribute to reduced susceptibility and increased survival of hematogenously disseminated BC cells mediated by increased expression of several target genes including \textit{ZEB1}. Both, miR-205-5p and potentially \textit{ZEB1} are promising candidates as markers for metastatic potential of disseminated ductal BC cells.
2. Results

2.1. miRNA and mRNA Expressions vs. Controls

In the group of 31 patients with invasive ductal breast cancer, we evaluated the expressions of seven miRNAs regulating CDH1 and EMT repressors (miR-9-5p, miR-124-3p, miR-200c-3p, miR-203a-3p, miR-205-5p, miR-221-3p, and miR-720) and mRNA expression for CDH1 and associated EMT genes (TWIST1, SNAI1, SNAI2, ZEB1, and ZEB2). The analyses were performed in TU-C, TU-IF and LNM samples, and in CD45-DB fractions enriched by CTCs with and without identified epithelial and/or mesenchymal markers (CTC+ and CTC−, respectively). The controls for all patients’ tissue samples and depleted fractions were non-neoplastic breast specimens (C-breast) and CD45-depleted fractions from healthy women (C-blood). In TU-C, significant upregulation was detected for miR-9-5p (fold change, FC 7.915, \( p = 0.042 \)) and miR-203a-3p (FC 2.356, \( p = 0.042 \)) and downregulation for CDH1 (fold change, FC 0.125, \( p < 0.001 \)) genes compared to controls. Similarly, TU-IFs were downregulated for CDH1 (FC 0.108, \( p = 0.001 \)), SNAI2 (FC 0.102, \( p = 0.002 \)) and ZEB2 (FC 0.086, \( p < 0.001 \)) genes and LNM samples were downregulated for miR-205-5p (FC 0.21, \( p = 0.012 \)), and upregulated for ZEB1 (FC 22.08, \( p = 0.043 \)) and ZEB2 (FC 0.122, \( p = 0.003 \)). In CTC-depleted bloods, miR-124-3p (FC 9.766, \( p = 0.036 \)) was upregulated and in CTC+ samples, miR-221-3p (FC 0.289, \( p = 0.017 \)) and ZEB2 (FC 0.395, \( p = 0.037 \)) were downregulated compared to controls (Figure 2, Table 1).

Figure 2. Significantly different expressions of miRNAs and mRNA of CDH1 and EMT genes in tumor centrum (TU-C), tumor invasive front (TU-IF) and lymph node metastasis (LNM), and in CD45-DB fractions enriched by CTCs compared to adequate controls (C-breast, non-neoplastic breast tissues and C-blood, CD45-depleted fractions from healthy women).
Table 1. Comparison of miRNA and mRNA of CDH1 and EMT gene expressions between controls and different types of BC patients’ specimens and between each other’s samples.

| miRNAs/Genes | TU-C vs. C-Breast | TU-IF vs. C-Breast | LNM vs. C-Breast |
|--------------|-------------------|-------------------|-----------------|
|              | FC                | p-Value           | 95% CI          | FC                | p-Value           | 95% CI          | FC                | p-Value           | 95% CI          |
| miR-9-5p     | 7.915             | 0.042             | 0.012–41.946    | 0.838             | 0.790             | 0.004–263.197    | 0.551             | 0.422             | 0.004–180.863    |
| miR-124-3p   | 1.789             | 0.553             | 0.001–4.238     | 1.632             | 0.557             | 0.002–1.277      | 0.649             | 0.634             | 0.002–624.915    |
| miR-200c-3p  | 1.686             | 0.382             | 0.054–45.865    | 2.137             | 0.092             | 0.215–59.281     | 1.821             | 0.271             | 0.156–48.471     |
| miR-203a-3p  | 2.356             | 0.042             | 0.077–79.574    | 1.556             | 0.294             | 0.042–38.469     | 1.287             | 0.593             | 0.039–29.094     |
| miR-205-5p   | 0.512             | 0.251             | 0.009–36.730    | 0.350             | 0.054             | 0.010–10.925     | 0.210             | 0.422             | 0.004–22.785     |
| miR-221-3p   | 1.335             | 0.423             | 0.081–17.503    | 1.365             | 0.290             | 0.168–13.709     | 1.096             | 0.802             | 0.091–19.943     |
| miR-720      | 1.152             | 0.735             | 0.050–33.593    | 1.398             | 0.245             | 0.130–15.221     | 1.033             | 0.939             | 0.042–22.785     |
| CDH1         | 0.123             | 0.002             | 0.004–2.366     | 0.001             | 0.004–2.015       | 0.630             | 0.754             | 0.005–461.981    |
| TWIST1       | 0.449             | 0.765             | 0.000–19.953    | 0.320             | 0.097             | 0.000–29.212     | 2.866             | 0.717             | 0.000–84.067.528 |
| SNAI1        | 0.711             | 0.586             | 0.027–23.670    | 0.706             | 0.658             | 0.020–55.154     | 1.460             | 0.740             | 0.024–246.709    |
| SNAI2        | 0.161             | 0.021             | 0.003–13.083    | 0.102             | 0.022             | 0.001–9.663      | 0.302             | 0.168             | 0.001–140.598    |
| ZEB1         | 0.482             | 0.117             | 0.032–9.616     | 0.474             | 0.052             | 0.064–6.320      | 22.08             | 0.043             | 0.003–522,995.072 |
| ZEB2         | 0.125             | <0.001            | 0.002–3.578     | 0.086             | <0.001            | 0.001–3.209      | 0.122             | 0.003             | 0.001–13.175     |

| Abbreviations: C-breast, non-neoplastic breast tissue controls; TU-C, tumor centrum; TU-IF, tumor invasive front; LNM, lymph node metastasis; C-blood, CD45-DB fractions from healthy women; CTC- and CTC+, CD45-DB fractions from patients without and with identified epithelial and mesenchymal markers, respectively. Significant results are highlighted in bold. |
2.2. miRNA and mRNA Expressions in Different Types of Samples

Expression of two miRNAs, and CDH1 and ZEB1 genes was found to be statistically different between BC samples. In both TU-IF and LNM, miR-9-5p was downregulated and in LNM, CDH1 and ZEB1 were upregulated compared to TU-C. Similarly, upregulated CDH1 and ZEB1 genes were found in LNM against expressions in TU-IF. CTC+ samples presented miR-205-5p downregulation compared to CTC- (Table 1, Figure 3).

![Figure 3](image-url)

**Figure 3.** Expression of miRNAs, and CDH1 and EMT genes between different types of BC patients' samples, namely in tumor centrum (TU-C), tumor invasive front (TU-IF) and lymph node metastasis (LNM), and in CD45-depleted blood (CD45-DB) fractions without and with identified epithelial and/or mesenchymal markers (CTC- and CTC+).

To evaluate the inhibitory function of miRNAs we analyzed the correlation between expressions of miRNAs, and CDH1 and EMT genes, and between CDH1 and each of EMT genes (Table 2 and Table S1). Negative correlation was found between miR-221-3p and TWIST1 gene in TU-Cs (correlation coefficient \( r \), \( r = -0.470, p = 0.015 \)), and miR-9 and SNAI1 gene in LNM’s (\( r = -0.607, p = 0.013 \)). In CD45-DB fractions the negative correlations were observed more frequently, in CTC- samples between miR-124-3p and TWIST1 (\( r = -0.883, p = 0.020 \)), and miR-221-3p and ZEB2 (\( r = -0.543, p = 0.024 \)). In CTC+ specimens, miR-9, miR-205-5p and miR-720 negatively correlated with both SNAI1 and ZEB1 (\( r \) ranged from \(-0.853 \) to \(-0.588 \) and \( p\)-value from 0.001 to 0.044), miR-221-3p with ZEB1 (\( r = -0.610, p = 0.035 \)) (Table 2). No negative correlations were detected between expressions of CDH1 and any of EMT genes in tumor, LNM and CTC samples (Table S1).

In this study, several associations between miRNAs and genes indicating regulation events previously documented by in vitro results were shown. In TU-Cs, upregulated miR-9 and downregulated CDH1, and upregulated miR-203a-3p, and downregulated SNAI2 were found compared to controls. LNM samples presented downregulation of miR-205-5p with upregulation of the ZEB1 gene (Table 1). However, in none of these associations were significant negative correlations between miRNA and mRNA expressions of CDH1 and EMT genes observed.
### Table 2. Significant correlations between miRNA and mRNA of CDH1, and EMT gene expressions in BC samples.

| Sample     | miRNA       | Gene     | Correlation Coefficient (r) | p-Value |
|------------|-------------|----------|----------------------------|---------|
| C-breast   | miR-124-3p  | CDH1     | 0.964                      | 0.036   |
|            | miR-203a-3p | ZEB1     | 0.479                      | 0.018   |
|            | miR-205-5p  | ZEB1     | 0.406                      | 0.036   |
|            | miR-221-3p  | ZEB1     | −0.470                     | 0.015   |
|            | miR-200c-3p | ZEB1     | 0.387                      | 0.046   |
| TU-C       | miR-200c-3p | SNAI1    | 0.527                      | 0.005   |
|            | miR-203a-3p | SNAI1    | 0.479                      | 0.018   |
|            | miR-221-3p  | SNAI1    | 0.527                      | 0.005   |
|            | miR-205-5p  | SNAI1    | 0.583                      | 0.048   |
|            | miR-200c-3p | SNAI1    | 0.800                      | 0.010   |
| TU-IF      | miR-200c-3p | SNAI1    | 0.797                      | 0.010   |
|            | miR-205-5p  | SNAI1    | 0.800                      | 0.010   |
|            | miR-221-3p  | SNAI1    | 0.800                      | 0.010   |
|            | miR-203a-3p | SNAI1    | 0.800                      | 0.010   |
| LNM        | miR-9       | TWIST1   | 0.967                      | 0.007   |
|            | miR-200c-3p | SNAI1    | 0.800                      | 0.010   |
|            | miR-203a-3p | SNAI1    | 0.800                      | 0.010   |
| C-blood    | miR-9       | SNAI1    | 0.527                      | 0.005   |
|            | miR-200c-3p | ZEB2     | 0.616                      | 0.043   |
|            | miR-205-5p  | ZEB2     | 0.682                      | 0.021   |
|            | miR-221-3p  | ZEB2     | 0.869                      | 0.025   |
| CTC-       | miR-9       | ZEB2     | 0.700                      | 0.001   |
|            | miR-200c-3p | ZEB2     | 0.700                      | 0.001   |
|            | miR-203a-3p | ZEB2     | 0.700                      | 0.001   |
| CTC+       | miR-9       | SNAI1    | −0.653                     | 0.021   |
|            | miR-200c-3p | SNAI1    | −0.653                     | 0.021   |
|            | miR-205-5p  | SNAI1    | −0.588                     | 0.044   |
|            | miR-221-3p  | SNAI1    | −0.610                     | 0.035   |
|            | miR-720     | ZEB1     | −0.623                     | 0.031   |

Abbreviations: C-breast, non-neoplastic breast tissue controls; TU-C, tumor centrum; TU-IF, tumor invasive front; LNM, lymph node metastasis; C-blood, CD45-DB fractions from healthy women; CTC- and CTC+, CD45-DB fractions from patients without and with identified epithelial and mesenchymal markers, respectively. Negative correlations are highlighted in bold.

### 2.3. Association between miRNA and mRNA Expression and Clinico-Pathological Parameters

Using univariate statistical analysis, we found spectrum of associations between up- or downregulated expressions of miRNAs and CDH1 and EMT genes and relevant clinico-pathological characteristics for each group of samples (Table 3). In TU-C, downregulated levels of miR-124-3p and miR-203a-3p were detected in patients with MTS and TNM staging III and IV compared to those without MTS and lower TNM, respectively. Furthermore, miR-200c-3p was downregulated in HER2 positive BCs and reduced CDH1, SNAI1, and ZEB2 expression was identified in ER+ and /or PR+ tumors. TU-IF samples showed down-regulation for miR-200c-3p in ER+ tumors, decreased expression for TWIST1 in ER+ BCs and several combinations of downregulated EMT genes, namely SNAI2 and ZEB1 associated with lymph node metastasis (LNM) positive phenotype and higher TNM, respectively; SNAI2 and ZEB2 with distant metastasis (MTS) presence and SNAI2, and both, ZEB1 and ZEB2 with LVI. Decreased ZEB2 was also associated with tumors sized ≤20 mm. In LNM
tissues, upregulation of miR-124-3p, SNAI1 and ZEB1 was associated with ER+, higher TNM and MTS+ in the primary tumor, respectively. In CD45-DB samples, downregulation of SNAI1 and upregulation of miR-9 were found in patients with HER2+ tumors, and upregulation of CDH1 in grade 3 cancers, respectively. miR-205-5p were found upregulated in patients with LNM+ and higher TNM but significantly downregulated in patients with CTC+ phenotype (Figure 4). FCs and p-values for these associations are summarized in Table 3.

Immunohistochemical analyses in TU-C samples showed that 17 and 5 patients presented strong homogenous expression (3+) and heterogeneous strong and moderate expression (3+ and 2+) of E-cadherin in different portions in individual patients, respectively. These samples were considered as those with high E-cadherin expression. In nine patients with low E-cadherin expression, strictly heterogeneous phenotype with different portion of strong, moderate, poor, and no expression (3+/2+/1+/0 and 2+/1+) was observed. The associations between CDH1 gene expression in high and low E-cadherin expression were tested by univariate statistical analysis. An increasing trend in CDH1 expression in E-cadherin high compared to low expression was found; however, without upregulation and statistically significant difference in CDH1 expression between E-cadherin high and low groups (FC 1.134, p = 0.777).

**Table 3.** Significant up- and downregulation of miRNA and mRNA of CDH1, and EMT gene expression in BC patients with different clinico-pathological parameters.

| Sample | Clinical Characteristics | miRNAs/Genes | FC     | p-Value | 95% CI       |
|-------|--------------------------|--------------|--------|---------|--------------|
| TU-C  | MTS+ vs. MTS-            | miR-124-3p   | 0.075  | 0.049   | 0.000–35.995 |
|       | HER2+ vs. HER2-           | miR-200c-3p  | 0.440  | 0.018   | 0.030–12.772 |
|       | TNM III and IV vs. TNM I and II | miR-203a-3p | 0.316  | 0.008   | 0.009–4.922  |
|       | ER+ vs. ER-               | SNAI1        | 0.465  | 0.007   | 0.049–7.093  |
|       | ZEB2                      | 0.486        | 0.017  | 0.113–5.252 |
|       | PR+ vs. PR-               | CDH1         | 0.366  | 0.025   | 0.028–3.448  |
|       |                            | SNAI1        | 0.336  | 0.008   | 0.049–6.071  |
| TU-IF | ≤20 mm vs. >20 mm         | ZEB2         | 0.343  | 0.011   | 0.024–4.821  |
|       | LNM+ vs. LNM-             | SNAI2        | 0.326  | 0.023   | 0.002–2.962  |
|       | ZEB1                      | 0.497        | 0.035  | 0.079–4.408 |
|       | MTS+ vs. MTS-             | SNAI2        | 0.432  | 0.041   | 0.034–94.834 |
|       | ZEB2                      | 0.344        | 0.030  | 0.006–7.949 |
|       | TNM III and IV vs. TNM I and II | SNAI2 | 0.246  | 0.007   | 0.002–4.248  |
|       | ZEB1                      | 0.438        | 0.007  | 0.059–2.126 |
|       | LVI+ vs. LVI-             | SNAI2        | 0.294  | 0.015   | 0.002–4.602  |
|       | ZEB1                      | 0.482        | 0.034  | 0.079–3.844 |
|       | ZEB2                      | 0.350        | 0.026  | 0.024–3.722 |
|       | ER+ vs. ER-               | miR-200c-3p  | 3.795  | 0.048   | 0.684–24.343 |
|       |                            | TWIST1       | 0.420  | 0.049   | 0.011–15.353 |
| LNM   | MTS + vs. MTS-            | ZEB1         | 824.73 | 0.018   | 0.283–1,584,361.881 |
|       | TNM III and IV vs. TNM I and II | SNAI1 | 5.959  | 0.025   | 0.227–432.565 |
|       | ER+ vs. ER-               | miR-124-3p   | 6.819  | 0.018   | 0.091–887.828 |
| CD45-DB | CTC+ vs. CTC-             | miR-205-5p   | 0.041  | 0.010   | 0.000–69.820 |
|       | LNM+ vs. LNM-             | miR-205-5p   | 22.961 | 0.035   | 0.012–400,412.929 |
|       | TNM III and IV vs. TNM I and II | miR-205-5p | 39.056 | 0.006   | 0.039–692,990.143 |
|       | HER2+ vs. HER2-           | miR-9        | 10.321 | 0.027   | 0.088–1098.021 |
|       |                            | SNAI1        | 0.439  | 0.041   | 0.061–9.288  |
|       | Grade 3 vs. Grade 1 and 2 | CDH1         | 1.468  | 0.033   | 0.151–11.647 |

Abbreviations: FC, fold change; CI, confidence interval; TU-C, tumor centrum; TU-IF, tumor invasive front; LNM, lymph node metastasis; CD45-DB, CD45-depleted blood; CTC, circulating tumor cell; MTS, metastatic; TNM, TNM staging system (T, tumor; N, lymph node; M, metastasis); LN, lymph node; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.
In this study, several associations between miRNAs and genes indicating regulation were documented by in vitro results. In TU-Cs, upregulated miR-205-5p with upregulation of the SNAI2 gene (Table 1). However, in none of these events previously documented by in vitro results were shown. In TU-Cs, upregulated miR-205-5p were found in patients with HER2+ tumors, and upregulation of SNAI2 was associated with tumors sized ≥20 mm. In LNM tissues, upregulation of miR-124-3p, and both, miR-203a-3p, and downregulated CDH1 expression was identified in ER+ and/or PR+ tumors.

Firstly, logistic regression was applied to predict the potential influence of miR-205-5p expression on the dissemination of tumor cells characterized by CTC positivity. Multivariate models, the clinical parameters of age, tumor size, histological grade, and HER2 status were included to control for potential confounders. The multivariate model correctly classified 88.9% of CTC negative and 91.7% of CTC positive patients, respectively, with an overall success rate of 90.0%. The presence of CTC positivity was negatively correlated with miR-205-5p expression in CD45-DB fractions (Table 4). Multivariate models for LNM, LVI, and MTS risk prediction were not significant.

Table 4. Risk estimation of miR-205-5p expression and clinical status for the presence of CTC in CD45-DB of BC patients (logistic regression adjusted for age).

| Risk Factor        | Variables  | p-Value | OR          | 95% CI          |
|--------------------|------------|---------|-------------|-----------------|
| CTCs presence      | Age        | 0.061   | 0.893       | 0.792–1.005     |
|                    | High grade (G3) | 0.075   | 46.197      | 0.683–3124.868  |
|                    | miR-205-5p * | 0.028   | 4.326       | 1.170–15.995    |
|                    | Tumor size > 20 mm | 0.047   | 96.081      | 1.066–8661.849  |
|                    | HER2 positivity § | 0.036   | 2153.786    | 1.628–2,849,343.631 |
|                    | Constant    | 0.126   | 0.000       |                 |

* Cut-off 10%. Abbreviations: CI, Confidence interval; CTCs, circulating tumor cells; HER2, Human epidermal growth factor receptor 2; OR, Odds ratio. Model summary: –2 Log likelihood 18.870; R squared (Cox & Snell) 0.512; R squared (Nagelkerke) 0.692. Input variables to model: age, histological grade, miR-205-5p expression, tumor size, and HER2 status.

Multivariate analysis was used to test for risk factors influencing hematogenous and lymphogenous dissemination, namely CTC, LNM, and LVI positivity, and the presence of distant MTS. Variables that were significant in univariate analysis were used in multivariate analysis.

Figure 4. Individual expression levels of miR-205-5p in CD45-depleted peripheral blood of CTC negative (CTC-) and positive (CTC+) BC patients. The median is depicted by a horizontal line within each bar. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles of individual fold change values. Values more than 3 IQRs from the end of the box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). Abbreviations: BC, breast cancer; CTC, circulating tumor cells.
3. Discussion

Based on the generally accepted knowledge of the key role of E-cadherin (encoded by \textit{CDH1} gene) in cancer cell spread, we investigated the influence of aberrant expression of several EMT genes and their regulating miRNAs, in addition to miRNAs which directly targeted \textit{CDH1} gene expression on ductal BC development in several stages of disease.

Our results showed decreasing levels of \textit{CDH1} expression in both TU-C and TU-IF compared to C-breast, but without a statistically significant difference between the two. LMN samples were characterized by a similar expression of \textit{CDH1} gene as in C-breast, but its upregulation compared to TU-C and TU-IF indicates a possible role of \textit{CDH1} gene in lymphogenous cancer spread. On the other hand, similar \textit{CDH1} expression was found in CTC- and CTC+ samples compared to C-blood.

In our patients, immunohistochemical E-cadherin expression was carefully evaluated in TU-C. In all tumors, full E-cadherin expression (3+) was found in 17 samples; however, 14 presented locally decreased levels of E-cadherin. Among them, increasing \textit{CDH1} levels were observed in those with high E-cadherin (3+ and 3+/2+ phenotype) compared to low levels (3+/2+/1+, 3+/2+/1+/0 and 2+/1+), but without a statistically significant difference. The reason we were not able to show \textit{CDH1}/E-cadherin association could the variability of immunohistochemistry results in ductal BCs. In technical terms, it may be that qualitative analyses of \textit{CDH1} expression and semiquantitative E-cadherin immunohistochemistry cannot be performed in the same region of tumor. However, the association between expression of \textit{CDH1} and E-cadherin has been documented by other authors [50] and the upregulated \textit{CDH1} levels in LNM tissues identified in our study could correspond with the previously published increase in E-cadherin expression in LNM tissues [47].

In this study, the relatively complicated scheme of \textit{CDH1} regulation by miRNAs and EMT genes was used to investigate possible regulators of \textit{CDH1} in several types of samples from invasive ductal BC patients. All evaluated associations were previously identified in vitro [30,33,38,40,42–46]. Among them, only upregulated miR-9 with downregulated \textit{CDH1} associating with invasive phenotype and upregulated miR-203a-3p with downregulated SNAI2 gene indicating inactive EMT process were detected in TU-C. Our results were in accordance with other studies showing upregulation of miR-203a-3p in cell lines and primary BCs. According to the findings, a negative association between downregulated miR-203a-3p and upregulated SNAI2 was observed in metastatic cells [43,51,52]. LNM samples presented downregulated levels of miR-205-5p with upregulation of the \textit{ZEB1} gene, indicating the important role of \textit{ZEB1} in invasion. However, a negative correlation between the expression of these miRNAs and associated genes was not detected. On the other hand, we found several negative correlations that were not investigated in vitro. They could designate the new regulating connection as in the case of miR-9 and SNAI1 expression observed in both, LNM and CTC+ samples. The other possibility is that these findings show only the independent co-existence observed in particular stages of BCs in relation to their functions (Table 2).

Similarly to LNM tissues, CTC+ samples showed miR-205-5p downregulation, and a negative correlation between miR-205-5p and \textit{ZEB1} expression was detected.

The \textit{ZEB1} transcription factor is regulated by multiple signaling pathways and molecules as TGF-\(\beta\), \(\beta\)-catenin and miRNAs, and it alone regulates a high number of genes, as was found in TNBCs [53,54]. In addition to EMT promotion, \textit{ZEB1} overexpression contributes to maintenance of stem-like features, immune evasion, and epigenetic reprogramming [55]. Moreover, \textit{ZEB1} initiates chemo-resistance but inhibits the apical–basal polarity of cancer cells and antiestrogen sensitivity [53]. All these functions could contribute to the insusceptibility and survival of disseminated BC cells located in LNM samples due to elevated levels of the \textit{ZEB1} gene found in our study.

To evaluate the association between aberrant expression of \textit{CDH1}, and regulation of miRNAs and EMT genes and selected clinico–pathological parameters, we found a varying combination of changes in individual types of patient’s samples. Patients with ER+ tumors showed downregulation of \textit{SNAI1} and \textit{TWIST1} in TU-C and TU-IF, respectively. Similarly,
downregulation on protein and mRNA levels were observed in primary BCs by other authors, respectively [56,57]. In our study, upregulation of miR-200c-3p and miR-124-3p in TU-IF and LNM samples was observed in ER+ patients compared to ER-, respectively. The identical finding for miR-200c-3p was described also in other studies [58,59]. Differences in PR status were observed only in TU-C where PR+ samples were characterized by downregulated CDH1 and SNAI1 genes. Other authors identified a similar association between decreased SNAI1 protein and the PR+ phenotype [60]. In addition, HER2+ against HER2- tumors showed downregulation miR-200c-3p, and upregulation of miR-9 and downregulation of SNAI1 were found in TU-C and CD45-DB fractions, respectively. In patients with advanced BCs in advanced stage (TNM III and IV), downregulated miR-203a-3p, and SNAI2 with ZEB1 were detected in TU-C and TU-IF, and upregulation of SNAI1 and miR-205-5p was found in samples with disseminated cancer cells, LNM and CD45-DB fractions, respectively. Finally, in TU-F, tumors >20 mm presented downregulated ZEB2 and in CD45-DB fractions, upregulation of CDH1 in Grade 3 tumors compared to patients with smaller and highly or moderately differentiated BCs, respectively.

For evaluation of the influence of expression change on hematogenous or lymphogenous dissemination, the presence of LVI, LNM, CTC, and MTS were crucial parameters that were consequently used for the creation of multivariate models. In patients with LVI+, downregulation of SNAI2, ZEB1, and ZEB2 was observed in TU-IF. The presence of LNM was associated with downregulation of SNAI2 and ZEB1 in TU-IF and upregulation of miR-205-5p in the CD45-DB fraction. Patients with distant metastasis showed downregulated miR-124-3p in TU-C, and SNAI2 and ZEB2 in TU-IF. Consistent with these findings, in vitro and in vivo studies showed that miR-124-3p inhibit the metastasis process [61,62]. The markedly upregulated levels of ZEB1 in LMN tissues from patients with MTS could indicate its previously hypothesized role in metastasis [63]. The presence of CTC was associated with downregulation of miR-205-5p in the CD45-DB fractions that was verified in the multivariate risk model for CTC risk prediction (Table 4).

Regardless of the many questions remaining about the role of miR-205-5p in normal breast physiology, tumor-suppressor activities of this miRNA were documented in many studies. Decreasing levels of miR-205-5p were observed from less aggressive BC subtypes and ER+/PR+ tumors to more aggressive cases as TNBCs and those with high metastatic capabilities, poor response to therapy and patient survival [41,64]. To date, more than 20 genes targeted by miR-205-5p associating with processes and pathways involved in breast tumorigenesis were identified [64,65]. Decreased levels of miR-205-5p expression in CTC+ samples allow higher expression of the ZEB1 gene, which could contribute to better condition and protection of cancer cells by several processes as previously discussed. Expression of other target genes ITGA5 and NOTCH2 could improve the stemness and metastatic potential of hematogenously disseminated cancer cells [66,67]. Moreover, after the reduction in miR-205-5p, CTCs could acquire chemoresistance features resulting from overexpression of VEGF-A and FGF2, leading to increased apoptosis upon chemotherapy treatment [68].

To our knowledge, miRNA expression analyses in CD45-DBs have been published very rarely. We found only one in silico study, in which specific differentially expressed miRNAs were identified, miR-99a and miR-151-3p for ductal BCs in situ, miR-145 and miR-210 for invasive BCs, and miR-361-5p and miR-205 for metastatic BCs [41]. Our study therefore brings original results. On the other hand, gene expression profiles were investigated in CTC samples by several research groups. In these studies, the gene expression profiles in CTCs obtained from patients with metastatic BCs were different compared to primary tumors that can be utilized for characterization of CTCs, and evaluation of prognosis and therapeutic prediction. However, expression profiles of mesenchymal CTCs were omitted for EpCAM separation of CTC-enriched fractions [69–72]. In our study, CTC+ samples were characterized by epithelial and/or mesenchymal features; therefore, we consider our results to be more objective.
Generally, the positive expression of E-cadherin is used to discriminate between ductal and lobular subtypes of BC. However, detailed examination reveals different levels of E-cadherin inhibition in the many regions of invasive ductal tumor tissues. In this BC subtype, genetic changes in the E-cadherin encoding gene CDH1 are very rare; therefore, we investigated the influence of aberrant expression of CDH1 and regulating miRNA and EMT genes on invasive and metastatic features in samples which represent several stages of BC cell dissemination. In this study, we showed a variable spectrum of upregulated or downregulated expressions of the CDH1 gene and associated miRNAs and EMT genes and did not verify any regulating relationships, which were previously described in cell line studies, except an association between miR-205-5p and ZEB1 expressions in the CTC+ fraction. However, we did observe extremely high ZEB1 expression in LMN samples obtained from patients with distant metastases that was not explained by miR-205-5p decreasing. This finding indicates that ZEB1 overexpression could enhance the metastatic potential of cancer cells disseminated through the lymphatic circulation. In CD45-DB fractions, the samples with the identified presence of CTCs showed downregulation of miR-205-5p expression that could contribute to maintaining the stemness and initiation of such protective features as immune evasion and chemoresistance through the increased expression of several target genes including ZEB1. Together, we identified miR-205-5p and ZEB1 as potential markers for metastatic behavior of disseminated BC cells originating from a ductal tumor; however, their clinical relevance needs to be widely investigated.

4. Materials and Methods

4.1. Patients

We analyzed patient’s RNA samples isolated from CD45-DB fractions and FFPE specimens from the central region and invasive front of tumor and lymph node metastases. The controls were non-neoplastic breast tissues and CD45-DB fractions of age-matched women. At the Department of Senology and Department of Pathology, St. Elisabeth Cancer Institute, Bratislava, 69 patients suspected of an invasive type of breast cancer were pre-selected and blood samples were collected. After the evaluation of post-operation tumor samples, 31 patients with invasive ductal BC were included in this study. 13 non-neoplastic breast tissues and 12 CD45-DB from healthy women at matched age were used as controls. This study was approved by St. Elizabeth Cancer Institute Review Board in Bratislava and written informed consent was obtained from all patients and controls. The age of patients ranged from 42 to 86 years, (median 65 years), controls were aged between 52 and 79 years (median 67 years) and between 54 and 66 years (median 59 years) in breast tissue and CD45-DB samples, respectively. No statistical differences in age were found between patients and controls. The clinical and histopathological characteristics and immunohistochemical data (tumor size, histological grade, LN and MTS status, TNM stage, LVI, hormone receptor (ER, PR) and HER2 status, Ki-67 and E-cadherin expression) were obtained from patients records and tumors were defined according to TNM classification (Table 5). No patient underwent preoperative radiotherapy or chemotherapy before specimen collection, and control women had no signs or symptoms of cancer or other serious diseases.
Table 5. Clinical characteristics.

| Variables                      | n  | %    |
|--------------------------------|----|------|
| All                            | 31 | 100.0|
| Age                            |    |      |
| ≤50                            | 3  | 9.68 |
| >50                            | 28 | 90.32|
| Histological grade             |    |      |
| 1 and 2                        | 14 | 45.16|
| 3                              | 17 | 54.84|
| Tumor size (mm)                |    |      |
| ≤20                            | 12 | 38.71|
| >20                            | 19 | 61.29|
| LNM status a                   |    |      |
| 0                              | 9  | 29.03|
| ≥1                             | 22 | 70.97|
| MTS status                     |    |      |
| Negative                       | 24 | 77.42|
| Positive                       | 7  | 22.58|
| TNM stage                      |    |      |
| I. and II.                     | 13 | 41.94|
| III. and IV.                   | 18 | 58.06|
| CTC occurrence b               |    |      |
| Negative                       | 18 | 58.06|
| Positive                       | 13 | 41.94|
| LVI                            |    |      |
| Negative                       | 7  | 22.58|
| Positive                       | 24 | 77.42|
| ER status c                    |    |      |
| Negative                       | 5  | 16.13|
| Positive                       | 26 | 83.87|
| PR status c                    |    |      |
| Negative                       | 11 | 35.48|
| Positive                       | 20 | 64.52|
| HER2 status d                  |    |      |
| Negative                       | 23 | 74.19|
| Positive                       | 8  | 25.81|
| Ki-67 proliferative index e    |    |      |
| Low                            | 4  | 12.90|
| High                           | 27 | 87.10|
| E-cadherin expression f        |    |      |
| Low                            | 9  | 29.03|
| High                           | 22 | 70.97|

Abbreviations: LNM, lymph node metastasis; MTS, metastatic; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. a LNM status was categorized according to the number of metastatic LNs; b CTC occurrence was evaluated in CD45-depleted blood (CD45-DB) fractions through the absence or presence of epithelial and mesenchymal markers; c ER, PR was considered as positive in cases with ≥ 1% of positively responding cells; d HER2 positive cases were those that showed strong homogeneous and circumferential membrane expression in more than 10% of tumor cells (i.e., 3+ intensity) or those that showed a 2+ intensity and subsequent FISH analysis demonstrated amplification of the HER2 gene. HER2-negative cases were with a response intensity of 0 or 1+, or cases with a response intensity of 2+ without proven amplification; e Low and high Ki-67 expression according to the number of stained cancer cell with a cut-off 15%; f High E-cadherin expression was classified as homogenous 3+ and heterogeneous 3+ and 2+ staining; low E-cadherin expression were defined in samples with heterogeneous staining covering 1+ and no expression regardless of portion of cells with 3+, 2+, 1+ and no E-cadherin expression.

4.2. CD45 Depletion of Peripheral Blood and CTC Detection

Preparation of CD45-negative blood fractions was performed by RosetteSep Human CD45 Depletion Cocktail (StemCell Technologies, Vancouver, BC, Canada) based on depletion of CD45+ peripheral blood cells. Quantitative real-time polymerase chain reaction (qRT-PCR) was used for CTCs detection in CD45-DB samples as has been previously described [73,74]. RNA extraction from CD45-DB fractions was exposed to detection of EMT-inducing transcription factors gene transcripts (TWIST1, SNAIL1, SLUG and ZEB1) and epithelial antigen (CK19) by TaqMan assays (LifeTechnologies, Carlsbad, CA, USA). The higher expression levels of either epithelial and/or mesenchymal gene transcripts than those of healthy donors were considered as CTCs positive.

4.3. miRNA and mRNA Isolation and Real-Time PCR

For gene expression analyses, miRNA and mRNA from CD45-DB fraction and FFPE breast tissues were used. miRNAs from CD45-DB fraction were isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and miRNAs from FFPE breast tissues were isolated using the miRNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. miRNAs from CD45-DB fractions were isolated using the miRNeasy Mini Kit–RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) and miRNAs from FFPE samples were extracted using the PureLink FFPE Total RNA Isolation Kit following the provided protocol (Invitrogen Corporation, Carlsbad, CA, USA). miRNAs and mRNA samples were reversely
transcribed into cDNA using the miScript II RT Kit (Qiagen, Hilden, Germany) and RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Vilnius, Lithuania), respectively.

For real-time polymerase chain reaction (RT-PCR) Custom miScript miRNA PCR Array (CMHS02741, Qiagen, Germany) was used. For expression analyses of mature forms of hsa-miR-9-5p, hsa-miR-124-3p, hsa-miR-203a-3p, hsa-miR-200c-3p, hsa-miR-205-5p, hsa-miR-221-3p, and hsa-miR-720, the miScript SYBR Green PCR Kit (Qiagen, Germantown, MD, USA) was used. Reactions were performed in AriaMx Real-Time PCR System (Agilent, Santa Clara, CA, USA) using the following conditions: pre-denaturation at 95 ºC for 15 min, followed by 40 cycles at 94 ºC 15 s, 55 ºC for 30 s, and 70 ºC for 30 s, followed by melt cycle at 95 ºC for 30 s, 65 ºC for 30 s, and 95 ºC for 30 s. Among three reference controls (Snord61, Snord72, and Snord95), Snord95, with the most stable expression, was selected for normalization of Ct values.

qRT-PCR detection and expression of CDH1, TWIST1, SNAI1, SNAI2, ZEB1, ZEB2 and 18S were performed using TaqMan Gene Expression Assays—single tube assays (Thermo Fisher Scientific, Pleasanton, CA, USA): CDH1–Hs01013959_m1, TWIST1–Hs00361186_m1, SNAI1–Hs00195591_m1, SNAI2–Hs00161904_m1, ZEB1–Hs01566408_m1, ZEB2–Hs002007691_m1, 18S–Hs_9999991_s1. qRT-PCR reactions were carried out in an AriaMx Real-Time PCR System (Agilent, Santa Clara, CA, USA) at following settings: uracil-N-glycosylase incubation 1 cycle at 50 ºC for 2 min, enzyme activation 1 cycle at 95 ºC for 20 s, 40 cycles at 95 ºC for 30 s denaturation and 60 ºC for 30 s annealing. For all fluorescence-based RT-PCR, fluorescence was detected between 10 and 40 cycles for the reference (18 S) and target genes. Fold change was calculated as normalized relative gene expression using formula $2^{-\Delta\Delta Ct}$.

4.4. Immunohistochemical Analyses of E-Cadherin

Immunohistochemistry for detection of E-cadherin was performed on paraffin sections with ready to use reagents using an automated immunostainer, Autostainer Link 48 (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Primary E-cadherin antibody (FLEX Monoclonal Mouse, clone NCH-38, RTU, IR05961) was supplied by Dako; Agilent Technologies, Inc. (Santa Clara, CA, USA). Antigen retrieval was performed using EnVision TM FLEX Target Retrieval Solution High pH (pH 9.0) for 20 min. at 97–98 ºC in PT Link instrument (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Endogenous peroxidase activity was blocked by incubation for 10 min. in 3% hydrogen peroxide, followed by antibody incubation for 20 min. at room temperature. EnVision TM FLEX/HRP, High pH kit (K8000, Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) was used as a detection system according to the manufacturer’s instructions. High E-cadherin expression was classified as homogenous 3+ and heterogeneous 3+ and 2+ staining; low E-cadherin expression was defined in samples with heterogeneous staining covering 1+ and no expression regardless of the portion of cells with 3+, 2+, 1+, and no E-cadherin expression.

4.5. Statistical Analysis

IBM SPSS statistics 23.0 software was used for statistical analysis. qPCR data were analyzed using REST 2009 Software (Technical University Munich and Qiagen, Germany). The normality of distribution was assessed by the Shapiro–Wilk test. Normally distributed variables were tested using Student’s t-test. Non-normally distributed data were tested by nonparametric Mann–Whitney U test. Pearson’s or Spearman’s correlations were used to assess the correlations between miRNA and mRNA expression of tested genes. Binary logistic regression was used to evaluate the influence of selected gene and miRNA expression on hematogenous and lymphogenous dissemination of tumor cells and to control for confounders. This determination included enumeration of the risk estimate presented as estimated odds ratio (OR) and 95% confidence interval (CI) for the OR. $p$-value < 0.05 was defined as statistically significant.
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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the St. Elizabeth Cancer Institute Review Board (3-2019/EK OUSA, 20 March 2019).

Informed Consent Statement: Written informed consent has been obtained from all individual participants included in the study.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| BC | Breast cancer |
| C-blood | CD45-depleted blood fraction from healthy women |
| C-breast | Non-neoplastic breast tissue |
| CD45-DB | CD45-depleted blood |
| CDH1 | Cadherin 1 |
| CI | Confidence interval |
| CTC | Circulating tumor cells |
| EMT | Epithelial-to-mesenchymal transition |
| ER | Estrogen receptor |
| FC | Fold change |
| FFPE | Formalin-fixed paraffin-embedded tissue |
| HER2 | Human epidermal growth factor receptor 2 |
| LN | Lymph node |
| LNM | Lymph node metastasis |
| LVl | Lymphovascular invasion |
| mRNA | Messenger RNA |
| miRNA | microRNA |
| MTS | Distant metastasis |
| OR | Odds ratio |
| PR | Progesterone receptor |
| r | Correlation coefficient |
| RT-PCR | Real time- Polymerase chain reaction |
| SNAI1 | Snail Family Transcriptional Repressor 1 |
| SNAI2 | Snail Family Transcriptional Repressor 2 |
| TNBC | Triple-negative breast cancer |
| TNM | Tumor Node Metastasis staging |
| TU-C | Tumor centrum |
| TU-IF | Tumor invasive front |
| TWIST1 | Twist Family BHLH Transcription Factor 1 |
| ZEB1 | Zinc Finger E-Box Binding Homeobox 1 |
| ZEB2 | Zinc Finger E-Box Binding Homeobox 2 |
26. Petridis, C.; Shinomiya, I.; Kohut, K.; Gorman, P.; Caneppele, M.; Shah, V.; Troy, M.; Pinder, S.E.; Hanby, A.; Tomlinson, I.; et al. Germline CDH1 mutations in bilateral lobular carcinoma in situ. *Br. J. Cancer* 2014, 110, 1053–1057. [CrossRef] [PubMed]

27. Caldeira, J.R.F.; Prando, E.C.; Quevedo, F.C.; Neto, F.A.M.; Rainho, C.A.; Ragatto, S.R. CDH1 promoter hypermethylation and E-cadherin protein expression in infiltrating breast cancer. *BMC Cancer* 2006, 6, 48. [CrossRef]

28. Sebova, K.; Zmetakova, I.; Bella, V.; Kajo, K.; Stankovicova, I.; Kajabova, V.; Krivulcik, T.; Lasabova, Z.; Tomka, M.; Galbavy, S.; et al. RASSF1A and CDH1 hypermethylation as potential epimarkers in breast cancer. *Cancer Biomark.* 2012, 10, 13–26. [CrossRef]

29. Fridrichova, I.; Zmetakova, I. MicroRNAs Contribute to Breast Cancer Invasiveness. *Cells* 2019, 8, 1361. [CrossRef] [PubMed]

30. Ma, F.; Li, W.; Liu, C.; Li, W.; Yu, H.; Lei, B.; Ren, Y.; Li, Z.; Fang, D.; Qian, C. MiR-23a promotes TGF-β1-induced EMT and tumor metastasis in breast cancer cells by directly targeting CDH1 and activating Wnt/β-catenin signaling. *Oncotarget* 2017, 8, 69538–69550. [CrossRef]

31. Zhou, X.; Marian, C.; Makambi, K.H.; Kallakury, B.V.; Loffredo, C.A.; Zheng, Y.-L. MicroRNA-9 as potential biomarker for breast cancer local recurrence and tumor estrogen receptor status. *PLoS ONE* 2012, 7, e39011. [CrossRef]

32. Lambertini, E.; Lolli, A.; Vezzali, F.; Penolazzi, L.; Gambari, R.; Piva, R. Correlation between Slug transcription factor and miR-221 in MDA-MB-231 breast cancer cells. *BMC Cancer* 2012, 12, 445. [CrossRef]

33. Pan, Y.; Li, J.; Zhang, Y.; Wang, N.; Liang, H.; Liu, Y.; Zhang, C.Y.; Zen, K.; Gu, H. Slug-upregulated miR-221 promotes breast cancer progression through suppressing E-cadherin expression. *Sci. Rep.* 2016, 6, 25798. [CrossRef] [PubMed]

34. Niu, X.-Y.; Zhang, Z.-Q.; Ma, P.-L. MiRNA-221-5p promotes breast cancer progression by regulating E-cadherin expression. *Eur. Rev. Med. Pharm. Sci.* 2019, 23, 6983–6990. [CrossRef]

35. Stinson, S.; Lackner, M.R.; Adai, A.T.; Yu, N.; Kim, H.J.; O’Brien, C.; Spoerke, J.; Jhunjhunwala, S.; Boyd, Z.; Januario, T.; et al. TRPS1 targeting by miR-221/222 promotes the epithelial-to-mesenchymal transition in breast cancer. *Sci. Signal.* 2011, 4, ra41. [CrossRef]

36. Castilla, M.; Díaz-Martín, J.; Sarrió, D.; Romero-Pérez, L.; López-García, M.Á.; Veites, B.; Biscuola, M.; Ramiro-Fuentes, S.; Isacce, C.M.; Palacios, J. MicroRNA-200 family modulation in distinct breast cancer phenotypes. *PLoS ONE* 2012, 7, e47709. [CrossRef] [PubMed]

37. Hurteau, G.J.; Carlsson, J.A.; Spivack, S.D.; Brock, G.J. Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin protein expression in infiltrating breast cancer. *BMC Cancer* 2007, 7, 9792–9796. [CrossRef]

38. Sundararajan, V.; Gengenbacher, N.; Stemmler, M.P.; Kleemann, J.A.; Brabietz, T.; Brabietz, S. The ZEB1/miR-200c feedback loop regulates invasion via actin interacting proteins MYLK and TSK5. *Oncotarget* 2015, 6, 27083–27096. [CrossRef] [PubMed]

39. Chen, H.; Li, Z.; Zhang, L.; Zhang, L.; Zhang, Y.; Wang, Y.; Xu, M.; Zhong, Q. MicroRNA-200c inhibits the metastasis of triple-negative breast cancer by targeting ZEB2, an epithelial-mesenchymal transition regulator. *Ann. Clin. Lab. Sci.* 2020, 50, 519–527. [PubMed]

40. Lee, J.-Y.; Park, M.K.; Park, J.-H.; Lee, H.J.; Shin, D.H.; Kang, Y.; Lee, C.H.; Kong, G. Loss of the polycomb protein Mel-18 enhances the epithelial-mesenchymal transition by ZEB1 and ZEB2 expression through the downregulation of miR-205 in breast cancer. *Oncogene* 2014, 33, 1325–1335. [CrossRef] [PubMed]

41. Sun, E.-H.; Zhou, Q.; Liu, K.-S.; Wei, W.; Wang, C.-M.; Liu, X.-F.; Lu, C.; Ma, D.-Y. Screening miRNAs related to different subtypes of breast cancer with miRNAs microarray. *Eur. Rev. Med. Pharm. Sci.* 2014, 18, 2783–2788.

42. Moes, M.; Le Bèche, A.; Crespo, I.; Laurini, C.; Halavatyi, A.; Vetter, G.; Del Sol, A.; Friederich, E. A novel network integrating a miR-203/SNAI1 feedback loop that regulates epithelial to mesenchymal transition. *PLoS ONE* 2012, 7, e35440. [CrossRef] [PubMed]

43. Zhang, Z.; Zhang, B.; Li, W.; Fu, L.; Fu, L.; Zhu, Z.; Dong, J.-T. Epigenetic silencing of miR-203 upregulates SNAI2 and contributes to the invasiveness of malignant breast cancer cells. *Genes Cancer* 2011, 2, 782–791. [PubMed]

44. Ji, H.; Sang, M.; Liu, F.; Ai, N.; Geng, C. miR-124 regulates EMT based on ZEB2 target to inhibit invasion and metastasis in triple-negative breast cancer. *Pathol. Res. Pr.* 2019, 215, 697–704. [CrossRef] [PubMed]

45. Liang, Y.-J.; Wang, Q.-Y.; Zhou, C.-X.; Yin, Q.-Q.; He, M.; Yu, X.-T.; Cao, D.-X.; Chen, G.-Q.; He, J.-R.; Zhao, Q. MiR-124 targets Slug to regulate epithelial-mesenchymal transition and metastasis of breast cancer. *Carcinogenesis* 2013, 34, 713–722. [CrossRef]

46. Li, L.-Z.; Zhang, C.Z.; Liu, L.-L.; Yi, C.; Lu, S.-X.; Zhou, X.; Zhang, Z.-J.; Peng, Y.-H.; Yang, Y.-Z.; Yun, J.-P. miR-720 inhibits tumor invasion and migration in breast cancer by targeting TWIST1. *Carcinogenesis* 2014, 35, 469–478. [CrossRef] [PubMed]

47. Jeschke, U.; Mylonas, I.; Kuhn, C.; Shabani, N.; Kunert-Keil, C.; Schindlbeck, C.; Gerber, B.; Friese, K. Expression of E-cadherin in human ductal breast cancer carcinoma in situ, invasive carcinomas, their lymph node metastases, their distant metastases, carcinomas with recurrence and in recurrence. *Anticancer Res.* 2007, 27, 1969–1974. [PubMed]

48. Borcherding, N.; Cole, K.; Kluz, P.; Jorgensen, M.; Kolb, R.; Bellizzi, A.; Zhang, W. Re-evaluating E-cadherin and β-catenin: A pan-cancer proteomic approach with an emphasis on breast cancer. *Am. J. Pathol.* 2018, 188, 1910–1920. [CrossRef]

49. Padmanaban, V.; Krol, I.; Suhail, Y.; Szczersba, B.M.; Aceto, N.; Bader, J.S.; Ewald, A.J. E-cadherin is required for metastasis in triple-negative breast cancer. *Oncotarget* 2015, 6, 27083–27096. [CrossRef] [PubMed]

50. Prasad, C.P.; Mirza, S.; Sharma, G.; Prashad, R.; DattaGupta, S.; Rath, G.; Ralhan, R. Epigenetic alterations of CDH1 and APC genes: Relationship with activation of Wnt/beta-catenin pathway in invasive ductal carcinoma of breast. *Life Sci.* 2008, 83, 318–325. [CrossRef] [PubMed]
