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Original Article

High miR-99b expression is associated with cell proliferation and worse patient outcomes in breast cancer

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Abstract: Although miR-99b is a known suppressive microRNA (miRNA) in several cancers, its role in breast cancer has not been elucidated. In this study, we examined the clinical relevance of miR-99b expression in breast cancer. We analyzed miRNA and mRNA expression and their relationships with clinical parameters in 1,961 breast cancer samples from two independent large cohorts, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA). Several algorithms, including gene set enrichment analysis (GSEA) and xCell, have been used to investigate biological functions and the tumor microenvironment. High miR-99b expression significantly enriched the mTORC1 signaling gene set in breast cancer (NES = 1.63, FDR = 0.03, and NES = 1.58, FDR = 0.10, in METABRIC and TCGA, respectively). No other mechanisms, including the epithelial mesenchymal transition, NFκB, and TGF-β signaling, were consistently enriched in both cohorts. MiR-99b-high breast cancer was associated with high homologous recombination deficiencies, intratumor heterogeneity, and high rates of mutation and neoantigens. In agreement, miR-99b-high breast cancer was associated with increased cell proliferation, correlating with Nottingham histological grade, and significant enrichment of E2F targets, G2/M checkpoint, and mitotic spindle gene sets consistently in both cohorts ($P = 0.01$, $P < 0.001$). High miR-99b levels were also associated with low stromal cell fractions in the tumor microenvironment, including adipocytes, keratinocytes, and lymphatic endothelial cells ($P < 0.001$). However, in both cohorts, miR-99b expression was not associated with significant infiltration of immune cells, except dendritic cells ($P = 0.006$, 0.020). Finally, in both cohorts, breast cancer with high miR-99b expression was significantly associated with worse disease-free survival (DSS) and overall survival (OS), particularly in estrogen receptor (ER)-positive/human epidermal growth factor (HER)2-negative breast cancer (DSS hazard ratio (HR) 1.29, 95% confidence interval (CI) 1.10-1.51, $P < 0.001$ in the METABRIC cohort and HR 1.82, 95% CI 1.12-2.98, $P = 0.017$ in the TCGA cohort). In conclusion, breast cancer with high miR-99b expression was significantly associated with mTORC1 signaling, cell proliferation, and decreased patient survival, particularly in the ER-positive/HER2-negative subtype.

Keywords: Cell proliferation, gene expression, microRNA, signaling, tumor microenvironment, survival, biomarker, breast cancer

Introduction

MicroRNAs (miRNAs) are single-stranded RNA that regulate gene expression by inhibiting messenger RNA (mRNA) translation and promoting mRNA degradation [1]. They are a class of non-coding RNAs that regulate many aspects of cancer biology, including cell proliferation, cell differentiation, angiogenesis, and disease progression [2]. MiRNAs can be broadly divided

into two categories: suppressive miRNAs that impede cancer progression and oncogenic miRNAs (onco-miRs) that promote cancer progression. Representative suppressive miRNAs include miR-34 [3] which inhibits cell proliferation [4], miR-195 that suppresses cell proliferation and glycolysis [5], and miR-143, which correlates to a tumor microenvironment (TME) with improved prognosis in breast cancer patients [6]. A representative onco-miR gene is miR-155. MiR-155 promotes cell proliferation by suppressing gene suppressor of cytokine signaling (SOCS1), resulting in Janus kinase/signal transducer and activation of transcription protein (JAK/STAT) pathway signaling [7].

MiR-99b inhibits mechanistic target of rapamycin (mTOR) signaling, which regulates gene transcription and protein synthesis involved in cell proliferation, immune cell differentiation, and tumor metabolism [8]. It inhibits prostate cancer cell growth via autophagy induction [9, 10]. MiR-99b also negatively regulates insulin-like growth factor (IGF)-1 receptor expression and, in turn, suppresses gastric cancer growth [11]. However, miR-99b has been reported to function as an onco-miR in leukemia by preventing transforming growth factor (TGF)-β mediated cell cycle arrest and apoptosis [12]. In breast cancer, miR-99b is induced by breast cancer type 1 susceptibility protein (BRCA1) and commonly regulates TNF receptor-associated factor 2 (TRAF2), a key regulator of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cell (NFκ) pathways in *in vitro* assays [13]. MiR-99b was also reported to modulate the complex network that regulates transforming growth factor (TGF)-β-induced epithelial to mesenchymal transition (EMT) in breast tumors, as well as the proliferation and migration of breast cancer cells [14]. Thus, although miR-99b is experimentally associated with increased cancer pathogenesis in multiple signaling pathways, its relevance in the progression and outcomes of breast cancer patients remains unknown.

We have previously reported the clinical relevance of several miRNAs using *in silico* translational research approaches [4-6]. Utilizing various computational algorithms, we revealed many relationships between miRNA expression and cancer biology, tumor immune microenvironment (TIME), and patient outcomes. For example, miR-195 expression correlates with adverse patient survival, especially in estrogen receptor (ER)-positive breast cancer [5], whereas miR-143 expression is associated with better survival in ER-positive breast cancer [6].

Herein, we investigated the clinical relevance of miR-99b using two large multiple independent cohorts, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) and The Cancer Genome Atlas (TCGA), as the testing and validation cohorts of breast cancer.

Methods

Data acquisition

Breast cancer datasets of female patients consisting of 1,961 cases were downloaded from the METABRIC [15] and TCGA [16] databases through cBioportal (https://www.cbioportal.org) [17]. Data extraction included miRNA-99b and mRNA expression levels, and clinicopathological parameters. The Roswell Park Institutional Review Board approval requirements were waived because all the data obtained from the METABRIC and TCGA databases were de-identified and available in the public domain.

Biological enrichment analysis

Functional enrichment analysis of miR-99b expression was performed by Gene Set Enrichment Analysis (GSEA) [18], referencing the Molecular Signatures Database Hallmark collection (http://www.gsea-msigdb.org) [19], as we previously reported [20-25]. Gene sets with a false discovery rate (FDR) < 0.25 were chosen to specify enriched signaling per GSEA indications.

Other scores

The xCell algorithm (http://xcell.ucsf.edu) [26] was used to examine the correlation between miR-99b expression levels and the infiltrating fraction of TME stromal cells and immune cells. xCell estimates the composition of 64 cell populations within tumors. The downloaded mRNA expression data were scored using the xCell algorithm for several stromal cell (adipocytes, keratinocytes, fibroblasts, microvascular (mv) endothelial, and lymphatic (ly) endothelial cells), immune cell ($CD4^+$ and $CD8^+$ T cells,

T-helper (Th)1 and Th2 cells, regulatory T cells (Tregs), M1 and M2 macrophages, dendritic cells (DC), and B cells) populations, as we previously reported [27-30].

To investigate immune cytolytic activity (CYT) levels in the TME, we calculated the score as the geometric mean of perforin (*PRF1*) and granzyme A (*GZMA*) mRNA expression, which reflects the ability of cytotoxic T cells to eliminate cancer cells [31], as we previously reported [32-37]. To investigate the relationship between miR-99b expression and mutation load, we used DNA alteration-related metrics. These include homologous recombination deficiency (HRD) and intratumoral genomic heterogeneity, non-silent and silent mutation rates, fraction of genome altered (FGA), insertion and deletion (indel), and single-nucleotide variant (SNV) neoantigens, as previously published [38].

Statistical analyses

Statistical analyses were conducted using R 4.1.0 software (https://www.R-project.org). Graphics were generated using R software and Excel (Microsoft Corporation, Redmond, Washington, USA). The Mann-Whitney U test was used for comparisons between the low and high miR99b groups. For comparisons across ≥ 3 groups, the Kruskal-Wallis test was used. The R software survival package was used to analyze the relationship in breast cancer patients between miR-99b expression levels and disease-specific (DSS) and overall (OS) survival of breast cancer patients using Cox proportional hazards regression. Statistical significance was set at $P < 0.05$ (Figure S1).

Results

Mechanistic target of rapamycin complex 1 (mTORC1) signaling was the only pathway significantly enriched in miR-99b high breast cancer among the pathways previously reported in in vitro experiments

To investigate whether miR-99b functions similarly in breast cancer patients as *in vitro* studies, we performed gene set enrichment analysis (GSEA) dichotomized by miR-99b expression in breast cancer using the Hallmark gene sets. mTORC1, EMT, and tumor necrotizing factor (TNF)-α signaling via NFκ and TGF-β signaling were shown to be activated by miR-99b *in vitro*. Here, we found that high miR-99b expression in breast cancer specimens correlated significantly with enriched mTORC1 gene sets but not with other reported signaling pathways. This finding was consistent in both the METABRIC and TCGA cohorts (**Figure 1**). These results suggest that the mTOR pathway, but not EMT, NFκB, or TGF-β, was activated in miR-99b high-expressing patient breast cancer specimens.

Rates of mutation were significantly higher in miR-99b expression-high breast cancer

Since high miR-99b expression correlated with enriched mTOR signaling gene sets, we examined whether miR-99b expression was related to mutational burden, an indicator of cancer aggressiveness [39]. Relationships between miR-99b expression and mutation-related scores, intratumor heterogeneity, HRD, mutation rates, FGA, and neoantigens were evaluated using the list of values calculated for each patient in TCGA by Thorsson *et al.* [38]. We found that miR-99b-high expression correlated with significantly increased levels of HRD, intratumor heterogeneity, silent and non-silent mutation rates, FGA, and SNV neoantigens, but not with indel neoantigens, in the TCGA cohort (**Figure 2**). These results suggest that high miR-99b expression in breast cancer correlated with abundant mutations that indicate tumor aggressiveness.

Cell proliferation was enhanced in miR-99b expression-high breast cancer

Based on our previous report that breast cancer mutation rate correlated with cell proliferation [39], we examined the association of miR-99b expression within cell proliferation-related gene sets and breast cancer clinical parameters including subtype, American Joint Committee on Cancer (AJCC) stage, and Nottingham histological grade. We found that miR-99b-high breast cancer specimens were significantly enriched in three genes related to cell proliferation in both cohorts: E2F targets, the G2/M checkpoint, and mitotic spindle gene sets (**Figure 3A**). Among the clinical parameters, miR-99b expression was lowest in triple negative breast cancer (TNBC) in the METABRIC cohort, but this result was not substantiated in the TCGA cohort (**Figure 3B**). Therefore, there

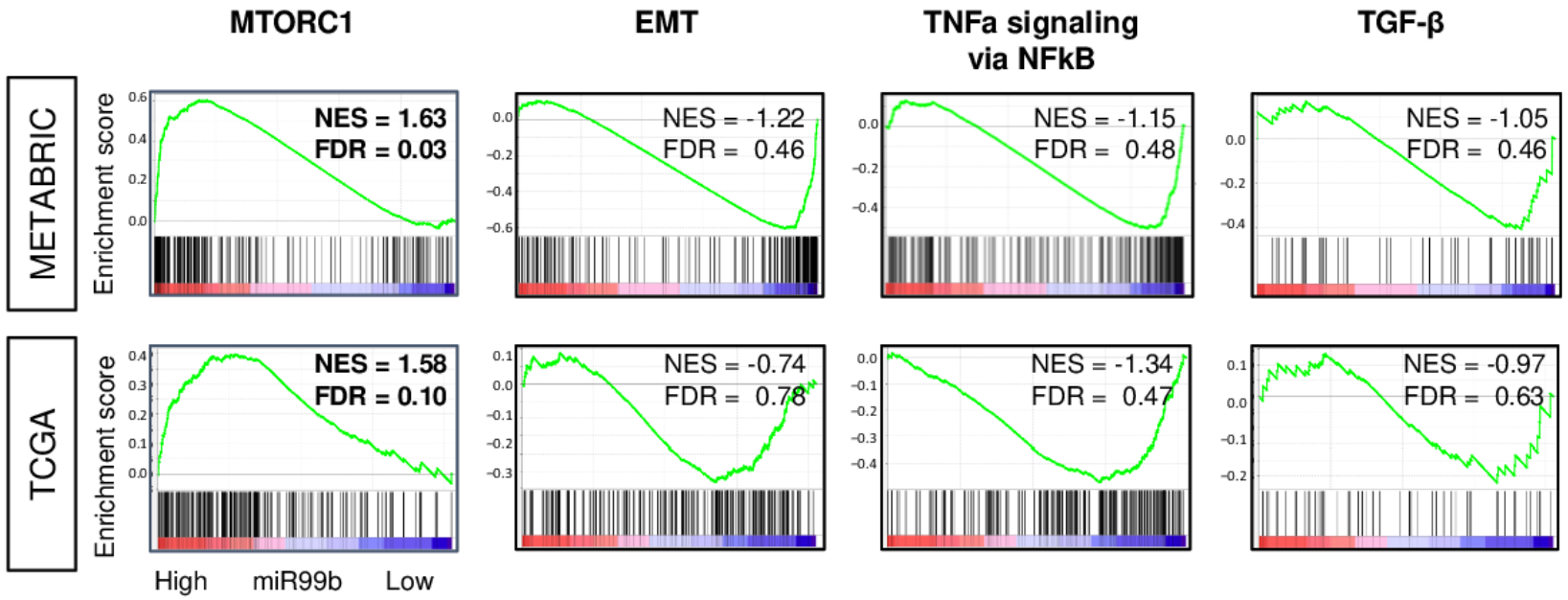

Figure 1. Biological functions of miR-99b-high expressing patient breast cancer. Enrichment correlation plots of gene set enrichment analysis (GSEA) between high and low miR-99b expression in the Hallmark collection (mTORC1, EMT, TNF-α, and TGF-β), in the METABRIC and TCGA cohorts. The top tertile dichotomizes high- or low-miR-99b breast cancer groups. FDR, False Discovery Rate; NES, Normalized Enrichment Score.

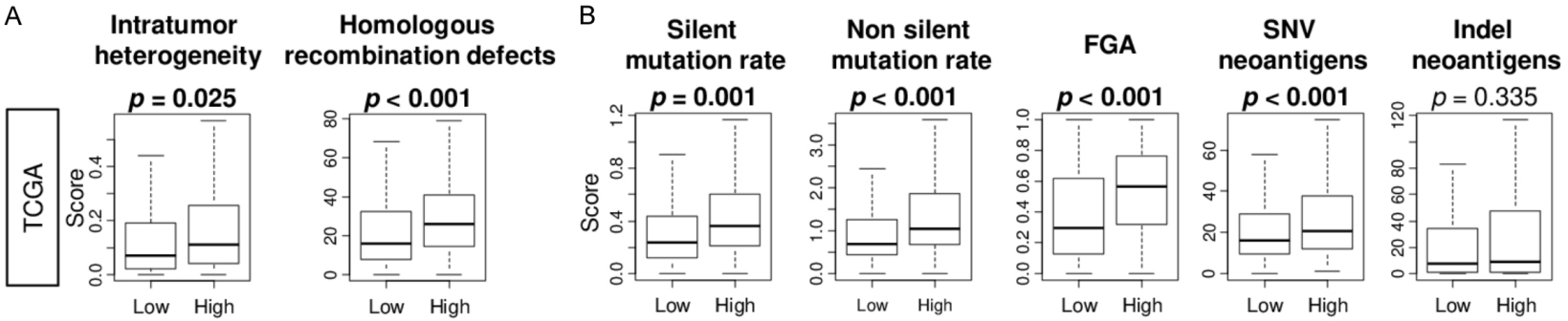

Figure 2. MiR-99b expression correlation with breast cancer mutations. Box plots of intratumor heterogeneity, homologous recombination defects, and silent and non-silent mutation rate, fraction genome altered (FGA), single-nucleotide variant (SNV) and Indel neoantigens in the TCGA cohort, between low and high miR-99b expression breast cancer. The top tertile was used as the cutoff to divide high- or low-miR-99b breast cancer groups.

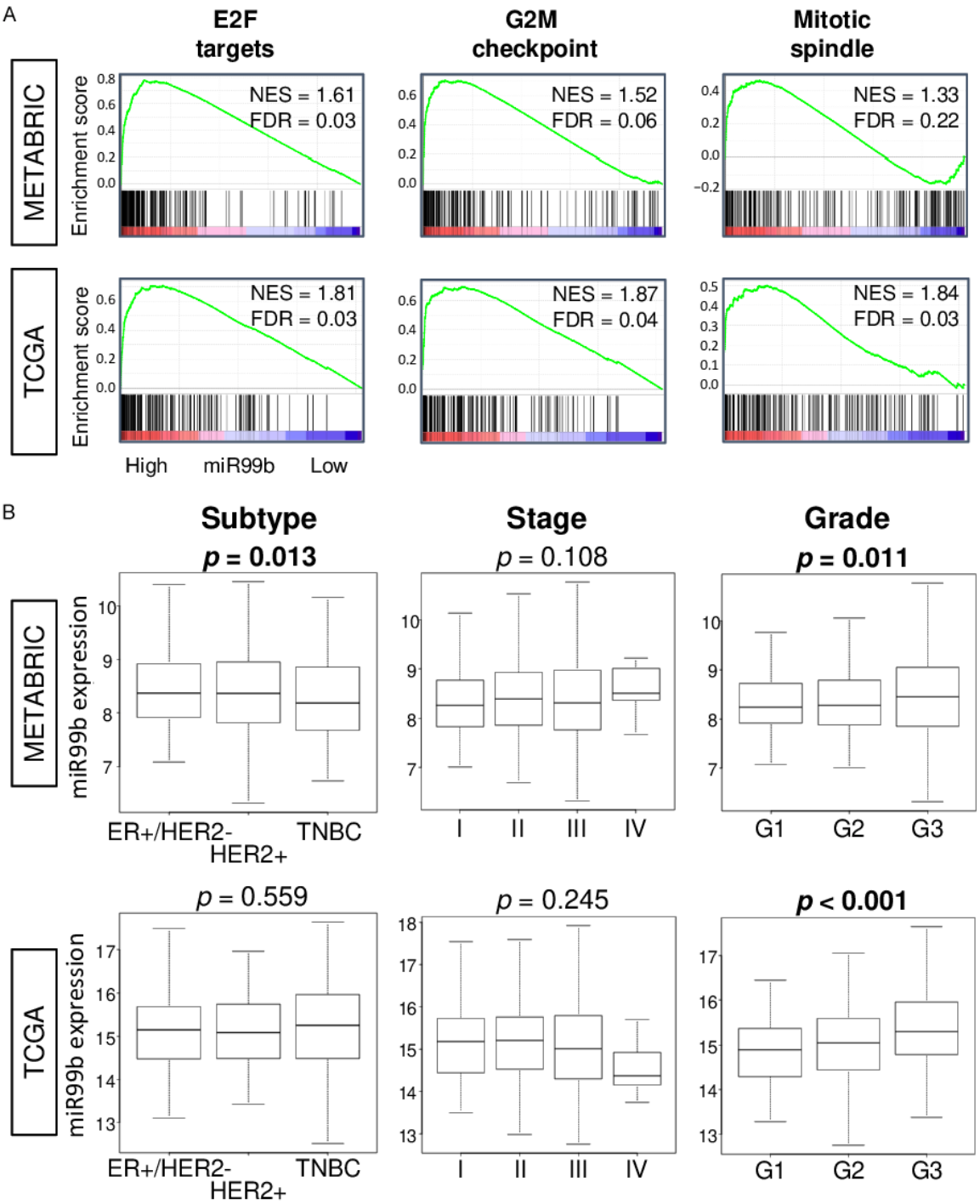

Figure 3. MiR-99b expression correlation with cell proliferation and clinical aggressiveness. A. Enrichment correlation plots of Hallmark gene sets between miR-99b-low and -high expression breast cancer, which showed significant differences in gene sets related to cellular proliferation in both METABRIC and TCGA cohorts. The top tertile dichotomizes high- or low-miR-99b breast cancer groups. B. Boxplots of miR-99b expression by subtype (ER+/HER2-, HER2+, and TNBC), AJCC stage (I-IV), and Nottingham histological grade (G1-3).

may not be a relationship between miR-99b expression and AJCC stage. However, miR-99b expression was significantly correlated with the histological grade in both cohorts (**Figure 3B**).

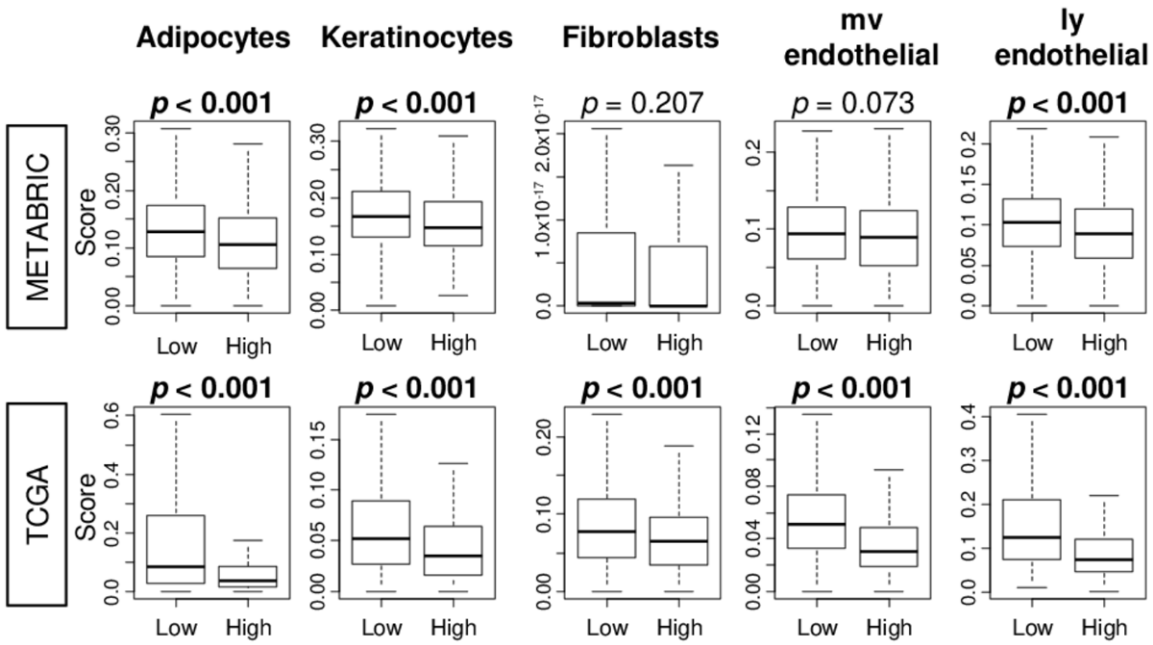

Figure 4. MiR-99b expression correlation with stromal cell fraction in the tumor microenvironment of breast cancer. Box plots of infiltrating fraction of adipocytes, keratinocytes, fibroblasts, microvascular (mv) endothelial, and lymphatic (ly) endothelial cells, between miR-99b-low and -high expression in the METABRIC and TCGA cohorts. Top tertile dichotomizes high- or low-miR-99b breast cancer groups.

Therefore, high miR-99b expression was associated with breast cancer cell proliferation in both bioinformatic and pathological assays.

MiR-99b-high breast cancer was significantly associated with low stromal cell fraction

We previously demonstrated that highly proliferative breast cancer has less infiltration of stromal cells including adipocytes [40]. Therefore, we investigated the association between miR-99b expression and stromal cell infiltration in the TME. MiR-99b-high expression breast cancer had significantly less infiltration of stromal cells, including adipocytes, keratinocytes, and lymphatic endothelial cells in both cohorts (**Figure 4**).

No definitive immune cell infiltrations associated with miR-99b expression in patient breast cancer

We previously reported that the predicted biological aggressiveness in breast cancer with high mutation rates is counterbalanced by anticancer immune cell infiltration in the TME [39]. Therefore, we investigated the association between miR-99b expression and immune cell infiltration. We found that miR-99b expression was inversely associated with the infiltration fractions of $CD8^+$ T cells, $CD4^+$ T cells, and dendritic cells (DC) in the METABRIC cohort. However, this was not validated in TCGA cohort (**Figure 5A**). The infiltration of pro-cancerous M2 macrophages positively correlated with miR-99b expression only in the METABRIC cohort (**Figure 5B**). In agreement with less infiltration of immune cells that promote an anticancer milieu and increased infiltration of immune cells that favor a pro-cancerous environment, miR-99b-high expression was significantly associated with less cytolytic activity in the METABRIC cohort (P = 0.008), but once again, it was not validated in the TCGA cohort (P = 0.070) (**Figure 5C**). In breast cancer, given that none of these findings have been validated in another cohort, definitive associations between immune cell infiltration and miR-99b expression may not exist.

MiR-99b-high expression is significantly associated with worse patient outcomes, particularly in ER-positive/HER2-negative breast cancer

To examine the clinical relevance of miR-99b expression in patients with breast cancer, we assessed the correlation between miR-99b

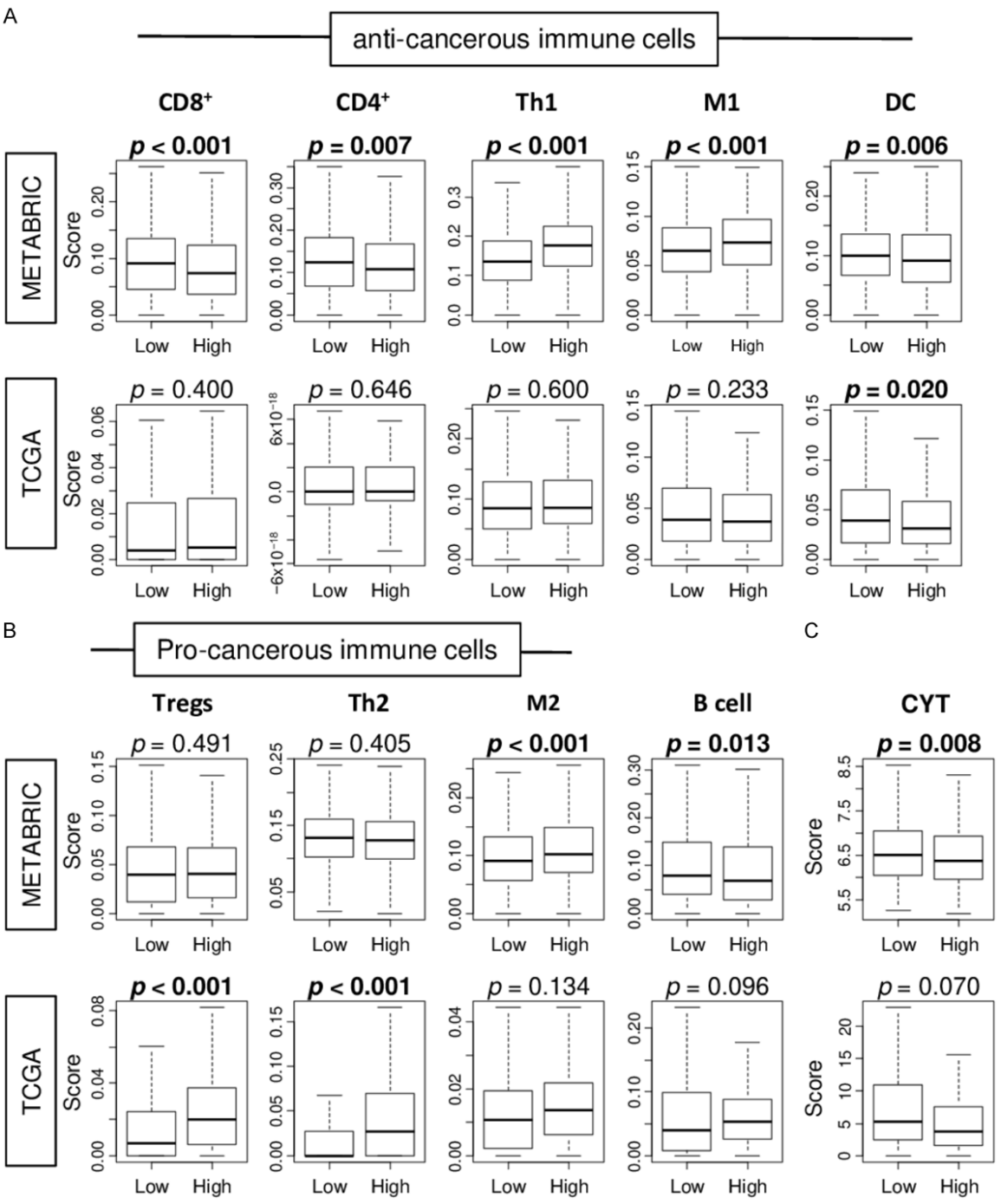

Figure 5. MiR-99b expression correlation with immune cell fraction in the breast cancer TME. Box plots of infiltrating fraction of (A) anti-cancerous immune cells ($CD8^+$ T cells, $CD4^+$ T cells, Th1 cells, M1 macrophages, and dendritic cells (DC)), (B) pro-cancerous immune cells (Tregs, Th2 cells, and M2 macrophages), and B cells, and (C) cytolytic activity (CYT) score between miR-99b-low and -high expression breast cancer. The top tertile dichotomizes high- or low-miR-99b breast cancer groups.

expression and OS and DSS in the two cohorts. MiR-99b-high expression was strongly correlated with unfavorable patient outcomes in both cohorts (**Figure 6A**). The survival relevance of each subtype was also investigated. In the METABRIC cohort only, miR-99b-high expression in TNBC was associated with worse DSS (**Figure 6B**; $P = 0.014$). However, in ER-positive/

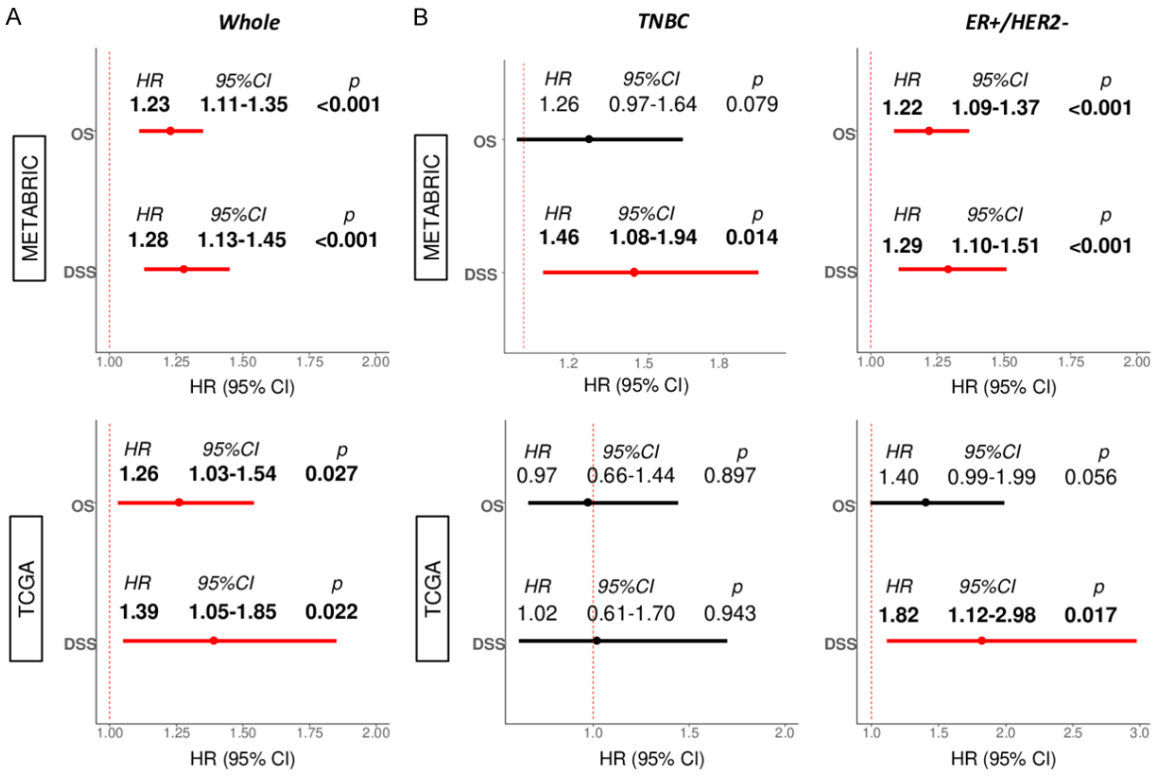

Figure 6. MiR-99b expression correlation with patient survival outcomes in breast cancer. Forest plots of miR-99b expression with overall survival (OS) and disease-specific survival (DSS) in (A) whole cohort, and (B) TNBC and ER-positive/HER2-negative subtypes in both cohorts.

HER2-negative tumors, miR-99b-high expression correlated with both worse OS and DSS in the METABRIC cohort (both $P < 0.001$) and DSS in the TCGA cohort ($P = 0.017$). MiR-99b-high expression was trended with worse OS in TCGA cohort ($P = 0.056$). These results suggest that miR-99b-high expression is associated with worse patient survival, particularly in ER-positive/HER2-negative tumors.

Discussion

We found that miR-99b-high expression in breast cancer has an enriched mTORC1 signaling gene set, but not EMT, NF-κB, or TGF-β signaling sets. MiR-99b-high expression breast tumors had enriched gene-sets related to cell proliferation. These include sets linked to E2F targets, G2/M checkpoint, and mitotic spindle signaling, which were also significantly associated with a higher histological grade, but not subtype or AJCC stage. In agreement, miR-99b-high expression coupled with both higher mutation rates and reduced stromal cell infiltration are consistent findings with highly proliferative tumors. In addition to dendritic cells, miR-99b expression was not associated with immune function or fraction in breast cancer. Finally, breast cancer with miR-99b-high expression correlated strongly with decreased OS and DSS, especially ER-positive/HER2-negative breast cancer in both cohorts.

MiRNAs are classified as suppressive miRNAs and onco-miRs, depending on their target tumor suppressor genes or oncogenes. For example, miR-34 is a well-known suppressive miRNA and its high bulk tumor expression is associated with less aggressive tumor biology in breast cancer [4]. MiR-34a-high tumors show increased apoptosis via the activation of the p53 pathway [41]. Other representative suppressive miRNAs include miR-30a and miR-200c [42]. Preclinical studies have reported that suppression of miR-200c results in higher expression of EMT-related genes, such as zinc finger E-box binding homeobox (ZEB)1, and increases the risk of breast cancer metastasis [43, 44]. These results were supported by our previous study, where we demonstrated that

low levels of miR-200c and miR-30a in breast cancer patients correlated with an enriched EMT-related gene set and were associated with poor prognosis compared with high expression tumors [42]. On the contrary, numerous studies demonstrated that the onco-miR miR-155 is upregulated in various malignancies including breast cancer [45, 46]. MiR-155 expression promotes angiogenesis, leading to more aggressive cancer biology [46].

MiRNAs modulate several biological activities. In particular, miRNA function depends on the site of cancer. An additional complexity arises because each miRNA can target multiple mRNAs. Furthermore, several miRNAs can target the same mRNA [47]. Therefore, it is possible that the same miRNAs play both unique and opposing roles in patient outcomes in different cancer types [48]. miR-99b is also known to act as both an onco-miRNA and a suppressive miRNA in different cellular contexts [12]. Wang *et al.* reported that overexpression of miR-99b inhibited cell growth and cervical cancer invasion and directly and negatively regulated mTOR expression in this cancer cell type [49]. However, our results demonstrated an opposite effect in breast cancer. The difference in the miR-99b phenotype may be due to the differing signaling effects between the two cancer types.

In addition to its functional roles in cancer, miR-99b has been implicated in the modulation of macrophage inflammatory responses and T-cell subsets, thereby playing critical roles in the maintenance of tissue homeostasis, peripheral tolerance regulation, and inflammatory reaction resolution [12]. In the current study, miR-99b expression was not associated with immune cell infiltration in the breast cancer TME. We speculate that this may be due to differences in biological features between the *in vivo* and *in vitro* scenarios and patient TME characteristics. As previously reported, animal models, including patient-derived xenografts, mimic the human TME to a certain extent [50]. To this end, we strongly feel that studies analyzing a large number of patient samples, such as this study, provide powerful insights into real patient tumor biology.

In this study we found that high miR-99b levels were associated with enhanced cancer cell proliferation and with low stromal cell fractions, particularly adipocytes. At first glance this finding appears to conflict with a well-known fact that intratumor cancer-associated adipocytes interact with cancer cells and secrete detrimental inflammatory cytokines that aggravate cancer progression [51]. We have previously addressed this issue by investigating the cancer biology of bulk tumor with high infiltrations of adipocytes in patient cancers [40, 52]. We found that intratumor cancer-associated adipocytes were consistently associated with less cancer cell proliferation in ER-positive breast cancer and in hepatocellular carcinoma, despite their correlation with inflammation and metastatic pathways. This relationship between highly proliferative breast cancer and low adipocyte infiltration was consistently and repeatedly reproduced regardless of the mechanism of increased cell proliferation, whether it is by increased unfolded protein response [53], activation of G2M checkpoint pathway [54], or proliferative tumors identified by a score of 5 gene expressions [35]. Given these findings, we speculate that less adipocyte infiltrations in high miR-99b expressing tumors are not due to a specific function of miR-99b but rather because highly proliferative cancer creates a highly dense tumor microenvironment packed with cancer cells that develop pressure too high for adipocytes to infiltrate. It will be interesting to prove this hypothesis using experimental models in the future. In terms of the mechanism of miR-99b in cancer progression, we did find that miR-99b expression was associated with activation of mTORC1 signaling, known to worsen cancer progression, but not EMT, TNF-α, or TGF-β signaling. Our findings in breast cancer patients that intratumor cancer-associated adipocytes were more associated with inflammation and metastatic pathways than cancer cell proliferation further support our analysis methodology [23, 30, 35, 52-54] in addition to experiments on cell lines and animal tumors, since results from model systems may not be applicable in human patients.

Since cell cycle inhibitors, such as cyclin-dependent kinase (CDK) 4/6 inhibitors, are clinically effective in ER-positive breast cancer, we speculate that suppression of miR-99b, which positively correlates with cell cycle impedance, may be a predictive and prognostic biomarker for breast cancer treatment and a potential target.

Our study has several limitations. First, as this was a retrospective study, our findings require cautious interpretation. While our results were

validated by independent cohorts, the treatments that the patients received were highly heterogeneous, which may have influenced the outcomes. Next, this study was analyzed using only a bioinformatics tool and lacked mechanistic insights. Although it is impossible to create a preclinical model that completely mimics the human TME, *in vivo/in vitro* experiments are necessary to elucidate the causal relationship between miR-99b and cell proliferation in the breast cancer TME. Improving these aspects in future in-depth studies will enable a comprehensive evaluation of the value of miR-99b expression in predicting clinical outcomes. In conclusion, we found that high expression of miR-99b in breast cancer was significantly associated with not only mTORC1 but also several cell proliferation-related signaling gene sets and worse patient outcomes, particularly in ER-positive/HER2-negative breast cancer.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

AJCC, American Joint Committee on Cancer; DSS, Disease-Specific Survival; ER, Estrogen Receptor; FDR, false Discovery Rate; GSEA, gene Set Enrichment Analysis; GSVA, Gene Set Variation Analysis; HER2, Human Epidermal Growth Factor Receptor 2; FGA, Fraction Genome Altered; HRD, Homologous Recombination Defects; INDEL, Interaction And Deletion; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; miRNA, microRNA; mRNA, messenger RNA; mTOR, mechanistic Target of Rapamycin; mTORC1, mechanistic Target of Rapamycin Complex 1; NES, Normalized Enrichment Score; NF-κB, Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; onco-miRs, oncogenic miRNAs; OS, Overall Survival; SNV, Single Nucleotide Variant; TCGA, The Cancer Genome Atlas; TGF-β, Transforming Growth Factor-β; TIME, Tumor Immune Microenvironment; TME, Tumor Microenvironment; TNBC, Triple-Negative Breast Cancer.

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References

[1] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[2] Rupaimoole R and Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov 2017; 16: 203-222.

[3] Misso G, Di Martino MT, De Rosa G, Farooqi AA, Lombardi A, Campani V, Zarone MR, Gullà A, Tagliaferri P, Tassone P and Caraglia M. Mir-34: a new weapon against cancer? Mol Ther Nucleic Acids 2014; 3: e194.

[4] Tokumaru Y, Katsuta E, Oshi M, Sporn JC, Yan L, Le L, Matsuhashi N, Futamura M, Akao Y, Yoshida K and Takabe K. High expression of miR-34a associated with less aggressive cancer biology but not with survival in breast cancer. Int J Mol Sci 2020; 21: 3045.

[5] Tokumaru Y, Oshi M, Patel A, Katsuta E, Yan L, Angarita FA, Dasgupta S, Nagahashi M, Matsuhashi N, Futamura M, Yoshida K and Takabe K. Low expression of miR-195 is associated with cell proliferation, glycolysis and poor survival in estrogen receptor (ER)-positive but not in triple negative breast cancer. Am J Cancer Res 2021; 11: 3320-3334.

[6] Tokumaru Y, Asaoka M, Oshi M, Katsuta E, Yan L, Narayanan S, Sugito N, Matsuhashi N, Futamura M, Akao Y, Yoshida K and Takabe K. High expression of microRNA-143 is associated with favorable tumor immune microenvironment and better survival in estrogen receptor positive breast cancer. Int J Mol Sci 2020; 21: 3213.

[7] Jiang S, Zhang HW, Lu MH, He XH, Li Y, Gu H, Liu MF and Wang ED. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. Cancer Res 2010; 70: 3119-3127.

[8] Zou Z, Tao T, Li H and Zhu X. MTOR signaling pathway and mTOR inhibitors in cancer: progress and challenges. Cell Biosci 2020; 10: 31.

[9] Niture S, Tricoli L, Qi Q, Gadi S, Hayes K and Kumar D. MicroRNA-99b-5p targets mTOR/AR axis, induces autophagy and inhibits prostate cancer cell proliferation. Tumour Biol 2022; 44: 107-126.
[10] Sun D, Lee YS, Malhotra A, Kim HK, Matecic M, Evans C, Jensen RV, Moskaluk CA and Dutta A. MiR-99 family of MicroRNAs suppresses the expression of prostate-specific antigen and prostate cancer cell proliferation. Cancer Res 2011; 71: 1313-1324.
[11] Wang Z, Zhao Z, Yang Y, Luo M, Zhang M, Wang X, Liu L, Hou N, Guo Q, Song T, Guo B and Huang C. MiR-99b-5p and miR-203a-3p function as tumor suppressors by targeting IGF-1R in gastric cancer. Sci Rep 2018; 8: 10119.
[12] Eniafe J and Jiang S. MicroRNA-99 family in cancer and immunity. Wiley Interdiscip Rev RNA 2021; 12: e1635.
[13] Tanic M, Zajac M, Gómez-López G, Benítez J and Martínez-Delgado B. Integration of BRCA1-mediated miRNA and mRNA profiles reveals microRNA regulation of TRAF2 and NFκB pathway. Breast Cancer Res Treat 2012; 134: 41-51.
[14] Turcatel G, Rubin N, El-Hashash A and Warburton D. MIR-99a and MIR-99b modulate TGF-β induced epithelial to mesenchymal plasticity in normal murine mammary gland cells. PLoS One 2012; 7: e31032.
[15] Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, Haffari G, Bashashati A, Russell R, McKinney S; METABRIC Group, Langerød A, Green A, Provenzano E, Wishart G, Pinder S, Watson P, Markowetz F, Murphy L, Ellis I, Purushotham A, Børresen-Dale AL, Brenton JD, Tavaré S, Caldas C and Aparicio S. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012; 486: 346-352.
[16] Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012; 490: 61-70.
[17] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-404.
[18] Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES and Mesirov JP. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.
[19] Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP and Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst 2015; 1: 417-425.
[20] Oshi M, Tokumaru Y, Angarita FA, Lee L, Yan L, Matsuyama R, Endo I and Takabe K. Adipogenesis in triple-negative breast cancer is associated with unfavorable tumor immune microenvironment and with worse survival. Sci Rep 2021; 11: 12541.
[21] Oshi M, Newman S, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. Inflammation is associated with worse outcome in the whole cohort but with better outcome in triple-negative subtype of breast cancer patients. J Immunol Res 2020; 2020: 5618786.
[22] Oshi M, Satyananda V, Angarita FA, Kim TH, Tokumaru Y, Yan L, Matsuyama R, Endo I, Nagahashi M and Takabe K. Angiogenesis is associated with an attenuated tumor microenvironment, aggressive biology, and worse survival in gastric cancer patients. Am J Cancer Res 2021; 11: 1659-1671.
[23] Oshi M, Takahashi H, Tokumaru Y, Yan L, Rashid OM, Nagahashi M, Matsuyama R, Endo I and Takabe K. The E2F pathway score as a predictive biomarker of response to neoadjuvant therapy in ER+/HER2- breast cancer. Cells 2020; 9: 1643.
[24] Oshi M, Tokumaru Y, Angarita FA, Yan L, Matsuyama R, Endo I and Takabe K. Degree of early estrogen response predict survival after endocrine therapy in primary and metastatic ER-positive breast cancer. Cancers (Basel) 2020; 12: 3557.
[25] Oshi M, Tokumaru Y, Patel A, Yan L, Matsuyama R, Endo I, Katz MHG and Takabe K. A novel four-gene score to predict pathologically complete (R0) resection and survival in pancreatic cancer. Cancers (Basel) 2020; 12: 3635.
[26] Aran D, Hu Z and Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. Genome Biol 2017; 18: 220.
[27] Oshi M, Tokumaru Y, Asaoka M, Yan L, Satyananda V, Matsuyama R, Matsuhashi N, Futamura M, Ishikawa T, Yoshida K, Endo I and Takabe K. M1 macrophage and M1/M2 ratio defined by transcriptomic signatures resemble only part of their conventional clinical characteristics in breast cancer. Sci Rep 2020; 10: 16554.
[28] Oshi M, Newman S, Tokumaru Y, Yan L, Matsuyama R, Kalinski P, Endo I and Takabe K. Plasmacytoid dendritic cell (pDC) infiltration correlate with tumor infiltrating lymphocytes, cancer immunity, and better survival in triple negative breast cancer (TNBC) more strongly than conventional dendritic cell (cDC). Cancers (Basel) 2020; 12: 3342.

[29] Oshi M, Asaoka M, Tokumaru Y, Yan L, Matsuyama R, Ishikawa T, Endo I and Takabe K. CD8 T cell score as a prognostic biomarker for triple negative breast cancer. Int J Mol Sci 2020; 21: 6968.
[30] Oshi M, Asaoka M, Tokumaru Y, Angarita FA, Yan L, Matsuyama R, Zsiros E, Ishikawa T, Endo I and Takabe K. Abundance of regulatory T cell (Treg) as a predictive biomarker for neoadjuvant chemotherapy in triple-negative breast cancer. Cancers (Basel) 2020; 12: 3038
[31] Rooney MS, Shukla SA, Wu CJ, Getz G and Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell 2015; 160: 48-61.
[32] Oshi M, Kawaguchi T, Yan L, Peng X, Qi Q, Tian W, Schulze A, McDonald KA, Narayanan S, Young J, Liu S, Morris LG, Chan TA, Kalinski P, Matsuyama R, Otsuji E, Endo I and Takabe K. Immune cytolytic activity is associated with reduced intra-tumoral genetic heterogeneity and with better clinical outcomes in triple negative breast cancer. Am J Cancer Res 2021; 11: 3628-3644.
[33] Oshi M, Gandhi S, Tokumaru Y, Yan L, Yamada A, Matsuyama R, Ishikawa T, Endo I and Takabe K. Conflicting roles of EGFR expression by subtypes in breast cancer. Am J Cancer Res 2021; 11: 5094-5110.
[34] Oshi M, Gandhi S, Huyser MR, Tokumaru Y, Yan L, Yamada A, Matsuyama R, Endo I and Takabe K. MELK expression in breast cancer is associated with infiltration of immune cell and pathological compete response (pCR) after neoadjuvant chemotherapy. Am J Cancer Res 2021; 11: 4421-4437.
[35] Oshi M, Gandhi S, Angarita FA, Kim TH, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. A novel five-gene score to predict complete pathological response to neoadjuvant chemotherapy in ER-positive/HER2-negative breast cancer. Am J Cancer Res 2021; 11: 3611-3627.
[36] Oshi M, Angarita FA, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. A novel three-gene score as a predictive biomarker for pathologically complete response after neoadjuvant chemotherapy in triple-negative breast cancer. Cancers (Basel) 2021; 13: 2401.
[37] Oshi M, Angarita FA, Tokumaru Y, Yan L, Matsuyama R, Endo I and Takabe K. High expression of NRF2 is associated with increased tumor-infiltrating lymphocytes and cancer immunity in ER-positive/HER2-negative breast cancer. Cancers (Basel) 2020; 12: 3856.
[38] Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, Porta-Pardo E, Gao GF, Plaisier CL, Eddy JA, Ziv E, Culhane AC, Paull EO, Sivakumar IKA, Gentles AJ, Malhotra R, Farshidfar F, Colaprico A, Parker JS, Mose LE, Vo NS, Liu J, Liu Y, Rader J, Dhankani V, Reynolds SM, Bowlby R, Califano A, Cherniack AD, Anastassiou D, Bedognetti D, Mokrab Y, Newman AM, Rao A, Chen K, Krasnitz A, Hu H, Malta TM, Noushmehr H, Pedamallu CS, Bullman S, Ojesina AI, Lamb A, Zhou W, Shen H, Choueiri TK, Weinstein JN, Guinney J, Saltz J, Holt RA, Rabkin CS; Cancer Genome Atlas Research Network, Lazar AJ, Serody JS, Demicco EG, Disis ML, Vincent BG and Shmulevich I. The immune landscape of cancer. Immunity 2019; 51: 411-412.
[39] Takahashi H, Asaoka M, Yan L, Rashid OM, Oshi M, Ishikawa T, Nagahashi M and Takabe K. Biologically aggressive phenotype and anticancer immunity counterbalance in breast cancer with high mutation rate. Sci Rep 2020; 10: 1852.
[40] Tokumaru Y, Oshi M, Katsuta E, Yan L, Huang JL, Nagahashi M, Matsuhashi N, Futamura M, Yoshida K and Takabe K. Intratumoral adipocyte-high breast cancer enrich for metastatic and inflammation-related pathways but associated with less cancer cell proliferation. Int J Mol Sci 2020; 21: 5744.
[41] Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, Bentwich Z and Oren M. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell 2007; 26: 731-743.
[42] Kawaguchi T, Yan L, Qi Q, Peng X, Gabriel EM, Young J, Liu S and Takabe K. Overexpression of suppressive microRNAs, miR-30a and miR-200c are associated with improved survival of breast cancer patients. Sci Rep 2017; 7: 15945.
[43] Bai WD, Ye XM, Zhang MY, Zhu HY, Xi WJ, Huang X, Zhao J, Gu B, Zheng GX, Yang AG and Jia LT. MiR-200c suppresses TGF-β signaling and counteracts trastuzumab resistance and metastasis by targeting ZNF217 and ZEB1 in breast cancer. Int J Cancer 2014; 135: 1356-1368.
[44] Hurteau GJ, Carlson JA, Spivack SD and Brock GJ. Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. Cancer Res 2007; 67: 7972-7976.
[45] Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Ménard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M and Croce CM. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005; 65: 7065-7070.
[46] Kong W, He L, Richards EJ, Challa S, Xu CX, Permuth-Wey J, Lancaster JM, Coppola D, Sellers TA, Djeu JY and Cheng JQ. Upregulation

of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer. Oncogene 2014; 33: 679-689.
[47] Sayad A, Najafi S, Kashi AH, Hosseini SJ, Akrami SM, Taheri M and Ghafouri-Fard S. Circular RNAs in renal cell carcinoma: functions in tumorigenesis and diagnostic and prognostic potentials. Pathol Res Pract 2022; 229: 153720.
[48] Si W, Shen J, Zheng H and Fan W. The role and mechanisms of action of microRNAs in cancer drug resistance. Clin Epigenetics 2019; 11: 25.
[49] Wang L, Chang L, Li Z, Gao Q, Cai D, Tian Y, Zeng L and Li M. MiR-99a and -99b inhibit cervical cancer cell proliferation and invasion by targeting mTOR signaling pathway. Med Oncol 2014; 31: 934.
[50] Oshi M, Okano M, Maiti A, Rashid OM, Saito K, Kono K, Matsuyama R, Endo I and Takabe K. Novel breast cancer brain metastasis patient-derived orthotopic xenograft model for preclinical studies. Cancers (Basel) 2020; 12: 444.
[51] Zhu Z, Zhu X, Yang S, Guo Z, Li K, Ren C, Zhou Y and Dou J. Yin-yang effect of tumour cells in breast cancer: from mechanism of crosstalk between tumour-associated macrophages and cancer-associated adipocytes. Am J Cancer Res 2020; 10: 383-392.
[52] Mukhopadhyay S, Tokumaru Y, Oshi M, Endo I, Yoshida K and Takabe K. Low adipocyte hepatocellular carcinoma is associated with aggressive cancer biology and with worse survival. Am J Cancer Res 2022; 12: 4028-4039.
[53] Oshi M, Roy AM, Gandhi S, Tokumaru Y, Yan L, Yamada A, Endo I and Takabe K. The clinical relevance of unfolded protein response signaling in breast cancer. Am J Cancer Res 2022; 12: 2627-2640.
[54] Oshi M, Takahashi H, Tokumaru Y, Yan L, Rashid OM, Matsuyama R, Endo I and Takabe K. G2M cell cycle pathway score as a prognostic biomarker of metastasis in estrogen receptor (ER)-positive breast cancer. Int J Mol Sci 2020; 21: 2921.

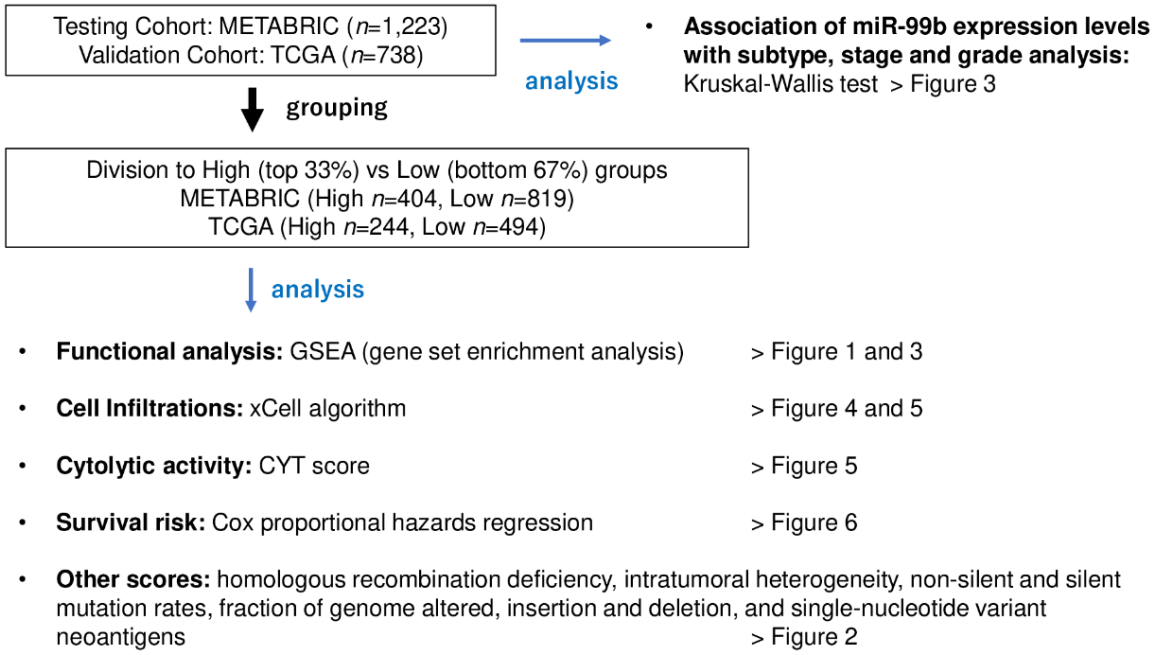

Figure S1. A flowchart of the study.