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# MiR-640 and MiR-525-5p as Relapse-Associated MicroRNAs in Systematically Untreated Individuals with Low-Risk Primary Breast Cancer

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MicroRNAs have been suggested to be signatures for predicting recurrence in breast cancer. This study aimed to identify miR-640 and miR-525-5p as relapse-associated microRNAs in systematically untreated and lymph node negative patients with primary breast cancer. GEO datasets GSE126125 and GSE103161 were analyzed to find the differential microRNAs in primary breast cancer with and without relapse. Systematically untreated patients (n = 180) with low-risk primary breast cancer were selected in this independent validation set, and 48 patients developed relapse within 5 years. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to detect miR-640 and miR-525-5p expressions in tumor tissues. Based on the retrieved data from GSE126125, miR-640 and miR-525-5p expressions were significantly lower in tumors from patients with relapse compared to tumors from patients without relapse, accompanying by highly predictive potential to discriminate cases with and without relapse. The decreased miR-640 and miR-525-5p expressions were revealed in dead patients as compared to alive patients. In the independent validation set, patients with low expression of miR-640 and miR-525-5p showed poor outcome as compared to those with high expression. The receiver operating characteristic (ROC) curves of miR-640 and miR-525-5p for evaluation as diagnostic markers were depicted with the area under curves (AUCs) of 0.940 and 0.886, respectively. Tumors with larger size and higher grade had lower expression of miR-640 and miR-525-5p. MiR-640, miR-525-5p and histologic grade were significant predictors of relapse-free survival (RFS). Our study validated the values of miR-640 and miR-525-5p in predicting relapse of systematically untreated and lymph node negative patients with primary breast cancer.

**Keywords:** breast cancer; lymph node negative; miR-525-5p; miR-640; relapse
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## Introduction

Breast cancer, regardless of sex, remains one of the most common cancers worldwide, and is also the first most frequently diagnosed cancer in women, accounting for 25.2% of all newly-diagnosed cancers and 14% of all cancer-related deaths (Jemal et al. 2011; Siegel et al. 2013). According to global statistics by International Agency for Research on Cancer (IARC) and World Health Organization (WHO) in 2020 (China, both sexes, all ages), the estimated number of new cases and deaths were 3,703,961 and 1,148,179, respectively (Sung et al. 2021). Generally speaking, primary breast cancer originates from breast epithelium, including lobular invasive carcinoma and ductal invasive carcinoma (Bonacho et al. 2020). Breast cancer begins with local invasion of surrounding tissues, spreads to other areas of the body such as blood or lymphatic vessels, and then ends with the spread of tumor cells to distant organs, resulting in a fatal diagnosis (Scully et al. 2012; Kim 2021). Early stage of breast cancer is usually successfully treated by surgery (Early Breast Cancer Trialists' Collaborative Group 2005; Hickey et al. 2013). Although significant breakthroughs have been made in the treatment strategy of breast cancer, the mortality of breast cancer is still high due to metastasis and recurrence (Waks and Winer 2019).

As a group of non-coding small RNA molecules, microRNAs (miRNAs) have become a promising bio-

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marker for cancer diagnosis and prognosis (Lee and Dutta 2009). MiRNAs have also been recognized as a vital role in development of breast cancer, including metastatic breast cancer and relapsed breast cancer. For instance, microRNA-21 promotes breast cancer proliferation and metastasis by targeting leucine zipper transcription factor like 1 (LZTFL1) (Wang et al. 2019). Various miRNAs such as miR-29b-3p and miR-20b-5p were found to be downregulated in breast cancer patients with recurrence (Sueta et al. 2017). Recently, studies indicated that miR-640 was significantly downregulated in hepatocellular carcinoma (Zhai et al. 2019), and miR-525-5p played a tumor inhibitory role in cervical cancer mainly by inhibiting UBE2C (ubiquitin conjugating enzyme E2 C)/ZEB1/2 (zinc finger E-box binding homeobox 1/2) signal transduction (Chen and Liu 2020). However, the role of miR-640 and miR-525-5p in breast cancer recurrence remains to be explored.

In our study, we attempted to explore diagnostic value of miR-640 and miR-525-5p in patients with primary breast cancer developing recurrence.

## Materials and Methods

*Microarray-identified microRNAs*

The Gene Expression Omnibus (GEO) datasets from platform GPL23960 were analyzed using the GEO2R, including GSE126125 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126125) and GSE103161 (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE103161). Primary breast tumors from patients with and without relapse were analyzed (n = 52) in GSE126125. Among them, 44 patients (84.62%) were breast invasive ductal carcinoma (IDC), and 41 cases (78.85%) were estrogen receptor (ER) negative. In GSE103161, the analysis of microRNA profiles was determined in primary breast cancer tumors of ER positive patients (n = 110). Both datasets included microRNA profiles of untreated and lymph node negative patients. After surgical removal, total RNA was isolated from tumor samples, and the sample labelling, hybridization, washing and scanning were then performed by miRCURY Power labeling kit, miRCURY LNA Array hybridization buffer, miRCURY LNA Array Washing buffer kit, and Agilent G2565CA Microarray scanner, respectively. Scanned images were imported into GenePixPro6.0 software (Molecular Devices, San Jose, CA, USA).

*Patient selection and tumor samples*

Freshly frozen tumor biopsies were collected from individuals (n = 180) who were diagnosed with low-risk primary breast cancer by pathological examination with no lymph node metastases and a tumor diameter ≤ 3 cm (Fohlin et al. 2020). All patients underwent surgery to remove the primary tumor, but none of the patients received systemic neoadjuvant or adjuvant therapy. The patients had informative follow-up data. Patient tissues for miR-640 and miR-525-5p expression analysis were approved by the Ethics Committee of Hunan Aerospace Hospital with no informed consent obtained from patients due to retrospective nature.

*Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)*

The total RNA was extracted from frozen tumor biopsies using the TRIzol (Catalog #: 15596026 Thermo Fisher Scientific, Shanghai, China), and the concentration was determined using a Nanodrop 2000 spectrophotometer. The cDNA synthesis was then performed using TaqMan Advanced miRNA cDNA kit (Catalog #: A28007, Thermo Fisher Scientific). qRT-PCR was performed using Applied Biosystems TaqMan MicroRNA (Catalog #: 4427975, Thermo Fisher Scientific) on QuantStudio 7 thermal cycler (Applied Biosystems, Bedford, MA, USA). The primer sequences have been provided in Table 1. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method after normalization with internal control (RNU6B).

*Target genes analysis*

The lists of validated target genes of miR-640 and miR-525-5p were obtained from 4 databases, including Targetscan (McGeary et al. 2019), miRDB (Chen and Wang 2020), microT-CDS (Paraskevopoulou et al. 2013), and miRWalk (Sticht et al. 2018). The breast cancer-associated genes were retrieved from the Genecards database (Stelzer et al. 2016). EVenn (Chen et al. 2021) was used to identify overlapped genes.

*Statistical analysis*

The Shapiro Wilk test was used to assess data normality, and one way ANOVA/student's t-test to analyze continuous variables with normal distribution [presented as mean ± standard deviation (SD)]. Fisher's exact or $\chi^2$ tests were

Table 1. The primer sequences in this study.

| | | Sequences |
|---|---|---|
| *miR-640* | Forward | 5'-CGCGATGATCCAGGAACCT-3' |
| | Reverse | 5'-AGTGCAGGGTCCGAGGTATT-3' |
| *miR-525-5p* | Forward | 5'-GCGCTCCAGAGGGATGCA-3' |
| | Reverse | 5'-AGTGCAGGGTCCGAGGTATT-3' |
| *U6* | Forward | 5'-TCACTTCCTATCGGATCGGC-3' |
| | Reverse | 5'-CTGTACCGACAAAAACACAAGC-3' |

used to examine the differences in categorical variables. The receiver operating characteristic (ROC) curve was used for investigating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy using Youden's index (Regev et al. 2016), and the area under the receiver operating characteristic curve (AUC) provided an estimate of the ability of miR-640 and miR-525-5p to discriminate the groups compared. The Kaplan-Meier method, log-rank test and multivariable Cox regression model were applied to describe the relapse-free survival (RFS) with hazard ratio (HR) and 95% confidence interval (CI), which was defined as the time after surgical treatment to the first distant recurrence, second primary tumors, or death from any cause. All *P*-values below 0.05 were considered statistically significant using GraphPad Prism Software Version 8.0 (San Diego, CA, USA).

## Results

*Analysis of microarray-identified microRNAs associated with relapse of systematically untreated and lymph node negative patients with primary breast cancer*

Firstly, we found no significant microRNAs associated with relapse in GSE103161 uploaded by Block I et al. using GEO2R (Supplementary Fig. S1). Based on the retrieved data (GEO accession GSE126125), miR-640 and miR-525-5p showed significantly different expression levels between cases with and without relapse in Fig. 1A, B according to the GEO2R. In detail, the expressions of miR-640 (t = −6.629, Log fold change = −0.463, *P* = 2.53E-05) and miR-525-5p (t = −4.837, Log fold change = −0.481, *P* = 0.004) were significantly lower in tumors from patients with relapse compared to tumors from patients without relapse (Fig. 1C). Regarding the potential of miR-640 and miR-525-5p to discriminate cases with and without relapse, the AUC of ROC curve of miR-640 and miR-525-5p were computed to be 0.928 (Sensitivity 88.46%; Specificity 88.46%; *P* < 0.001, Fig. 2A) and 0.862 (Sensitivity 92.31%; Specificity 73.08%; *P* < 0.001, Fig. 2B), respectively, validating their values in predicting relapse of systematically untreated and lymph node negative patients with primary breast cancer. Moreover, the decreased miR-640 (t = 3.845, *P* = 3.00E-04) and miR-525-5p (t = 3.845, *P* = 3.00E-04) expressions were revealed in dead patients as compared to alive patients (Fig. 2C, D).

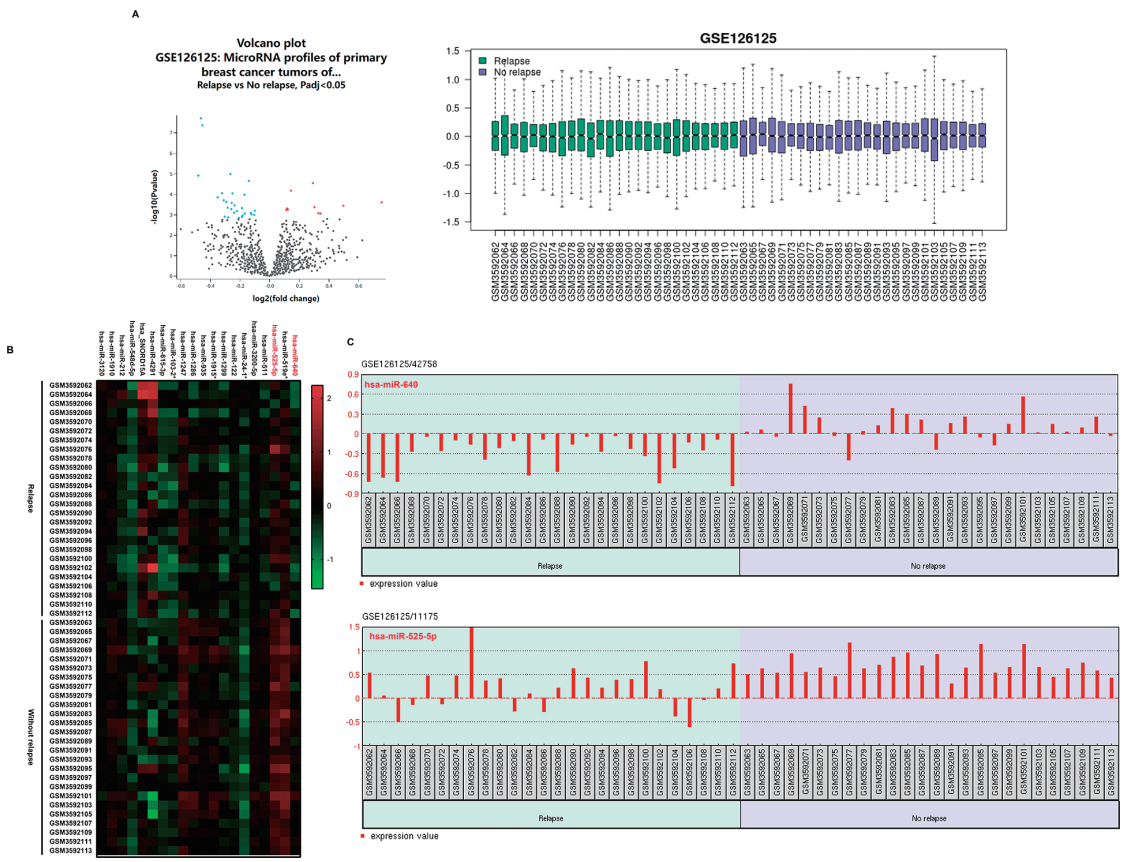


Fig. 1. Analysis of microarray-identified microRNAs associated with relapse of systematically untreated and lymph node negative patients with primary breast cancer retrieved from GEO accession GSE126125.
(A) The data uploaded by Block I et al. (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126125) (GEO accession GSE126125, Platform GPL23960) was determined by GEO2R; Volcano plots presenting the differences microRNAs, and Box plot analysis indicating the comparable defined groups. (B) Heatmap of top 20 different microRNAs in tumors from patients with and without relapse. (C) Statistical analysis in GEO2R by using the Bayes test showed expression of miR-640 and miR-525-5p.

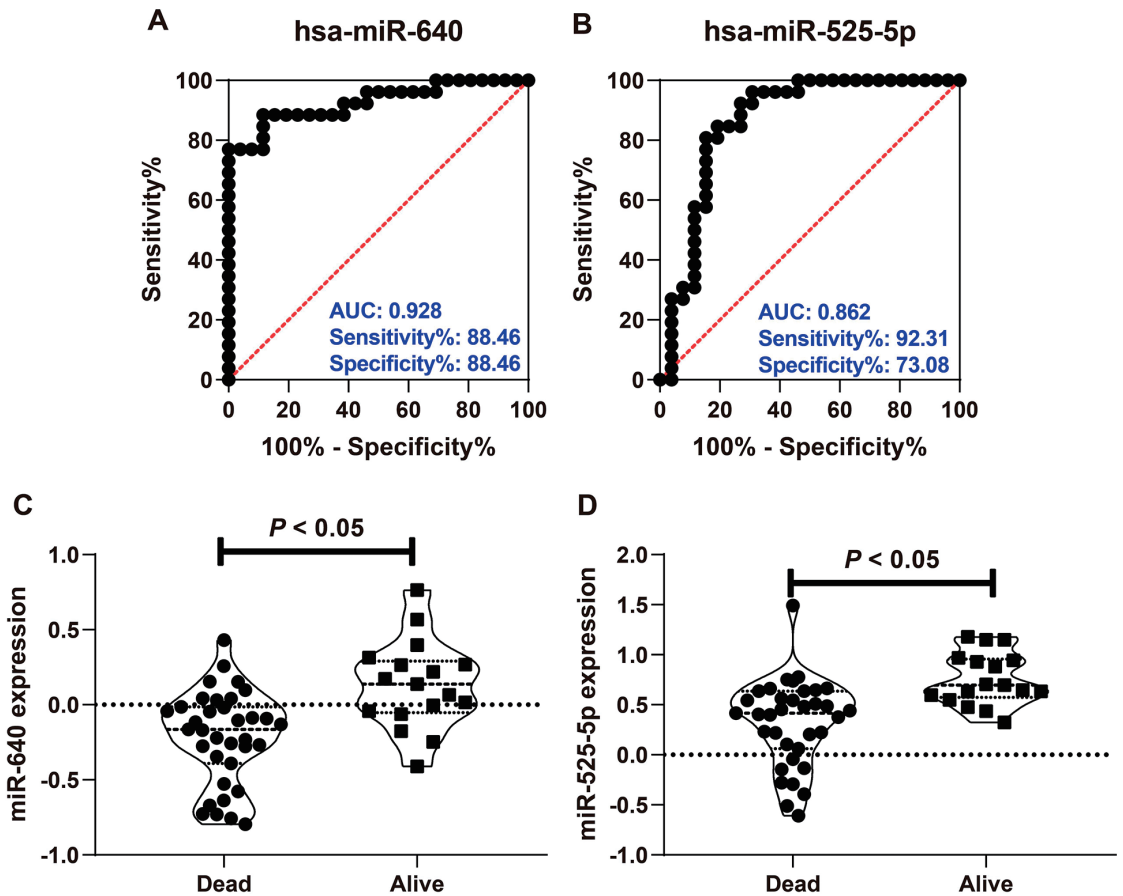


Fig. 2. The miR-640 and miR-525-5p expressions in predicting relapse of systematically untreated and lymph node negative patients with primary breast cancer, and their correlation with prognosis.
(A and B) Receiver Operating Characteristic (ROC) curve analyses of miR-640 (A) and miR-525-5p (B) to discriminate between patients with relapse and those without relapse from uploaded data by Block I et al. (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126125) (GEO accession GSE126125, Platform GPL23960). (C and D) The decreased miR-640 (C) and miR-525-5p (D) expressions were revealed in dead patients as compared to alive patients.

*Characteristics of study population in an independent validation set*

To match the patient characteristics and analysis parameters, a total of 180 patients with primary breast cancer were selected in this independent validation set. Forty-eight patients developed relapse (locoregional, n = 37; distant metastasis, n = 11) within 5 years after diagnosis, while 132 patients did not experience relapse. The characteristics of the study subjects are presented in Table 2. There were no significant differences between cases with and without relapse regarding age at diagnosis ($P$ = 0.570), tumor size ($P$ = 0.680), ER status ($P$ = 0.134), tumor type ($P$ = 0.576), and histologic grade ($P$ = 0.345).

*Validation of miR-640 and miR-525-5p expressions in primary tumors of patients who developed relapse*

We found expressions of miR-640 (0.549 ± 0.203 vs. 0.924 ± 0.106, $P$ = 1.68E-17) and miR-525-5p (0.737 ± 0.189 vs. 1.020 ± 0.122, $P$ = 5.88E-14) significantly down-regulated in primary tumors of patients who developed relapse than those who did not (Fig. 3A, B). Moreover, the ROC curves of miR-640 and miR-525-5p for evaluation as diagnostic markers were depicted in Fig. 3C, D. The AUCs for miR-640 and miR-525-5p were 0.940 (sensitivity = 85.42%, specificity = 92.42%, PPV = 80.39%, NPV = 94.57%, accuracy = 90.56%, at the optimal cutoff point of 0.7705; Fig. 3C) and 0.886 (sensitivity = 79.17%, specificity = 89.39%, PPV = 73.08%, NPV = 92.19%, accuracy = 86.67%, at the optimal cutoff point of 0.8755; Fig. 3D), respectively.

*Investigation of miR-640 and miR-525-5p associated with clinicopathological characteristics and relapse in an independent validation set*

As shown in Table 3, tumors with larger size and higher grade had lower expression of miR-640 ($P$ = 0.001 and $P$ = 1.00E-06) and miR-525-5p ($P$ = 0.005 and $P$ = 2.00E-06) in an independent validation set, respectively. There were weak positive correlations between miR-640 or miR-525-5p expression and other clinicopathological characteristics, such as age ($P$ = 0.355 and $P$ = 0.290), ER status ($P$ = 0.298 and $P$ = 0.199) and tumor type ($P$ = 0.172 and $P$ = 0.121). Furthermore, the expressions of miR-640 and miR-525-5p in breast cancer were closely associated with

Table 2. Baseline characteristics of study patients.

| | N | Relapse (n = 48) | No relapse (n = 132) | *P* |
|---|---|---|---|---|
| Age at diagnosis | | | | |
| ≤ 50 years | 47 | 14 | 33 | |
| > 50 years | 133 | 34 | 99 | 0.57 |
| Tumor size | | | | |
| ≤ 1 cm | 72 | 18 | 54 | |
| 2-3 cm | 108 | 30 | 78 | 0.68 |
| Estrogen receptor status | | | | |
| Positive | 170 | 43 | 127 | |
| Negative | 10 | 5 | 5 | 0.134 |
| Tumor type | | | | |
| Invasive ductal carcinoma | 159 | 40 | 119 | |
| Mucinous carcinoma | 9 | 3 | 6 | |
| Papillary carcinoma | 8 | 3 | 5 | |
| Carcinoma with metaplasia | 4 | 2 | 2 | 0.576 |
| Histologic grade | | | | |
| G1 | 52 | 14 | 38 | |
| G2 | 81 | 18 | 63 | |
| G3 | 47 | 16 | 31 | 0.345 |

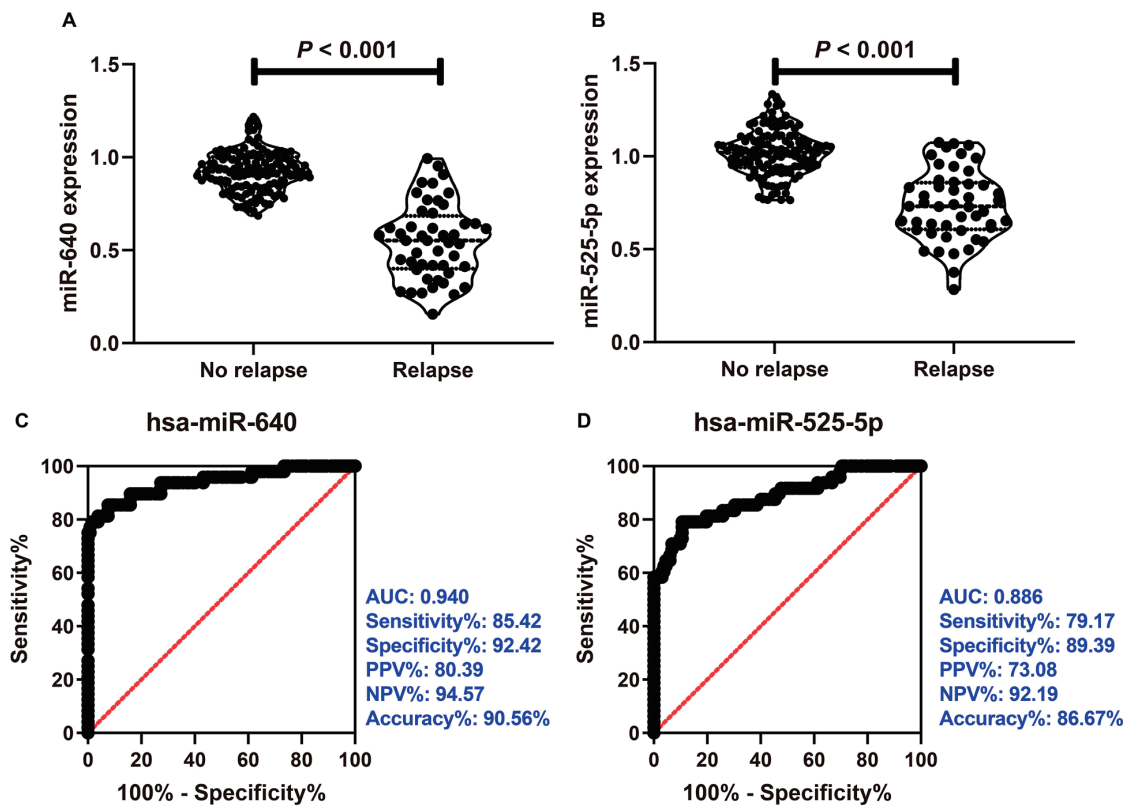


Fig. 3. Investigation of miR-640 and miR-525-5p associated with breast cancer relapse in an independent validation set. (A and B) Quantitative real time-polymerase chain reaction (qRT-PCR) validation of miR-640 and miR-525-5p in recurrent and non-recurrent breast tumors. (C and D) ROC analysis in the validation set for discriminating breast cancer cases with and without relapse. PPV, Positive predictive value; NPV, Negative predictive value.

RFS (Fig. 4). Based on the median values in breast tumors, patients with low expression of miR-640 (HR = 20.29, 95% CI: 11.45-35.96, $\chi^2 = 52.49$, $P < 0.001$) and miR-525-5p (HR = 7.45, 95% CI: 4.22-13.18, $\chi^2 = 33.91$, $P < 0.001$) showed poor outcome as compared to those with low expression. Multivariate analyses (Cox model, Table 4) identified miR-

Table 3. Investigation of miR-640 and miR-525-5p associated with clinicopathological characteristics of study patients in an independent validation set.

| | | miR-640 expression | | miR-525-5p expression | |
|---|---|---|---|---|---|
| | N | mean ± SD | *P* | mean ± SD | *P* |
| Age at diagnosis | | | | | |
| ≤ 50 years | 47 | 0.799 ± 0.236 | | 0.919 ± 0.188 | |
| > 50 years | 133 | 0.833 ± 0.209 | 0.355 | 0.953 ± 0.190 | 0.290 |
| Tumor size | | | | | |
| ≤ 1 cm | 72 | 0.887 ± 0.202 | | 0.993 ± 0.214 | |
| 2-3 cm | 108 | 0.782 ± 0.216 | 0.001 | 0.912 ± 0.165 | 0.005 |
| Estrogen receptor status | | | | | |
| Positive | 170 | 0.828 ± 0.213 | | 0.949 ± 0.180 | |
| Negative | 10 | 0.755 ± 0.263 | 0.298 | 0.869 ± 0.316 | 0.199 |
| Tumor type | | | | | |
| Invasive ductal carcinoma | 159 | 0.832 ± 0.206 | | 0.953 ± 0.180 | |
| others | 21 | 0.763 ± 0.279 | 0.172 | 0.884 ± 0.248 | 0.121 |
| Histologic grade | | | | | |
| G1 | 52 | 0.923 ± 0.157 | | 1.038 ± 0.143 | |
| G2 | 81 | 0.831 ± 0.198$^{*}$ | | 0.940 ± 0.170$^{*}$ | |
| G3 | 47 | 0.703 ± 0.245$^{*,\#}$ | 1.00E-06 | 0.849 ± 0.219$^{*,\#}$ | 2.00E-06 |

$^{*}P < 0.05$, as compared to G1; $^{\#}P < 0.05$, as compared to G2.

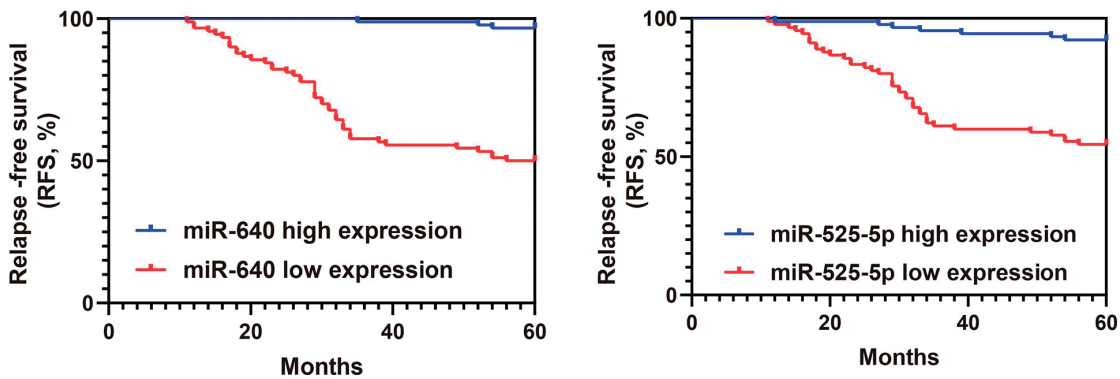


Fig. 4. Kaplan-Meier estimates of relapse-free survival (RFS) for systemically untreated patients with differential expressions of miR-640 and miR-525-5p.

640 (HR = 0.005, 95% CI: 0.001~0.028, *P* = 6.92E-10) and miR-525-5p (HR = 0.022, 95% CI: 0.003~0.184, *P* = 4.40E-04) were significant predictors of better RFS, while histologic grade was a significant predictor of worse RFS (G2 vs. G1: HR = 2.791, 95% CI: 1.104~7.055, *P* = 0.030).

*Potential target genes of miR-640 and miR-525-5p*

Online databases (Targetscan, miRDB, microT-CDS, and miRWalk) were used to predict the potential targets of miRNAs (miR-640 and miR-525-5p). Meanwhile, we also downloaded the breast cancer-related genes from the Genecards database. As illustrated in Supplementary Fig. S2, the results showed 181 targets of miR-525-5p and 67 targets of miR-640 using Venn diagram. Moreover, two overlapped genes, including transmembrane protein 33 (TMEM33) and protein tyrosine phosphatase non-receptor type (PTPN14), were found to be targeted by both miR-640 and miR-525-5p.

## Discussion

In present study, we performed analysis of GEO dataset GSE126125, and identified that expressions of miR-640 and miR-525-5p were significantly lower in tumors from patients with relapse compared to tumors from patients without relapse, accompanying by highly predictive potential to discriminate cases with and without relapse. Moreover, the decreased miR-640 and miR-525-5p expressions were revealed in dead patients as compared to alive patients. Subsequently, this study collected a total of 180 patients with primary breast cancer, of which 48 patients developed relapse. Both miR-640 and miR-525-5p expressions were closely associated with tumor size, histologic

Table 4. Results of multivariate Cox proportional hazards model for patient prognosis.

| | B | SE | Wald | *P* | Hazard Ratio (HR) | 95% CI |
|---|---|---|---|---|---|---|
| miR-640 expression | −5.218 | 0.846 | 38.044 | 6.92E-10 | 0.005 | 0.001~0.028 |
| miR-525-5p expression | −3.832 | 1.090 | 12.356 | 4.40E-04 | 0.022 | 0.003~0.184 |
| Age (> 50 years vs. < 50 years) | −0.375 | 0.356 | 1.109 | 0.292 | 0.687 | 0.342~1.381 |
| Tumor size (2-3 cm vs. < 1 cm) | 0.468 | 0.368 | 1.618 | 0.203 | 1.597 | 0.776~3.283 |
| Estrogen receptor status (positive vs. negative) | −0.123 | 0.540 | 0.052 | 0.820 | 0.884 | 0.307~2.549 |
| Tumor type (Invasive ductal carcinoma vs. others) | −0.263 | 0.477 | 0.304 | 0.581 | 0.769 | 0.302~1.957 |
| Histologic grade | | | | | | |
| G2 vs. G1 | 1.026 | 0.473 | 4.706 | 0.030 | 2.791 | 1.104~7.055 |
| G3 vs. G1 | −0.095 | 0.378 | 0.064 | 0.801 | 0.909 | 0.433~1.908 |

SE, standard error; CI, confidence interval.

grade, and RFS. Besides, these two miRNAs had high diagnostic value for cancer recurrence in breast cancer patients without systemic neoadjuvant or adjuvant therapy.

In recent years, miR-640 has been investigated in several diseases, including acute liver injury (Wang et al. 2020), intervertebral disc degeneration (Dong et al. 2019; Sherafatian et al. 2019), and tumors (Sun and Yang 2022; Zheng et al. 2022). Besides, miR-640 in bile were significantly different between patients with primary sclerosing cholangitis (PSC) and cholangiocarcinoma complicating PSC (Voigtlander et al. 2015). In addition, miR-640 significantly inhibited angiotensin I-induced cell migration and tube formation via directly targeting Zinc Finger Protein 91 (ZFP91) (Harel et al. 2020). In our study, higher miR-640 showed better RFS in patients with breast cancer, being similar with a previous study (Li et al. 2013). Wnt activation has been observed in breast malignancies and contributes to tumor recurrence (Krishnamurthy and Kurzrock 2018). As reported, the miR-640-SLIT1 axis that regulates the Wnt/$\beta$-catenin signaling pathway might be possible therapeutic option for the effective treatment of glioma in combination with radiotherapy (Zheng et al. 2022). Worth mentioning, miR-640 could suppress breast cancer cells tumorigenesis via inhibition of Wnt/$\beta$-catenin signaling pathway (Tang et al. 2021). Furthermore, tumor hypoxia greatly influenced breast cancer progression, which was generally linked to poor prognosis in breast cancer patients (Harrison et al. 2018). The main mediator in poorly oxygenated areas is hypoxia inducible factor 1-alpha (HIF1-$\alpha$), a predictor of tumor recurrence and survival (Simon et al. 2010; Harrison et al. 2018), which could be inhibited by miR-640 through binding to its 3'-UTR (Zhou et al. 2016). All mentioned above suggested miR-640 may suppress tumor recurrence by targeting genes (e.g., HIF1-$\alpha$) or regulating signaling pathway (e.g., Wnt/$\beta$-catenin).

Much fewer studies have been conducted on miR-525-5p (located in a copy number amplified region 19q13.42) in human malignancies, which seems to have oncogenic properties in laryngeal squamous cell carcinoma (Cybula et al. 2016). Being similar with our result, miR-525-5p expression level was significantly lower in tumor tissues from patients with non-small cell lung cancer when compared to normal tissues, showing a tumor-suppressor role in the proliferation and migration of non-small cell lung cancer (NSCLC) via negatively regulating fibroblast growth factor 11 (FGF11) (Wu et al. 2021). The function of miR-525-5p was also identified in human brain tumor and its overexpression contributed to delaying the growth of glioma cells (Xie et al. 2020). Moreover, miR-525-5p could be sponged by circ_0000423 to suppress colorectal cancer cell progression, which could be overturned by SGPP1 (Sphingosine-1-phosphate phosphatase 1) silencing (Kang et al. 2022). As revealed by Xiao et al. (2021), miR-525-5p inhibited breast cancer cell proliferation with the facilitated cell apoptosis via targeting MEIS2 (Meis homeobox 2) and affecting Wnt pathway. Furthermore, as a potential treatment target for breast cancer, miR-525-5p was associated with a good prognosis (Yu et al. 2022), being consistent with our study.

According to miRNA prediction target algorithms both miR-640 and miR-525-5p have the same potential target genes (PTPN14 and TMEM33). PTPN14 (also known as Pez, PTP36, or PTPD2) regulated cell-cell and cell-matrix adhesion, cell migration, and growth (Zhang et al. 2013), which has been indicated as candidate tumor suppressor in breast cancer. For example, Belle et al. (2015) found knocking down PTPN14 in tumor cells promoted the growth and metastasis of breast cancer xenografts by enhancing the trafficking of both soluble and membrane-bound proteins. Besides, overexpression of PTPN14 suppressed the cell migration and invasion induced by caveolin-1 in breast cancer cell line (Diaz-Valdivia et al. 2020). TMEM33 (Transmembrane protein 33), a stress-inducible endoplasmic reticulum transmembrane protein, was reported to show dysregulated expressions in cancers (Liu et al. 2021; Chen et al. 2022). A previous study by Sakabe et al. (2015) revealed high TMEM33 mRNA expression in endocrine-resistant breast cancer cells as compared with their matched sensitive control cells, and they also found that early recurrent breast cancer specimens showed high TMEM33 expression when compared with non-recurrent breast tumors, suggesting that TMEM33 might play a role

in tumorigenesis of breast cancer. As we all known, miRNAs negatively regulate gene expression at the post-transcriptional level (Petri et al. 2014). In our study, miR-640 and miR-525-5p expressions were significantly lower in relapse tumors, suggesting their roles of tumor suppressor miRNAs with therapeutic potential. Therefore, further mechanism experiments would be performed to find the regulation of miR-640 and miR-525-5p in breast cancer via targeting oncogene TMEM33. Besides, miRNAs were revealed to be easily isolated from different liquid biopsies with low invasiveness (e.g., whole blood, plasma, serum), which has been used in the detection of next-generation analytes in oncology (Finotti et al. 2018). However, circulating levels of miR-640 and miR-525-5p from breast cancer patients were not assessed due to time and funding constraints. Finally, we need to collect larger clinical samples to refine our findings.

In summary, this study approved that miR-640 and miR-525-5p were downregulated in primary breast cancer patients with recurrence, and might be able to potential biomarkers for recurrent primary breast cancer diagnosis.

## Conflict of Interest

The author declares no conflict of interest

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## Supplementary Files

Please find supplementary file(s);
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