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High expression of long non-coding RNA *MALAT1* in breast cancer is associated with poor relapse-free survival

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Abstract

Purpose—Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) has been identified as a prognostic marker for the metastasis of early-stage non-small cell lung cancer (NSCLCs). We studied *MALAT1* expression in breast cancer in relation to disease features and patient survival.

Methods—Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to measure *MALAT1* expression in tumor samples of 509 breast cancer patients. Hazards ratios (HRs) and 95% confidence intervals (CIs) were calculated to assess the association between *MALAT1* expression and breast cancer survival using the Cox proportional hazards regression model, and the analysis was adjusted for age at surgery, tumor grade, disease stage, and hormone receptor status. Meta-analysis of multiple microarray datasets from online databases and our own study was performed to evaluate the association of *MALAT1* with breast cancer survival.

Results—Patients with low grade or ER-positive tumors had higher expression of *MALAT1* compared to those with high grade (p=0.013) or ER negative (p=0.0002) tumors. Patients with PR-positive tumors also had higher *MALAT1* expression than those with PR-negative tumors (p<0.0001). In patients with positive hormone receptors or low tumor grade, tumors with high

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None

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The study involved human participants, but not animals

Informed consent
Study participants in our study provided informed consents

MALAT1 expression were more likely to recur. Survival analysis showed that patients with high expression of *MALAT1* had a 2-fold increase in risk of relapse (p=0.0083) compared to those with low expression. This association remained significant after adjustment for age at surgery, disease stage, tumor grade and hormone receptor status. Meta-analysis showed that high *MALAT1* expression was associated with poor relapse-free survival in patients with hormone receptor positive tumors (HR=1.44, 95%CI=1.08-1.92).

Conclusions—High expression of lncRNA *MALAT1* is associated with breast cancer relapse and may play a role in tumor progression.

Keywords

MALAT1; Breast Cancer; Estrogen Receptor; Survival; Meta-analysis

Introduction

Breast cancer is the most common female malignancy in the US, accounting for nearly 30% of all new cancer diagnoses in women [1, 2]. Despite extensive research, the tumorigenic process of the breast remains elusive, and effective therapies available for patients with metastatic diseases are limited. More studies are needed to identify and characterize new genes and their products which promote tumor progression and metastasis. This knowledge will not only improve our understanding of the disease, but also help to develop new biomarkers or targets for disease prognosis and treatment. Breast cancer research in the past has focused largely on proteins and protein-coding genes, with limited attention to non-coding genes and their transcripts. Protein-coding genes account only for 2% of the human genome. If we consider that cancer is a genetic disease, it is conceivable that we are overlooking a large part of the genome which may be involved in the disease.

More than 75% of the human genome are transcribed, but a majority of the transcripts do not encode proteins, known as non-coding RNAs (ncRNAs) [3]. Studies have shown that dysregulated ncRNAs, including long non-coding RNAs (lncRNAs), play a crucial role in tumorigenesis and in determination of malignant phenotypes [4]. LncRNAs are defined as non-coding RNAs with more than 200 nucleotides in length and no potential for protein translation. These transcripts are found to regulate important biologic processes, such as development, differentiation and transformation [5–9], as well as to interact with other functional molecules, including DNA, proteins and microRNAs [10]. Evidence also suggests that many lncRNAs have tissue-specific expression and cell-specific function [7]. Subcellular localization also determines the function of lncRNA, as many lncRNAs are localized preferentially in the nucleus, regulating gene expression, like X inactive specific transcript (*XIST*), BMP2-OP1-responsive gene (*BORG*), and nuclear enriched abundant transcript 1 (*NEAT1*). LncRNAs are found frequently to be dysregulated in human cancer, and the dysregulation is associated with disease recurrence, metastasis and prognosis [11–13].

Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), also known as noncoding nuclear-enriched abundant transcript 2 (*NEAT2*), is a lncRNA located on chr11q13 with more than 8.0 kb in length. *MALAT1* was initially found to be a prognostic

marker for the metastasis of early-stage non-small cell lung cancer (NSCLCs) [11]. *MALAT1* is localized to nuclear speckles, and regulates messenger RNA splicing by interaction with SR proteins and splicing factors [14–16]. *MALAT1* expression is upregulated in several solid tumors, including the breast, pancreas, lung, colon, prostate and liver [11], and is suspected to be involved in tumorigenesis or disease progression [17–20]. In the present study, we analyzed *MALAT1* expression in breast cancer and investigated its associations with clinical and pathological features and patient survival.

Material and methods

Study patients

Patients diagnosed with primary breast cancer were recruited from two hospitals affiliated with the University of Torino, the University Hospital between January 1998 and July 1999 and Mauriziano Hospital between October 1996 and August 2012. Tumor samples were collected from 509 consented patients during surgical resection of their primary cancer. Most of the patients were followed after surgery for a median of 82.8 months, ranging from 1.6 to 196.4 months. Of these patients, 433 (85%) had information on relapse-free and overall survival. Patient clinical and pathological data were extracted from their medical records. The study was approved by the Ethic Review Committee at each hospital.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from fresh frozen breast tumor samples (~30 mg) using the Allprep DNA/RNA Kit (Qiagen). About 1 μg total RNA was reverse-transcribed into cDNA using the Reverse Transcription Kit from LifeTech. The cDNA was amplified for a specific region of *MALAT1* after mixing with the SYBR Green Master Mix (LifeTech) and a pair of primers specific for *MALAT1. GAPDH* was used as reference. The PCR primers were designed using the sequence NR_002819.2 and synthesized by Integrated DNA Technologies. The primer sequences are: *MALAT1* forward 5'-GTTCTGATCCCGCTGCTATT-3' and reverse 5'-TCCTCAACACTCAGCCTTTATC-3' and *GAPDH* forward 5'-GTCAAGGCTGAGAACGGGAA-3' and reverse 5'-AAATGAGCCCCAGCCTTCTC-3'. PCR was run in the Roche LightCycler 480 system as previously described [12, 13]. Levels of *MALAT1* expression were calculated as an expression index (EI) using the formula $1{,}000 \times 2^{(-\Delta Ct)}$, where ΔCt = Ct (*MALAT1*) − Ct (*GAPDH*). Each sample was analyzed in triplicate. Tests with poor replicates (coefficient of variation >15%) were repeated.

Meta-analysis of *MALAT1* data

Datasets containing *MALAT1* expression in breast cancer were extracted from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds). All the data were generated from the Affymetrix Human Genome U133 plus 2.0 array and U133A array. Ten datasets were identified with at least 50 patients in each, including GSE1456, GSE4922, GSE6532, GSE19615, GSE20711, GSE20685, GSE16446, GSE31448, GSE3494 and GSE42568. Expression data on probe 224567_x_at (which targets *MALAT1*) were used for meta-analysis. Breast cancer provisional data in The Cancer Genome Atlas (TCGA) were retrieved using the web-based tool cBioPortal (http://www.cbioportal.org/index.do) [21, 22].

Using the study-specific tertile distribution as cutoff, *MALAT1* expression data were grouped into 3 categories named as "*MALAT1*_high" (*MALAT1* expression >high tertile cutoff), "*MALAT1*_mid" (low tertile cutoff <= *MALAT1* expression <high tertile cutoff), and "*MALAT1*_low" (*MALAT1* expression <=low tertile cutoff). For the Cox proportional hazards regression analysis, *MALAT1*_ high was compared with *MALAT1*_low across the studies. In meta-analysis, pooled risk ratio (hazards ratio) and 95% confidence interval were calculated using the random-effect model (the DerSimonian and Laird method) which considers both within- and between-study variation [16, 23]. Review Manager (RevMan 5.3, Cochrane Collaboration) was used for meta-analysis.

Statistical analysis

Differences of *MALAT1* expression classified by its tertile distribution (high, mid, and low) in relation to patient's clinical and pathological parameters, including age at surgery, disease stage, tumor grade, status of estrogen receptor (ER) and progesterone receptor (PR) were analyzed using the Chi-square test. For survival analysis, hazards ratios (HRs) and 95% confidence intervals (CIs) were calculated using the Cox proportional hazards regression model with and without adjustment for age at surgery, tumor grade, disease stage, ER, and PR status. Overall survival (OS) was defined as the time interval from the date of surgery to the date of death or last follow-up. Relapse-free survival (RFS) was the time interval from surgery to recurrence or last follow-up. Statistical Analysis System software (version 9.4, SAS Institute) was used for all the statistical analyses. *P* values <0.05 (two tailed) were considered significant.

Results

Table 1 shows *MALAT1* expression in association with clinical and pathological characteristics of breast cancer patients in our study. *MALAT1* expression was higher in low than in high grade tumors (p=0.013) and in ER positive than ER negative tumors (p=0.0002). Patients with PR-positive tumors also had higher *MALAT1* expression than those with PR-negative tumors (p<0.0001). However, compared to those without relapse, patients with relapse had higher expression of *MALAT1* even though they had low grade tumors or ER positive diseases (data not shown). No significant differences were found in *MALAT1* expression by age at surgery or disease stage.

Survival analysis showed that patients with high expression of *MALAT1* had a 2-fold increase in risk of relapse (HR=2.02, p=0.0083, Table 2) compared to those with low expression, and the survival curves were significantly different in patients with low, mid and high expression of *MALAT1* (p=0.0074, Figure 1A). The association with relapse-free survival remained significant after patient age at surgery, tumor grade, disease stage, ER and PR status were adjusted in the analysis (HR=3.04, p=0.0001, Table 2). A linear correlation was also significant between *MALAT1* expression and risk of relapse (HR=1.73, p<0.0001 after adjustment, Table 2). *MALAT1* expression was not associated with overall survival in our univariate analysis (Table 2, Figure 1B), but the association became significant after we adjusted for the clinical and pathological variables. Patients with high expression of *MALAT1* had significantly increased risk of death compared to those with low expression

(HR=2.51, p=0.0050, Table 2), and the increase in risk was dose-dependent (p=0.0046, Table 2).

Since high *MALAT1* expression was associated with low tumor grade and hormone receptor-positive tumors, we performed additional survival analysis in subgroups of patients who were stratified by tumor grade (Grade 1 or 2 versus 3) or hormone receptor status (ER or PR positive versus ER and PR negative). Results of the stratified analyses are shown in Table 3. The association between high *MALAT1* expression and poor relapse-free survival was observed consistently in two subgroups of patients classified by tumor grades, and the strength of the association appeared to be stronger in grade 1 and 2 tumors compared to grade 3 tumors. For hormone receptor status, the association was seen only in hormone receptor positive tumors; no significant association was found in hormone receptor negative tumors. For patients with low grade and hormone receptor positive tumors, *MALAT1* had a significant influence on relapse-free survival; patients with high *MALAT1* had higher risk for relapse than those with low *MALAT1.*

Meta-analysis of the association between *MALAT1* expression and breast cancer survival was performed on 8 GEO datasets plus the TCGA breast cancer provisional data and our study. A total of 10 datasets with 2,958 patients had information on relapse-free survival, and 7 studies with 2,225 patients had overall survival data. The summarized results showed slightly increased risks of relapse and death for patients with high expression of *MALAT1,* but none of the associations were statistically significant (relapse-free survival HR=1.19, 95%CI: 0.92-1.55, Figure 2A; overall survival HR=1.16, 95%CI: 0.92-1.45, Figure 2B). However, when survival analysis was performed only in ER positive patients, *MALAT1* expression was significantly associated with relapse-free survival. ER positive patients with high *MALAT1* had a 44% higher risk (95% CI: 1.08-1.92) for relapse compared to ER positive patients with low expression (Figure 3A). The association for overall survival was not significant (Figure 3B).

A CpG island was indicated in the genomic database annotated using the UCSC genome browser in an ER-positive cell line (MCF-7 cells) (Figure 4A). The relationship between *MALAT1* expression and DNA methylation of the gene was evaluated after we merged the expression and methylation data available from TCGA where 778 breast cancer patients had gene expression data by RNA sequencing and DNA methylation data by the HumanMethylation450 chip. There were 10 methylation probes designed for the CpG sites in the promoter region of *MALAT1*, and 5 of them were in the CpG island. Overall, methylation levels in these CpG sites were very low, less than 0.1, and a weak inverse correlation was suggested between methylation and expression of *MALAT1* (Spearman correlation coefficient=−0.122, p=0.00070, Figure 4B).

Discussion

In the study, we found that *MALAT1* levels in breast tumors were associated with breast cancer relapse and the association was independent of the established prognostic indicators, such as tumor grade, disease stage, and hormone receptor status. After adjustment for covariates, patients with high *MALAT1* expression had a 3-fold increase in risk of relapse

compared to those with low expression, and the increase in risk was significantly correlated with the level of expression. It was interesting to note that *MALAT1* expression did not seem to be associated with overall survival in univariate analysis, but after adjusting for tumor grade, disease stage and receptor status, a significant association was detected. Patients with high *MALAT1* had increased risk for death, which was consistent with the association for relapse. A significant association found after adjustment was quite unusual, suggesting that some covariates in the multivariate analysis may confound the relationship of *MALAT1* and overall survival. Among the adjusted variables, we knew that tumor grade and hormone receptor status were associated with *MALAT1* expression, and their associations with *MALAT1* were in an opposing direction as to the association of *MALAT1* with overall survival, i.e., high *MALAT1* expression associated with poor survival, but meanwhile low grade or hormone receptor positive tumors having high expression of *MALAT1*. To address the confounding effect, we evaluated the survival associations in subgroups of patients stratified by tumor grade or hormone receptor status, and the subgroup analyses showed that the *MALAT1*'s associations with survival were more evident in patients with favorable prognostic indicators (low or mid tumor grades or ER or PR positive tumors) than in those with unfavorable indicators (high tumor grade or ER and PR negative tumors). The observation suggests that *MALAT1* may have prognostic values only for certain patients. This limited value in prognosis may also explain why we did not find a consistent association between *MALAT1* and survival in our meta-analysis when we did not stratify the patients by their tumor grade or hormone receptor status. To test this possibility, we performed meta-analysis only on ER-positive patients, and the analysis showed that *MALAT1* expression was significantly associated with relapse-free survival, suggesting that *MALAT1* may have prognostic values mainly in patients with hormone receptor positive tumors.

We also compared the median values of *MALAT1* expression between patients with and without relapse in groups classified by tumor grade or ER status. In each group of comparisons, *MALAT1* levels were higher in patients with relapse than in those without relapse, suggesting that *MALAT1* may provide additional value of prognosis for patients who were initially considered to have good prognosis. Information on adjuvant chemo or hormonal therapy was available for 300 patients. We analyzed the treatment data with regard to the relationship between *MALAT1* expression and treatment response. Our analysis showed that *MALAT1* levels were not different between patients who responded and who did not respond, but for those who responded to adjuvant treatment low *MALAT1* rendered better relapse-free survival compared to high *MALAT1*. These results were consistent with the earlier observations that high levels of *MALAT1* in breast tumors were related to poor disease outcomes and that these relationships existed largely in patients who had a less aggressive disease or responded to adjuvant treatment.

Although *MALAT1* is one of the lncRNAs discovered first in cancer, our understanding of the lncRNA's functions is still limited. The *MALAT1* gene is highly conserved across mammals, suggesting that it may have important biological implications [9]. Evidence suggests that *MALAT1* may act like an oncogene in several malignancies, including lung cancer [17, 24], pancreatic cancer [19], liver cancer [20], bladder cancer [25], prostate cancer[26], colon cancer [27, 28], renal cell carcinoma [18], and oral squamous cell

carcinomas [29, 30]. Lowering *MALAT1* expression in hepatocellular carcinoma cells resulted in reduced cell proliferation and colony formation [20]. *MALAT1* was also found to promote colon cancer cell proliferation, invasion and metastasis *in vitro* and *in vivo* by increasing AKAP-9 expression [28]. For breast cancer, suppressing *MALAT1* expression led to increased differentiation of primary tumor cells and reduced metastasis [31]. Huang et al. reported that *MALAT1* expression was elevated in breast tumors compared to adjacent normal tissues and high expression was associated with ER or PR positive tumors as well as poor disease-free survival, findings very similar to our observations in the current study [32].

Many lncRNAs dysregulated in human cancer were involved in the signal pathways of oncogenes or tumor suppressor genes [33]. For example, human maternally expressed gene 3 (*MEG3*) could inhibit cell proliferation through both p53-dependent and p53-independent pathways [34]. *MALAT1* was found to be involved in the epithelial-mesenchymal transition (EMT)-mediated metastasis in oral squamous cell carcinoma by modulating the activation of β-catenin and NF-κB pathways [29]. *MALAT1* could also regulate the Wnt-β-catenin pathway by enhancing nuclear β-catenin levels and promoting c-Myc expression [18, 31]. Furthermore, *MALAT1* was reported to activate the mammalian target of rapamycin (mTOR) signaling pathway by regulating the alternative splicing of S6K1 [19]. *MALAT1* regulated cell proliferation and metastasis in gallbladder carcinoma through activating the MAPK-ERK signaling pathway [35].

Using the TCGA data and Ingenuity Pathway Analysis (IPA), we performed pathway enrichment analysis based on the genes correlated with *MALAT1* expression. The results of our analysis suggest that an active glycolytic pathway may be involved in the function of *MALAT1* in breast cancer. Targeting tumor cell metabolisms and their key regulatory enzymes has emerged as an alternative strategy to complement the conventional genotoxic stress-based cancer therapy [36–38]. Studies have shown that cancer cells rely on glycolysis for energy production, whereas normal cells depend on the oxidative pathway [39, 40]. Highly proliferating tumor cells display an altered, high glycolytic metabolism called the Warburg effect [40, 41]. To date, a few studies have reported the role of lncRNAs in regulation of cancer metabolism. Prostate cancer gene expression marker 1 (*PCGEM1*) which is an androgen-induced prostate-specific lncRNA could increase cancer cell proliferation by enhancing aerobic glycolysis [42]. Li et al. reported that lncRNA urothelial cancer-associated 1 (*UCA1*) promoted glycolysis by upregulating hexokinase 2 (HK2) in bladder cancer cells [43]. Yang et al. found that hypoxia-induced *LincRNA-p21* was critical for hypoxia-enhanced glycolysis [44]. Another study indicated that colorectal neoplasia differentially expressed (*CRNDE*) nuclear transcripts upregulated in colorectal cancer were involved in the regulation of cell metabolism [45]. Zhao et al. reported that *LINC00092* promoted metastasis by altering glycolysis and sustaining the local supportive function of cancer-associated fibroblasts (CAFs) through binding to glycolytic enzyme-the fructose-2,6-biphosphatase PFKFB2 [46]. Knockdown lncRNA ceruloplasmin (NRCP) could significantly decrease glycolysis and increases mitochondrial respiration in ovarian cancer cells [47]. These studies suggest that lncRNAs may play an important role in cancer cell metabolism and targeting lncRNAs or their regulated metabolisms may offer new therapeutic applications.

DNA methylation is one of the epigenetic mechanisms that regulate gene expression in mammals [48]. Growing evidence demonstrates that DNA methylation in the promoter of a lncRNA gene can regulate its expression [49–52]. A CpG island was indicated in the *MALAT1* promoter. To estimate if the CpG island plays a role in epigenetic regulation of *MALAT1*, we merged the DNA methylation and gene expression data from two breast cancer datasets in TCGA, and evaluated if *MALAT1* expression was correlated with promoter methylation in breast cancer. Our analysis suggested a possible inverse correlation between *MALAT1* methylation and expression, but the relationship was very weak, probably due to low methylation in the *MALAT1* promoter in breast tumor tissues. Given the low level of methylation, we consider that DNA methylation may not play a role in regulation of *MALAT1* expression in breast cancer.

In summary, we found that *MALAT1* expression in breast cancer was paradoxically associated with tumor grade, ER/PR status and patient survival. While low grade or hormone receptor positive tumors were more likely to express *MALAT1*, high expression was associated with poor relapse-free survival. *MALAT1* levels in breast tumors are indicative of breast cancer prognosis, but the prognostic value may be limited to patients with ER positive tumors.

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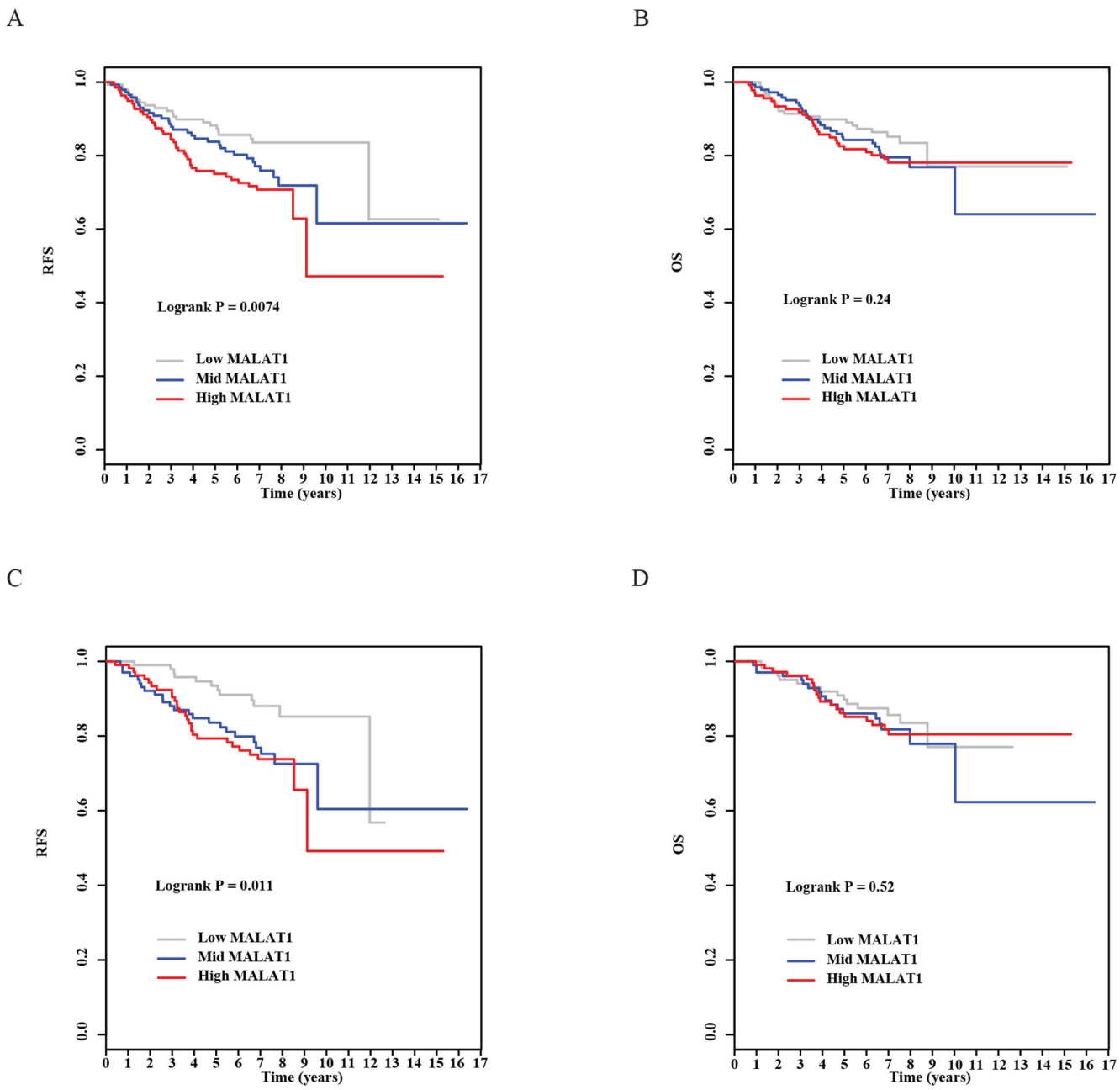
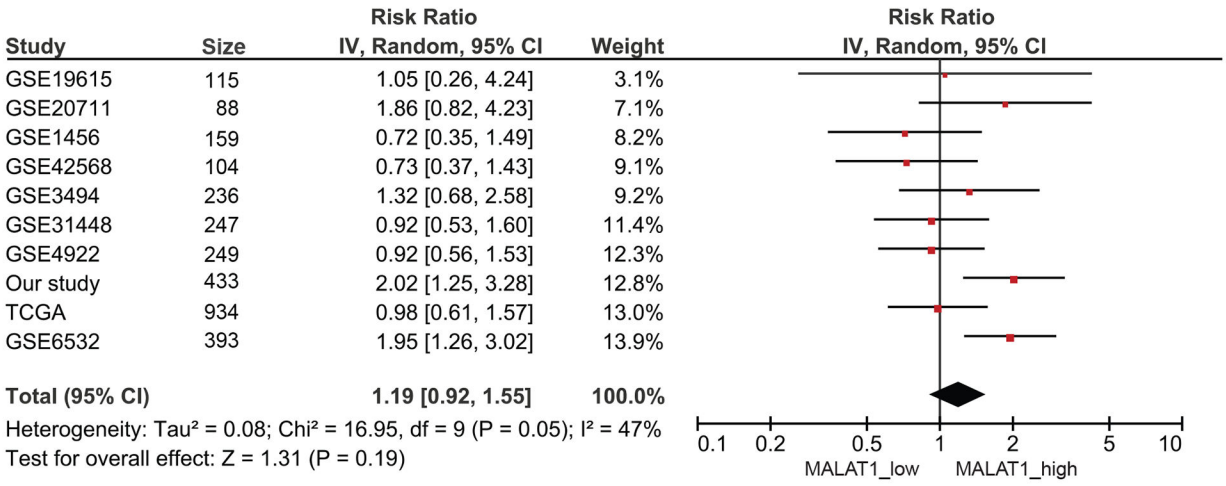


Figure 1. Associations of *MALAT1* expression with patient survival.

A) Kaplan-Meier estimates for relapse-free survival by high, mid, and low *MALAT1* expression. **B)** Kaplan-Meier estimates for overall survival by high, mid, and low *MALAT1* expression. **C)** Kaplan-Meier estimates for relapse-free survival by high, mid, and low *MALAT1* expression in hormone receptor positive patients. **D)** Kaplan-Meier estimates for overall survival by high, mid, and low *MALAT1* expression in hormone receptor positive patients.

A



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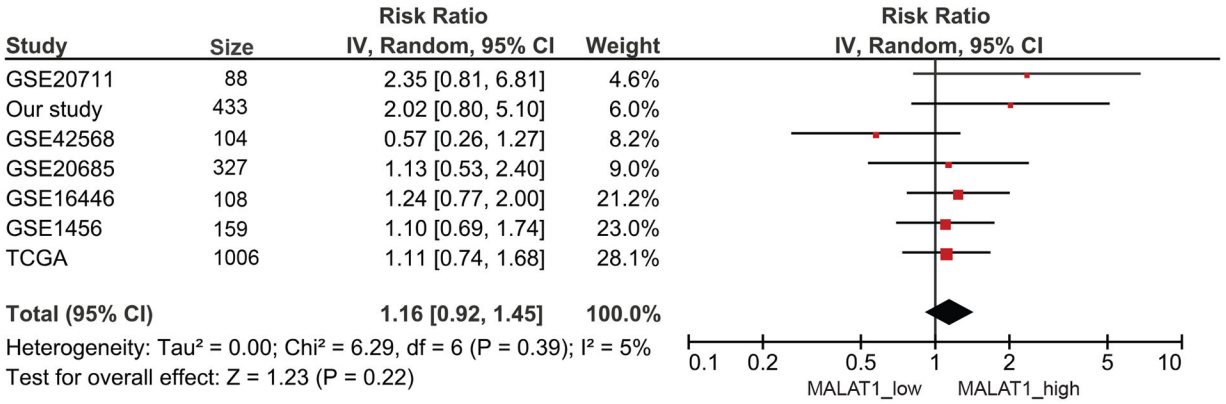
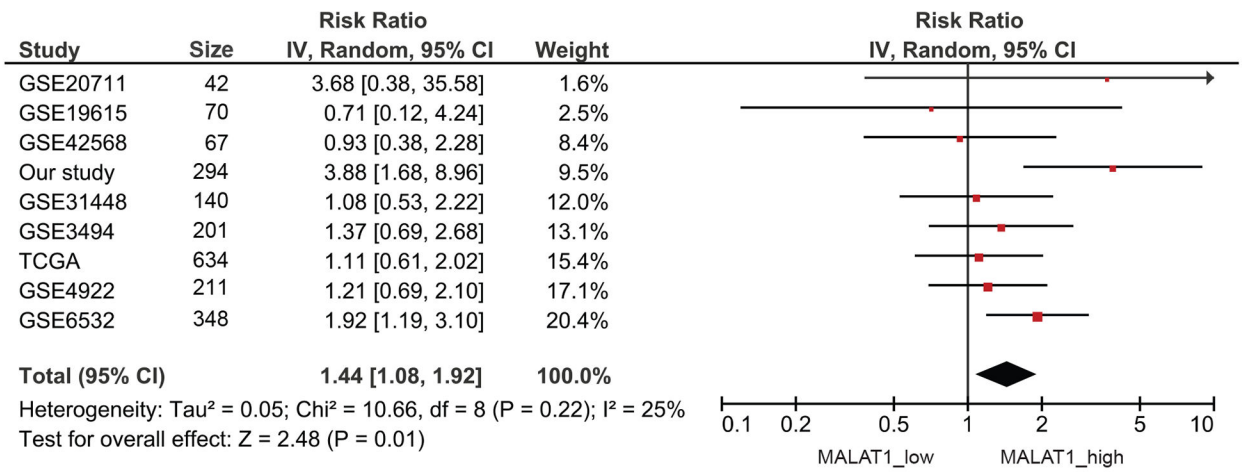


Figure 2. Meta-analysis of the association between *MALAT1* expression and breast cancer survival.

A) Meta-analysis of the association between *MALAT1* expression and relapse-free survival.

B) Meta-analysis of the association between *MALAT1* expression and overall survival.

A



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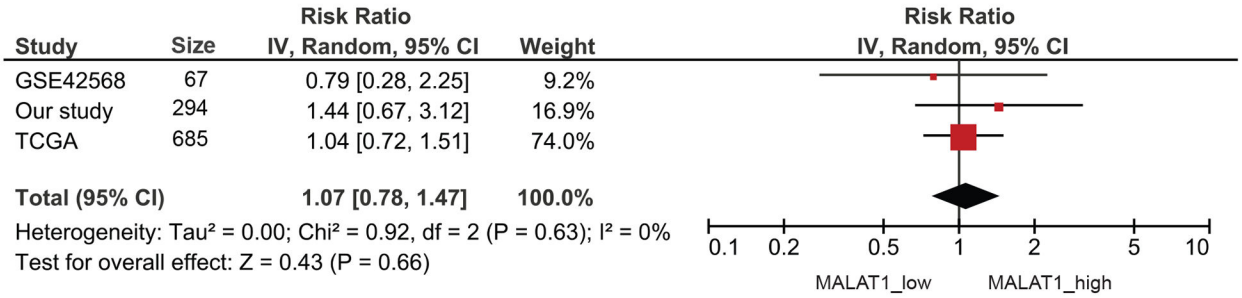
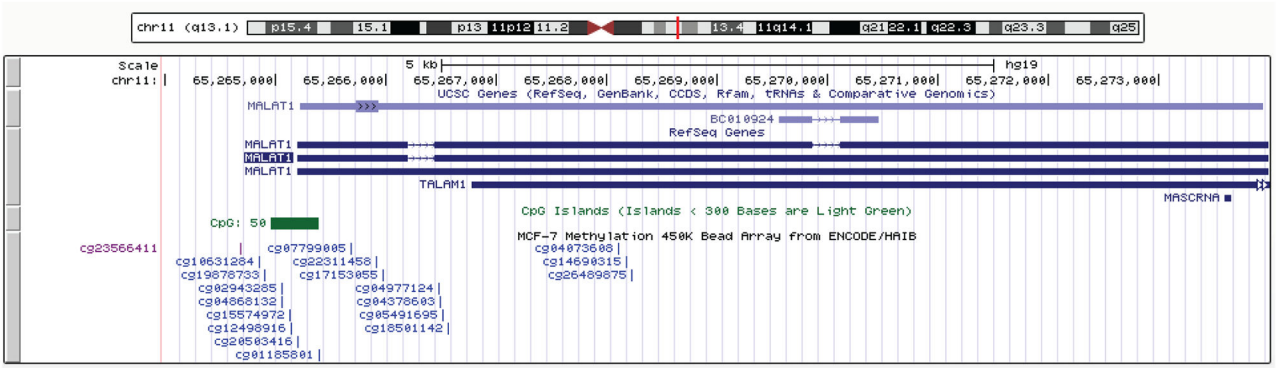


Figure 3. Meta-analysis of the association between *MALAT1* expression and breast cancer survival in ER-positive patients.

A) Meta-analysis of the association between *MALAT1* expression and relapse-free survival in ER-positive patients. B) Meta-analysis of the association between *MALAT1* expression and overall survival in ER-positive patients.

A



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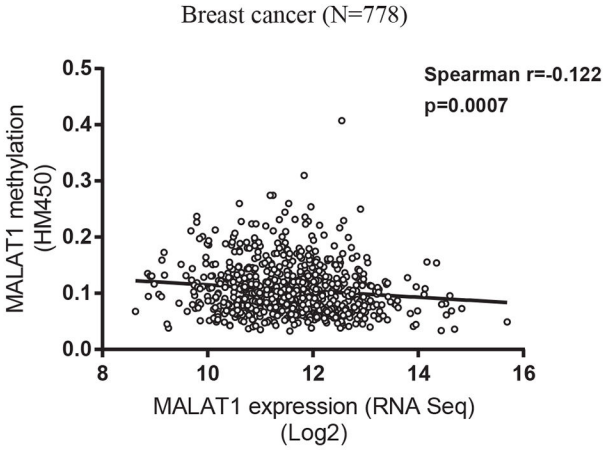

Figure 4. Methylation status and *MALAT1* expression.

A) A screenshot from the UCSC Genome Browser shows the CpG island in the *MALAT1* promoter and probes included in the Illumina HumanMethylation450 BeadChip for measuring methylation in the CpG sites. B) Scatter plot shows the correlation between *MALAT1* expression and promoter methylation in breast cancer using the TCGA data.

Table 1.

Associations of *MALAT1* expression with clinical and pathological factors of breast cancer

Variables	Total No. (N=509)	*MALAT1* expression			P value
		Low No. (%)	Mid No. (%)	High No. (%)	
Age at Surgery (year)					
≤57.80	253 (49.90)	88 (34.78)	86 (33.99)	79 (31.23)	0.57
>57.80	254 (50.10)	78 (30.71)	88 (34.65)	88 (34.65)	
Disease Stage					0.30
Stage 1	168 (34.93)	49 (29.17)	53 (31.55)	66 (39.29)	
Stage 2	241 (50.10)	83 (34.44)	78 (32.37)	80 (33.20)	
Stage 3 & 4	72 (14.96)	24 (33.33)	32 (44.44)	16 (22.22)	
Tumor Grade					**0.013**
Grade 1	60 (12.12)	13 (21.67)	19 (31.67)	28 (46.67)	
Grade 2	192 (38.79)	50 (26.04)	65 (33.85)	77 (40.10)	
Grade 3	243 (49.09)	101 (41.56)	83 (34.16)	59 (24.28)	
ER Status					**0.0002**
Positive	349 (70.08)	97 (27.79)	122 (34.96)	130 (37.25)	
Negative	149 (29.92)	68 (45.64)	48 (32.21)	33 (22.15)	
PR Status					**<0.0001**
Positive	295 (59.36)	75 (25.42)	111 (37.63)	109 (36.95)	
Negative	202 (40.64)	89 (44.06)	59 (29.21)	54 (26.73)	

Table 2.

Associations of *MALAT1* expression with breast cancer survival

Variable	RFS		P value	OS		P value	RFS*		P value	OS*		P value
	HR	95% CI		HR	95% CI		HR	95% CI		HR	95% CI	
MALAT1												
Low	1			1			1			1		
Mid	1.56	0.91-2.67	0.11	1.34	0.76-2.36	0.31	**1.88**	**1.06-3.33**	**0.03**	1.53	0.80-2.92	0.20
High	**2.02**	**1.20-3.40**	**0.0083**	1.41	0.80-2.48	0.24	**3.04**	**1.73-5.37**	**0.0001**	**2.51**	**1.32-4.77**	**0.0050**
Continuous	**1.41**	**1.09-1.81**	**0.0080**	1.18	0.89-1.56	0.24	**1.73**	**1.31-2.28**	**<0.0001**	**1.59**	**1.15-2.19**	**0.0046**

*Adjusted for age at surgery, tumor grade, disease stage, ER, and PR.

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Table 3.

Associations of *MATLA1* expression with breast cancer survival stratified by tumor grade and/or hormone receptor status

MALAT1	RFS		P value	OS		P value	RFS*		P value	OS*		P value
	HR	95% CI		HR	95% CI		HR	95% CI		HR	95% CI	
Grade 1or 2												
Low	1			1			1			1		
Mid	**4.17**	**1.53-11.35**	**0.0052**	2.36	0.95-5.87	0.064	**3.88**	**1.39-10.88**	**0.0099**	**3.46**	**1.20-0.02**	**0.022**
High	**3.45**	**1.26-9.45**	**0.016**	1.56	0.61-4.03	0.36	**2.64**	**1.26-9.76**	**0.016**	2.41	0.83-7.03	0.11
Continuous	**1.58**	**1.06-2.37**	**0.024**	1.19	0.79-1.81	0.41	**1.63**	**1.07-2.47**	**0.023**	1.41	0.89-2.21	0.14
Grade 3												
Low	1			1			1			1		
Mid	1.32	0.63-2.74	0.46	1.04	0.47-2.31	0.93	1.33	0.61-2.88	0.47	0.97	0.40-2.31	0.94
High	**2.15**	**1.11-4.16**	**0.023**	1.47	0.71-3.05	0.30	**2.57**	**126-5.23**	**0.0094**	2.02	0.91-4.49	0.085
Continuous	**1.49**	**1.07-2.07**	**0.019**	1.22	0.84-1.77	0.29	**1.64**	**1.15-2.34**	**0.0067**	1.46	0.96-2.21	0.075
ER+ or PR+												
Low	1			1			1			1		
Mid	**2.18**	**1.09-4.36**	**0.027**	1.30	0.65-2.57	0.46	**2.32**	**1.13-4.75**	**0.022**	**2.74**	**1.04-7.21**	**0.041**
High	**2.43**	**1.23-4.78**	**0.011**	1.26	0.64-2.48	0.51	**2.82**	**1.39-5.73**	**0.0042**	2.46	0.97-6.28	0.059
Continuous	**1.49**	**1.09-2.02**	**0.012**	1.12	0.80-1.55	0.52	**1.61**	**1.16-2.22**	**0.0042**	1.45	0.96-2.18	0.075
ER− and PR−												
Low	1			1			1			1		
Mid	1.77	0.69-4.57	0.24	1.45	0.50-4.17	0.22	1.59	0.61-4.19	0.35	1.06	0.34-3.27	0.92
High	1.86	0.72-4.79	0.20	1.70	0.60-4.77	0.51	1.81	0.64-5.12	0.26	1.30	0.39-4.29	0.67
Continuous	1.34	0.85-2.09	0.21	1.29	0.78-2.14	0.32	1.34	0.81-2.22	0.26	1.14	0.63-2.09	0.66
Grade 1or 2 and ER+ or PR+												
Low	1			1			1			1		
Mid	**3.55**	**1.28-9.81**	**0.015**	2.30	0.86-6.15	0.096	**3.50**	**1.21-10.16**	**0.021**	**4.09**	**1.24-13.44**	**0.020**
High	**2.92**	**1.05-8.15**	**0.041**	1.78	0.66-4.82	0.26	**3.13**	**1.08-9.04**	**0.035**	3.20	0.99-10.36	0.053
Continuous	1.50	0.99-2.29	0.058	1.26	0.81-1.97	0.30	**1.58**	**1.01-2.48**	**0.046**	1.56	0.95-2.57	0.078

*Adjusted for age at surgery and disease stage, as well as tumor grade or ER/PR status when they were not used for stratification.