Hypermethylation of TMEM240 Involved in Expression Deficiency Predicts Poor Hormone Therapy Response and Disease Progression in Breast Cancer

Ruo-Kai Lin  
Taipei Medical University

Chih-Ming Su  
Taipei Medical University-Shuang Ho Hospital

Shih-Yun Lin  
Taipei Medical University College of Pharmacy

Le Thi Anh Thu  
Quang Tri Medial College

Phui-Ly Liew  
Taipei Medical University-Shuang Ho Hospital

Jian-Yu Chen  
Taipei Medical University School of Pharmacy: Taipei Medical University College of Pharmacy

Huey-En Tzeng  
Taipei Medical University Hospital

Chin-Sheng Hung (hungcs@tmu.edu.tw)  
Taipei Medical University

Research

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Abstract

Background:

Approximately 25% of patients with early-stage breast cancer experience cancer progression throughout the disease course. Alterations in \textit{TMEM240} in breast cancer were identified and investigated to monitor treatment response and disease progression.

Methods:

Circulating methylated \textit{TMEM240} in the plasma of breast cancer patients was used to monitor treatment response and relapse events. Illumina methylation arrays were used to identify novel hypermethylated CpG sites and genes related to poor hormone therapy response. Quantitative methylation-specific real-time polymerase chain reaction (QMSP), quantitative real-time reverse transcription PCR, and immunohistochemical analyses were performed to measure DNA methylation, mRNA and protein expression levels in 335 breast samples from Taiwanese and Korean patients. Kaplan–Meier curves, Cox proportional hazards regression and receiver operating characteristic curves were used to analyze 10-year survival and disease progression. The Cancer Genome Atlas (TCGA) dataset was used to investigate \textit{TMEM240} alterations in Western countries. Transient transfection and knockdown of \textit{TMEM240} were performed to determine its biological functions and its relationship to hormone drug treatment response in breast cancer cells.

Results: Aberrant methylated \textit{TMEM240} was identified in breast cancer patients with poor hormone therapy response using genome-wide methylation analysis in the Taiwan and TCGA breast cancer cohorts. A cell model showed that TMEM240, which is localized to the cell membrane and cytoplasm, represses breast cancer cell proliferation and cell migration. TMEM240 protein expression was observed in normal breast tissues, but not detected in 88.2% (67/76) of breast tumors and in 90.0% (9/10) of metastatic tumors from breast cancer patients. Almost all triple-negative breast cancer patients (95.7%, 22/23) had deficient TMEM240 protein expression. QMSP revealed that in 54.5% (55/101) of Taiwanese breast cancer patients, the methylation level of \textit{TMEM240} was at least 2-fold higher in tumor tissues than in the matched normal breast tissues. Patients with hypermethylation of TMEM240 had poor 10-year overall survival (p = 0.003) and poor treatment response, especially hormone therapy response (p < 0.001). Prediction of disease progression based on circulating methylated \textit{TMEM240} was found to have 87.5% sensitivity, 93.1% specificity, and 90.2% accuracy, better than the currently used biomarkers CEA and CA-153.

Conclusions: Circulating methylated \textit{TMEM240} is a potential biomarker for treatment response and disease progression monitoring in breast cancer.

Background

Breast cancer is the most common cancer in women, surpassing lung cancer, worldwide [1-3]. The estrogen-dependent nature of breast cancer is the fundamental basis for hormone therapy. The hormone receptor-positive human epidermal growth factor receptor 2-negative (HR+/HER2−) subtype, which is characterized by the expression of estrogen receptor (ER) and/or progesterone receptor (PR) without HER2 overexpression/amplification, accounts for approximately 70% of breast cancer patients. However, estrogen-independent growth often exists de novo at diagnosis or develops during the course of hormone therapy. Nearly 20% – 30% of patients with early-stage disease become metastatic throughout the disease course [4]. Therefore, ER expression alone is insufficient in predicting endocrine therapy efficacy [5]. A significant number of these patients will develop either primary or secondary hormone resistance, prompting the need for tests that can predict treatment response before treatment options are chosen. The initiation and progression of cancer, which is conventionally considered a genetic disease, involve epigenetic abnormalities [6].

Genomic screening of 98 different primary human tumors revealed that on average, approximately 600 aberrantly methylated CpG islands exist in each tumor [7]. Combined analysis of data from Taiwanese individuals for whom both data on breast cancer tissue and data on clinical treatment response are available in the TCGA database has shown that hypermethylation of the gene encoding \textit{transmembrane protein gene 240 (TMEM240)} is a biomarker of poor hormone therapy response in breast cancer. \textit{TMEM240} encodes a transmembrane domain-containing protein found in the brain and cerebellum. In studies of patients from France, Germany, the Netherlands, Colombia, Japan, and China, mutations in \textit{TMEM240} have been found to cause spinocerebellar ataxia 21 (SCA21) with mental retardation, severe cognitive impairment, and hypokinetic and hyperkinetic movement disorders [8-11]. The pathogenic mechanism of SCA21 may be mediated through the induction of early gliosis and lysosomal impairment by mutant \textit{TMEM240} [12].

Advances in detection technology have reduced breast cancer death rates in several Western countries [15]. Therefore, developing biomarkers for treatment response can improve patient outcomes. For current disease progression monitoring for breast cancer patients, simultaneous use of the two serum markers CA-153 and carcinoembryonic antigen (CEA) shows that the early diagnosis of metastasis in up to 60%–80% of patients with breast cancer is not sensitive enough to monitor disease progression in real time [16-18]. Therefore, no dynamic monitoring system for accurately and sensitively measuring recurrence or metastasis events is available in current clinical practice. Circulating cell-free DNA (ccfDNA) in plasma can be used for the noninvasive sampling of cancer cells obtained from patients with breast cancer [19]. Cells release cell-free DNA through a combination of apoptosis, necrosis, and active secretion. Cancer cells, as well as cells in the tumor microenvironment, can produce ccfDNA. Multiple genetic and epigenetic alterations are found in ccfDNA [20]. Assays of circulating methylated DNA (cmDNA) could be used for outcome prediction in metastatic breast cancer patients treated with chemotherapy and/or multikinase inhibitors [21].

Circulating methylated \textit{TMEM240} can be
Genome-wide methylation analysis by bisulfite sequencing (Figure S1). The primers and probes used in QMSP are listed in Supplementary Table S1. The methylation level of TMEM240 was considered hypermethylated when the methylation level of TMEM240 relative to that of the ACTB gene was at least 2-fold higher in the breast tumor than in the paired normal breast tissue sample. The specificity of TMEM240 was considered hypermethylated when the methylation level of TMEM240 relative to that of the ACTB gene was at least 2-fold higher in the breast tumor than in the paired normal breast tissue sample. The specificity of TMEM240 methylation end products was confirmed by bisulfite sequencing (Figure S1). The primers and probes used in QMSP are listed in Supplementary Table S1.

Methods

Patients and tissue, plasma collection

A total of 335 Taiwanese clinical samples, including 137 human breast tumor tissues, 137 adjacent normal breast tissue samples and 61 plasma samples, were obtained from Taipei Medical University (TMU) Hospital, Shuang Ho Hospital and the TMU Joint Biobank. Three sets of tissue microarrays of breast cancer tissues were performed to analyze TMEM240 protein expression. Two sets of microarrays were performed in the Department of Pathology of Shuang Ho Hospital. The tissue microarrays contained breast tumor tissues and matched adjacent normal breast tissues obtained from 36 Taiwanese breast cancer cases. Three tissue microarrays representing a total of 131 tissues, including 76 breast carcinoma tissues, 10 matched metastatic carcinoma tissues and 45 matched normal tissues from South Korea, were purchased from SuperBioChips Laboratories (catalog number CBA4; South Korea), and tissues from Taiwanese breast cancer cases were obtained from the Department of Pathology, Shuang Ho Hospital (Taiwan). The pathologic diagnoses of these cases were microscopically confirmed by two researchers. Prior to the collection of clinical data and samples, written informed consent was obtained from all patients. Patients undergoing preoperative chemoradiotherapy or an emergent operative procedure were excluded from this study. Sections of cancerous tissue and corresponding noncancerous tissues were reviewed by a senior pathologist. Clinical data regarding age, sex, tumor type, TNM tumor stage, menopausal state, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) tumor markers, were prospectively collected and obtained from Taipei Medical University (TMU) Hospital, Shuang Ho Hospital and the TMU Joint Biobank. Following surgery, patients were monitored every 3 months for the first 2 years and semiannually thereafter.

Genomic DNA, circulating cell-free DNA and RNA extraction

Genomic DNA from matched pairs of primary tumors and adjacent breast tissues was extracted using the QIAamp DNA Mini Kit (Qiagen, Bonn, Germany, Cat. No. 51306) according to the manufacturer’s instructions. The tumor and normal specimens that were used for RNA extraction were frozen immediately after surgical resection and stored at −80 °C. Total mRNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany, Cat. No. 74134) according to the manufacturer’s instructions. Circulating cell-free DNA was extracted from plasma (3.5 mL) that had been isolated from 10 mL of peripheral blood within 2 hours of collection. Circulating cell-free DNA (ccfDNA) was extracted from 15 of the plasma samples using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Austin, TX, USA) according to the manufacturer’s recommended protocol [22-24]. CcfDNA was extracted from 46 of the plasma samples using the iCatcher Circulating cfDNA 1000 kit (CatchGene, New Taipei City, Taiwan) according to the manufacturer’s recommended protocol.

Reverse transcription PCR

To measure TMEM240 mRNA expression, real-time reverse transcription PCR (RT–PCR) was performed in a LightCycler 96 (Roche Applied Science, Penzberg, Germany). Real-time PCR was performed using the SensiFAST™ Probe No-ROX Kit (Bioline, London, UK, Cat. No. BIO-86020) with specific primers and the corresponding Universal Probe Library probe (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as a reference gene. The PCR conditions were as follows: preincubation at 95 °C for 10 minutes followed by 40 cycles of amplification at 95 °C for 10 seconds and 60 °C for 10 seconds. The normalized gene expression values obtained using LightCycler Relative Quantification software (Version 1.5, Roche Applied Science) were compared with those of the control group. TMEM240 mRNA expression was considered low if the mRNA expression level of TMEM240 relative to GAPDH was 0.5-fold lower in the breast tumor tissue than in the paired normal breast tissue. The primers and probes used in RT–PCR are listed in Table S1.

TaqMan quantitative methylation-specific PCR

After bisulfite conversion of DNA using the EpiTect Fast DNA Bisulfite Kit (Qiagen, Bonn, Germany, Cat. No. 59826), the DNA methylation level of TMEM240 was measured using TaqMan quantitative methylation-specific PCR (QMSP) in a LightCycler 96 (Roche Applied Science, Penzberg, Germany). QMSP was performed using the SensiFAST™ Probe No-ROX Kit (Bioline, London, UK, Cat. No. BIO-86020) with specific primers and methyl-TaqMan probe for TMEM240. Normalized DNA methylation values, which were calibrated to the control group, were obtained using LightCycler Relative Quantification software (Version 1.5, Roche Applied Science). The beta-actin (ACTB) gene was used as a reference gene. Primers and probes for TMEM240 methylation detection were designed to bind to the junction between the promoter and exon 1. The QMSP conditions were as follows: preincubation at 95 °C for 10 minutes followed by 50 cycles of amplification at 95 °C for 10 seconds and 60 °C for 10 seconds. TMEM240 was considered hypermethylated when the methylation level of TMEM240 relative to that of the ACTB gene was at least 2-fold higher in the breast tumor than in the paired normal breast tissue sample. The specificity of TMEM240 methylation end products was confirmed by bisulfite sequencing (Figure S1). The primers and probes used in QMSP are listed in Supplementary Table S1.

Genome-wide methylation analysis

Successfully detected in patients with CRC [13]. In this study, the promoter methylation level, the expression level and the biological functions of TMEM240 will be clarified. Whether circulating methylated TMEM240 can be detected in blood from individuals with breast cancer and its association with treatment response and disease progression will also be investigated.
Genome-wide methylation analysis of 5 paired breast cancer tissues and corresponding noncancerous breast tissues was performed using the Illumina Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA, USA) for one sample and the Infinium MethylationEPIC Kit (Illumina) for the remaining 4 samples, as previously reported [17]. The two arrays contain more than 450,000 and 850,000 methylation sites, respectively, and provide genome-wide coverage of the gene region and CpG island coverage, respectively, including 99% of RefSeq genes. Bisulfite conversion of 500 ng of genomic DNA was performed using the EpiTect Fast DNA Bisulfite Kit (Qiagen, Bonn, Germany, Cat. No. 59826). Methylation scores for each CpG site were represented as “beta” values ranging from 0 (unmethylated) to 1 (fully methylated) based on determination of the ratios of the methylated signal intensities to the sums of the methylated and unmethylated signal outputs.

**Cell lines, cell culture, and drug treatment**

The MDA-MB-231 and T47D breast cancer cell lines used in this study were obtained from the Bioresource Collection and Research Center (http://www.brcr.firdi.org.tw/). MDA-MB-231 cells were cultured in DMEM/F12 supplemented with human platelet lysate (hPL, American Red Cross, USA) and 1% penicillin/streptomycin. T47D cells were cultured in DMEM/F12 supplemented with human platelet lysate (hPL, American Red Cross, USA), 1% penicillin/streptomycin and 6 ng/ml insulin. For the **TMEM240 demethylation assay**, MDA-MB-231 cells were treated with dimethyl sulfoxide (DMSO) or with the demethylation agent decitabine (DAC, Sigma–Aldrich, St. Louis, MO, USA) for 96 hours. DAC was dissolved in DMSO. After treatment of the cells, DNA and RNA were extracted, and methylation and gene expression levels were analyzed. For the hormone therapy response assay, T47D cells were treated with DMSO or with a series of concentrations of Tamoxifen (0, 10 and 20 μM) for 48 hours (Sigma–Aldrich, St. Louis, MO, USA).

**Immunofluorescence assay**

For immunofluorescence staining assays, cells were seeded in 4-well glass chamber slides (Nunc). After TMEM240 plasmid overexpression, the DLD-1 cells were fixed in 4% formaldehyde and stained with anti-DDK (1:200, Abcam, Cambridge, UK). Imaging was performed using deconvolution fluorescence microscopy (Olympus).

**Immunohistochemical assay**

Immunohistochemical staining with an antibody against TMEM240 (1:35, Sigma–Aldrich, HPA066721, St. Louis, MO, USA) was performed using an iView DAB detection kit (Ventana, Tucson, AZ, USA) and a BenchMark XT autostainer. The assay included both positive and negative controls. The researchers who evaluated the immunohistochemical staining results were blinded to the clinical follow-up data. The intensity of TMEM240 expression was identified semiquantitatively as no expression, low expression (weaker than or equal to the expression intensity observed in normal colon epithelium), or high expression (stronger than the expression intensity observed in normal colon epithelium).

**Plasmid extraction, confirmation and purification**

Plasmid DNA was extracted using the Geneaid™ Midi Plasmid Kit (Geneaid Biotech Ltd., Cat. No. PI025) according to the manufacturer's instructions. The extracted DNA was subjected to preliminary length analysis by sequenced to confirm errorless production. The plasmid concentration was measured using a NanoDrop 2000C ultramicrowavelength spectrometer (Thermo Fisher Scientific, USA), and the plasmid was stored at -20 °C until further use.

**cDNA expression construct, RNAi, and transfection**

**TMEM240** interference RNA was obtained from Life Technologies Corporation. Transfections were performed using 10 nM si-TMEM240 or nontargeting siRNAs, and Lipofectamine-RNAiMax and Lipofectamine 3000 reagent (Invitrogen) was used to transfect MDA-MB-231 and T47D cells according to the manufacturer's protocol.

**Transwell assay**

Transwell assays were used to study cell migration. In the transwell assays, the upper and lower chambers of the culture wells were separated by a semipermeable membrane (Falcon) with a pore size of 8 μm. Approximately 2 × 10^4 and 1 × 10^5 treated and untreated MDA-MB-231 and T47D cells, respectively, were seeded in the upper chamber. Then, 300 μL of serum-free DMEM/F12 was added as culture medium, and 800 μL of serum-containing culture medium was added as a chemical attractant in the lower chamber. After 16 hours of incubation, the cells retained over the membrane were washed twice with PBS, fixed with 4% formaldehyde and stained with 1% crystal violet/ddH₂O for 60 minutes at room temperature. Five randomly chosen areas were photographed using a camera attached to a microscope (Nikon), and ImageJ was used to quantify the number of cells in each area.

**Wound healing assay**

The wound healing assays were performed using culture inserts (Ibidi, GmbH, Martinsried, Germany). After seeding 1 × 10^5 cells overnight, the cells were transfected with siRNA for 48 hours. The culture inserts were then removed, the wounded areas were photographed using a camera attached to a microscope (Nikon), and ImageJ was used to calculate the wound areas.
To investigate whether TMEM240 was knocked down in MDA-MB-231 and T47D cells using si-control (Figure 2B, left panel; and Figure S2). To determine whether decreased TMEM240 expression induces cell growth, microscopic observation revealed that TMEM240 overexpression repressed the growth of T47D and MDA-MB-231 cells compared with a vector cell viability assay, TMEM240 inhibited MDA-MB-231 and T47D cancer cell growth by 55.2% and 48.7%, respectively (Figure 2A-B, right panel). Resulted in abundant TMEM240 protein expression (Figure 2A, left panel) and mRNA expression (Figure 2A-B, middle panel). According to the SRB manipulation eciency was determined through real-time RT–PCR. Transfection of the TMEM240 plasmid into MDA-MB-231 and T47D cells and with and without drug treatment. Multivariate Cox proportional hazards regression analyses (adjusted for age, sex, race, tumor subtype, and tumor stage) were further used to analyze the correlation between TMEM240 hypermethylation and 10-year overall survival in breast cancer patients. Comparisons of hypermethylation and hypomethylation curves that yielded log-rank test p values of less than 0.05 were considered statistically significant. The TMEM240 methylation level and drug treatment response in breast cancer patients whose data were accessed through the TCGA portal were analyzed using the Mann–Whitney test. In addition to accuracy, other commonly used measures of evaluating the classification, such as the receiver operating characteristic curve (ROC) and area under the curve (AUC), sensitivity, specificity, false-positive rate and false-negative rate, are also reported.

Results

**TMEM240 was identified in samples from Taiwanese and Western breast cancer patients by genome-wide methylation analysis**

To identify a novel potential biomarker in breast cancer patients with poor hormone therapy response, we used five criteria to screen potential targets: (1) hypermethylation in Taiwanese breast cancer patients; (2) hypermethylation in Western breast cancer patients; (3) hypermethylation in breast cancer patients with poor hormone therapy response compared with complete response; (4) a methylation level in normal tissues that was close to 0; and (5) low expression in breast cancer patients (Figure 1A). First, to identify critical tumor suppressor genes, the Infinium Methylation Assay was applied to 5 breast cancer tissue samples and paired noncancerous breast tissue samples. A total of 2612 genes were hypermethylated according to the criterion \( \Delta \text{Avg}_\beta (\beta_{\text{Tumor}} - \beta_{\text{Normal}}) > 0.4 \). Second, we analyzed the TCGA Illumina Infinium HumanMethylation450 BeadChip array data of 87 paired Western breast cancer patients. A total of 6882 genes were found to be hypermethylated according to the criterion \( \Delta \text{Avg}_\beta (\beta_{\text{Tumor}} - \beta_{\text{Normal}}) > 0.4 \). Next, the top 20 genes with the highest methylation levels in 8 breast cancer patients with poor hormone therapy response compared with 21 patients with complete response to hormone therapy were identified. Next, we further found that 11940 genes showed much lower DNA methylation levels in breast, colon, rectal, lung, uterine, gastric, esophageal, pancreatic, liver, and prostate normal tissues. Finally, TCGA RNA sequencing data of 38 paired breast cancer samples from Western patients showed that the expression of 2474 genes were decreased by 50% in the breast cancer samples. The **TMEM240** gene was identified using InteractiVenn (Figure 1B). Few reports about TMEM240 in women cancer was found. Methylation of **TMEM240** was further analyzed in the TCGA cohort, and the gene was found to be highly methylated in breast cancer, endometrial and uterine cancer. The cluster analysis of the **TMEM240** methylation pattern was visualized as a heatmap (Figure 1C). The role of TMEM240 in breast cancer is unclear. Therefore, TMEM240 in breast cancer was selected for further analysis. A comprehensive analysis of its epigenetic alterations, mRNA and protein expression was performed, and the biological role of **TMEM240** was further studied.

**TMEM240 represses breast cancer cell proliferation and cell migratory ability**

Alterations in **TMEM240** and its functional roles during tumorigenesis have not been studied previously. To study the biological roles of the TMEM240 protein in breast cancer cells, TMEM240 was overexpressed or knocked down in MDA-MB-231 cells by electroporation. The gene manipulation efficiency was determined through real-time RT–PCR. Transfection of the TMEM240 plasmid into MDA-MB-231 and T47D cells resulted in abundant TMEM240 protein expression (Figure 2A, left panel) and mRNA expression (Figure 2A-B, middle panel). According to the SRB cell viability assay, TMEM240 inhibited MDA-MB-231 and T47D cancer cell growth by 55.2% and 48.7%, respectively (Figure 2A-B, right panel). Microscopic observation revealed that TMEM240 overexpression repressed the growth of T47D and MDA-MB-231 cells compared with a vector control (Figure 2B, left panel; and Figure S2). To determine whether decreased TMEM240 expression induces cell growth, **TMEM240** gene expression was knocked down in MDA-MB-231 and T47D cells using si-**TMEM240**. **TMEM240** mRNA expression was reduced after transfection of the MDA-MB-231 and T47D cells with si-**TMEM240** for 24 h compared with the ci-control (Figure 2C and 2D). Microscopic observation and SRB assay revealed that si-**TMEM240** induced proliferation of MDA-MB-231 and T47D cells compared with the si-control group. TMEM240 knockdown increased MDA-MB-231 and T47D cell proliferation by 1.4- and 1.5-fold, respectively (Figure 2C and 2D). To investigate whether **TMEM240** is associated with breast cancer cell migration, MDA-MB-231 cells were transfected with **TMEM240** or si-**TMEM240** for 24 h. The motility of the cells was then analyzed using transwell assays and wound healing assays. The data revealed that an increase in **TMEM240** expression suppressed the migration ability of MDA-MB-231 cells by 75.6% (Figure 2E). Knockdown of **TMEM240** in MDA-
MB-231 cells significantly induced cell migration by 63.0% and 62.7% based on the results obtained using transwell assays (Figure 2F) and wound healing assays (Figure 2G), respectively.

**TMEM240 protein is mainly distributed in cell membranes and in the cytoplasm**

Although TMEM240 is predicted to be a membrane protein, to date no study has reported the intracellular distribution of the TMEM240 protein. According to the structure of TMEM240 protein reported in the UniProtKB/Swiss-Prot database, the protein contains two transmembrane protein regions located between amino acid residues 5–25 and 90–110. Exogenous expression of TMEM240 and immunofluorescent staining were used to examine the distribution of the protein in the DLD-1 cell line. Deconvolution and 3D reconstruction of immunofluorescence images showed that the TMEM240 protein was mainly concentrated in the cytoplasm and cell membranes (Figure 4).

**Low TMEM240 protein expression in Taiwanese and South Korean breast cancer patients**

TMEM240 reveals the tumor suppressor potential for breast cancer cell growth and migration (Figure 2). To investigate whether TMEM240 protein expression is altered in cancerous breast tissues, TMEM240 protein expression in 76 breast tumors from 40 Korean breast cancer patients and 36 Taiwanese breast cancer patients was analyzed by immunohistochecmistry. TMEM240 was observed to be localized to the cell membrane and cytoplasm in normal breast tissues (Figure 3A). The protein was expressed at lower-than-normal levels in 88.2% (67/76) of the tumors from breast cancer patients and in 90.0% (9/10) of metastatic tumors from breast cancer patients (Figure 3B and 3C and Table 1). Almost all triple-negative breast cancer patients (95.7%, 22/23) had deficient TMEM240 protein expression (Table 1).

**Promoter hypermethylation and low TMEM240 mRNA expression in Taiwanese breast cancer patients**

Low expression of TMEM240 protein was observed in breast cancer patients. We investigated whether TMEM240 mRNA was also expressed at lower levels in breast cancer. We analyzed TMEM240 mRNA expression in 52 paired Taiwanese breast cancer tissues. In 50.0% (26/52) of these tissues, TMEM240 mRNA expression was lower in the breast cancer tumor tissue than in the normal breast tissue (Figure 3D, Table 1). We further analyzed the methylation patterns of TMEM240 in paired 101 Taiwan breast cancer patients, the methylation level of TMEM240 was at least 2-fold higher in 54.5% (55/101) breast tumor tissues than in the matched normal breast tissues (Figure 3E, 3F and Table 1). The DNA hypermethylation levels and mRNA expression levels of TMEM240 showed a significant negative correlation by Spearman rank correlation coefficient analysis ($p = 0.037$). To determine whether hypermethylation of TMEM240 is involve in the regulation of mRNA expression, TMEM240 mRNA expression was investigated using administration of the DNA demethylating drug decitabine (DAC) to T47D and MDA-MB-231 breast cancer cells. The cells were treated with DMSO and DAC for 48 h. In the DAC groups for the two cell lines, methylation of TMEM240 decreased to 28.36% and 7.8%, respectively, of that in the DMSO group ($p = 0.001$, Figure 3G and 3H, left panel), and TMEM240 mRNA expression increased by 150-fold and 337-fold, respectively ($p < 0.001$, Figure 3G and 3H, right panel), suggesting that hypermethylation of the TMEM240 promoter is the main mechanism through which TMEM240 silencing occurs.

**TMEM240 promoter hypermethylation and low mRNA expression in breast cancer tissues from the TCGA dataset**

To further evaluate alterations in TMEM240 hypermethylation and mRNA expression in Western breast cancer patients, we analyzed the TCGA data of the Illumina Infinium HumanMethylation450 BeadChip array for 78 breast cancer tumors, 78 matched normal tissues and 623 breast cancer tumor tissues and displayed the methylation levels in a heatmap. The exon 1 region of TMEM240 was hypermethylated in 40.3% (251/623) of the breast tumor tissues (Table 2). Analysis of RNA sequencing data from TCGA showed that TMEM240 mRNA expression was reduced by half in 51.4% (37/72) of the breast cancer tumor tissues compared with the matched normal breast tissues ($p = 0.019$, Figure S3) and in 60.2% (458/761) of tumors from breast cancer patients (Table 2). The DNA hypermethylation levels and mRNA expression levels of TMEM240 showed a significant negative correlation on Spearman rank correlation coefficient analysis ($p=0.049$). Hypermethylation of TMEM240 was associated with Asian, ER-negative, PR-negative and triple-negative breast cancer patients and patients with invasive ductal carcinoma (all $p < 0.001$, Table 2). In addition, Kaplan–Meier curves indicated that patients with hypermethylation of TMEM240 had a poor survival rate (Figure 4A, log rank test, $p = 0.003$). A Cox proportional hazards survival analysis further adjusted for race, age, tumor type, tumor stage and menopausal state showed that TMEM240 promoter hypermethylation was significantly and independently associated with 10-year overall survival (Table 3, $p = 0.002$).

**Hypermethylation of TMEM240 in breast cancer was associated with poor treatment response in the TCGA cohort set**

To further investigate whether hypermethylation of TMEM240 is associated with poor treatment response, we analyzed the relationship between hypermethylation of TMEM240 and clinical treatment response to chemotherapy, hormone therapy and targeted therapy in patients from the TCGA cohort. The results indicated that patients with hypermethylation of TMEM240 had poor chemotherapy response (Table 4, $p = 0.012$) and poor hormone therapy response (Table 4, $p < 0.001$). Better hormone therapy response was observed in 85.0% of patients with lower methylation of TMEM240 but in only 28.6% of patients with hypermethylation of TMEM240 (Table 4B, Mann–Whitney U test, $p = 0.005$). Higher methylation of TMEM240 was associated with poorer response to tamoxifen treatment (Figure 4C, Mann–Whitney U test, $p = 0.041$) and with poorer response to aromatase inhibitor treatment (Figure 4D, Mann–Whitney U test, $p = 0.037$).

**High TMEM240 expression enhance the tamoxifen treatment response in breast cancer cell lines**
To further investigate whether the expression of TMEM240 may be involved in the response to hormone drug treatment, a cell proliferation assay was performed after overexpression and/or knockdown of TMEM240 and tamoxifen treatment in T47D breast cancer cells. The proliferation of T47D (ER+/PR+) cells was significantly decreased by 62.9% when cells transfected with si-control were treated with 20 mM tamoxifen (p = 0.003), but only a 31.1% decrease in proliferation was observed in cancer cells transfected with si-TMEM240 (Figure 4E). The data indicate that lower expression of TMEM240 is related to resistance to tamoxifen treatment. Overexpression of TMEM240 in T47D cells induced 76.9% cancer cell death when the cells were treated with 20 mM tamoxifen but only a 46.0% decrease in the vector control cancer cells when treated with 20 mM tamoxifen (Figure 4F).

**Circulating methylated TMEM240 predicts disease progression and poor hormone therapy response in Taiwanese breast cancer patients**

Hypermethylation of TMEM240 was found in breast tumors of patients who displayed poor treatment response, especially in tumors from patients who received hormone therapy. Detection of circulating methylated TMEM240 in the plasma of patients with poor treatment response could provide a potential tool for real-time monitoring of clinical outcomes after medical treatment. Breast cancer patients were recruited from the Taipei Medical University Hospital and Shuang Ho Hospital and were followed up for at least 1 year. After these patients received treatment, circulating methylated DNA was extracted from their plasma at 3-6 month intervals and analyzed by QMSP. The patients with poor prognosis had significantly higher circulating methylated TMEM240 levels than other patients but did not display higher levels of CA-15-3 and CEA (Table 5). The level of circulating methylated TMEM240 dramatically and gradually decreased in breast cancer patients following treatment (Case 1 and Case 2, Figure 5A-B). When patients experienced disease progression, recurrence or metastasis, the levels of circulating methylated TMEM240 increased significantly (Case 3 and Case 4, Figure 5C-E, Mann–Whitney U test, p < 0.001). The circulating methylated TMEM240 test for poor prognosis prediction was found to have 87.5% sensitivity (28/32), 93.1% specificity (27/29), and 90.2% accuracy (55/61), values that are better than those obtained using the currently used biomarkers CEA and CA-153 (Table 5 and Figure 5).

Hypermethylation of TMEM240 in breast cancer was associated with poor response to hormone therapy in the TCGA cohort. We further investigated whether patients with an increase in circulating methylated TMEM240 in plasma experienced disease progression, recurrence or metastasis after hormone therapy. The results indicated that the patients with poor response after hormone therapy had significantly higher levels of circulating methylated TMEM240 (Table 5). The circulating methylated TMEM240 test for poor hormone therapy response prediction was found to have a sensitivity of 76.9% (10/13), a specificity of 95.7% (22/23), and an accuracy of 88.9% (32/36) (Figure 5E-F).

**Discussion**

Aberant promoter hypermethylation of CpG islands associated with TSGs can cause transcriptional silencing and contribute to tumorigenesis. In the present investigation, hypermethylation of TMEM240 in patients with poor hormone therapy response was identified using genome-wide methylation array analysis. QMSP confirmed the presence of TMEM240 hypermethylation in Taiwanese breast cancer tumor tissues compared with normal tissues. In the TCGA cohort, hypermethylation of the promoter region of TMEM240 was found in 40.3% of tumors. It occurs more frequently in Asian patients (54.8%). Results similar to those found in the Asian TCGA cohort were consistently found in Taiwanese breast cancer patients (54.5%). Low expression of TMEM240 protein was found in most Taiwanese and Korean breast cancer patients. Moreover, patients with hypermethylation of TMEM240 had poor 10-year overall survival. In addition, hypermethylation of TMEM240 was observed in patients with progressive disease, especially in patients treated with hormone therapy. The results obtained that the patients with poor response after hormone therapy had significantly higher levels of circulating methylated TMEM240 (Table 5). The circulating methylated TMEM240 test for poor hormone therapy response prediction was found to have a sensitivity of 76.9% (10/13), a specificity of 95.7% (22/23), and an accuracy of 88.9% (32/36) (Figure 5E-F).

In addition to its association with progressive disease and poor prognosis, hypermethylated TMEM240 was found to be strongly associated with ER/PR negative breast cancer, TNBC and poor hormone therapy response. Almost all triple-negative breast cancer patients (95.7%, 22/23) displayed deficient TMEM240 protein expression. Patients with hypermethylation of TMEM240 often had poor hormone therapy response (Table 3, p < 0.001). Patients who had circulating hypermethylated TMEM240 also experienced disease progression (Figure 7). The data indicate that TMEM240 deficiency is involved in breast tumorigenesis through pathways other than the ER/PR and HER2 pathways, leading to a poor hormone therapy response. Even patients with hypermethylation of TMEM240 and positive ER/PR expression exhibited poor hormone therapy responses, including treatment with tamoxifen or aromatase inhibitors (AIs) (Table 4). Only in patients in which TMEM240 was expressed at sufficient levels in the cancer cells did hormone therapy produce a good therapeutic response (Figure 2 and Figure 3). Ki-67 as a parameter and multigene analysis (MGA) have been used to predict the response to hormone therapy [25, 26]. Methylation of TMEM240 may play a role in determining resistance to AI drugs such as Ki-67 or MGA. The mechanisms and pathways that are modulated by TMEM240 are worth investigating further. TNBC represents a group of breast cancers with heterogeneous genomic features. There are several different subtypes of...
TNBC, including the Vanderbilt subtype and the Baylor subtype [27, 28]. Each subtype carries a different set of mutant genes [27, 28]. Further study may focus on the relationship between TMEM240 and specific subtypes of TNBC.

Advances in detection technology have reduced breast cancer death rates in several Western countries [15]. Therefore, the development and use of biomarkers of treatment response can improve patient outcomes. The presence of breast-derived circulating DNA is indicative of residual disease after treatment [29]. Circulating methylated TMEM240 dramatically and gradually decreases and then diminishes in patients with various subtypes of breast cancer who do not show disease progression (Figure 7), suggesting that measurement of circulating methylated TMEM240 could be used to detect the presence of residual disease. In addition, the level of circulating methylated TMEM240 in plasma increased further in breast cancer patients with recurrence or metastasis (Figure 7). In these patients, the concentrations of CEA and CA15-3 in serum remained normal or increased much later than did the circulating methylated TMEM240. The detection of CEA and CA15-3 was incapable of revealing disease progression and poor treatment response in several patients (Table S). Measurement of circulating methylated TMEM240 could be used to monitor and detect early disease progression after treatment and during long-term follow-up. Although hypermethylation of TMEM240 also occurs in other types of cancer, its high alteration in cancers may assist the detection of disease progression. Combining measurement of TMEM240 hypermethylation with the measurement of additional breast cancer-specific methylated DNA biomarkers that are associated with disease progression will improve detection sensitivity and cancer specificity.

**Conclusion**

Deficiency in TMEM240 expression plays an important role during cancer progression in breast cancer patients. Circulating hypermethylated TMEM240 may represent a potential biomarker for disease progression and poor hormone therapy response.

**Abbreviations**

ACTB, beta-actin; CA15-3, cancer antigen 15-3; ccfDNA, circulating cell-free DNA; CEA, carcinoembryonic antigen; cmDNA, circulating methylated DNA; breast cancer; breast cancer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase gene; Ki-67, marker of proliferation Ki-67; QMSP, quantitative methylation specific real-time polymerase chain reaction; QPCR, Quantitative real-time reverse transcription polymerase chain reaction; SAC21, spinocerebellar ataxia 21; SRB, sulforhodamine B; TNBC, Triple negative breast cancer; TCGA, The Cancer Genome Atlas; TMEM240, transmembrane Protein 240; TSG, tumor suppressor gene; TMU, Taipei Medical University; QS, overall survival.

**Declarations**

**Ethics approval and consent to participate**

The study has been approved by the Taipei Medical University - Joint Institutional Review Board and the Institutional Review Board. Written informed consent was obtained from all patients.

**Consent for publication**

Not applicable

**Availability of data and materials**

The data generated in this study are available from the corresponding author upon reasonable request.

**Competing interests**

There are no conflicts of interest.

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**Authors’ contributions**

CSH and RKL designed the studies.

CSH, CMS and HET collected and provided the clinical samples.

SYL, LTAT, PLL and JYC performed the experiments.

RKL, CMS and CSH analyzed and interpreted the data.
RKL and CSH drafted the manuscript. All authors read and approved the manuscript.

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Authors’ information

1 Division of Colon and Rectal Surgery, Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan. 2 Department of Pathology, Shuang Ho Hospital, Taipei Medical University, New Taipei, Taiwan. 3 Department of Pathology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan. 4 Ph.D. Program for the Clinical Drug Discovery from Botanical Herbs, Taipei Medical University, Taipei, Taiwan. 5 Department of Surgery, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan. 6 Division of General Surgery, Department of Surgery, Shuang Ho Hospital, Taipei Medical University, New Taipei City, Taiwan. 7 Ph.D. Program in Biotechnology Research and Development, College of Pharmacy, Taipei Medical University, Taipei, Taiwan. 8 School of Pharmacy, College of Pharmacy, Taipei Medical University, Taipei, Taiwan. 9 Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan. 10 Master Program for Clinical Pharmacogenomics and Pharmacoproteomics, Taipei, Taiwan. 11 Clinical trial center, Taipei Medical University Hospital, Taipei, Taiwan, R.O.C.

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Tables

Table 1. TMEM240 protein, mRNA expression and promoter hypermethylation in relation to the clinical parameters of Taiwan breast cancer.¹
| Characteristics                      | All | All | All | All |
|--------------------------------------|-----|-----|-----|-----|
|                                     | All | Protein expression | mRNA expression | DNA Methylation |
|                                     | Low n(%) | High n(%) | Low n(%) | High n(%) | Low n(%) | High n(%) |
| Overall                             | 76  | 67  (88.2) | 9   (11.8) | 52  | 26  (50.0) | 26  (50.0) | 101 | 46  (8.9) | 55  (54.5) |
| Tumor Type                          |     |     |     |     |     |     |     |
| IDC                                 | 60  | 52  (86.7) | 8   (13.3) | 52  | 26  (50.0) | 26  (50.0) | 98  | 43  (43.9) | 55  (56.1) |
| ILC                                 | 1   | 1   (100.0) | 0   (0.0)  | 0   | 0   (0.0)  | 0   (0.0)  | 2   | 2   (100.0) | 0   (0.0)  |
| Others                              | 5   | 5   (100.0) | 0   (0.0)  | 0   | 0   (0.0)  | 0   (0.0)  | 1   | 1   (100.0) | 0   (0.0)  |
| Tumor Stage                         |     |     |     |     |     |     |     |
| 0, I and II                         | 48  | 41  (85.4) | 7   (14.6) | 9   | 5   (55.6) | 4   (44.4) | 19  | 7   (36.8) | 12  (63.2) |
| III and IV                          | 18  | 17  (94.4) | 1   (5.6)  | 41  | 21  (51.2) | 20  (48.8) | 79  | 38  (48.1) | 41  (51.9) |
| Tumor Size                          |     |     |     |     |     |     |     |
| T0-T1                               | 11  | 10  (90.9) | 1   (9.1)  | 40  | 24  (60.0) | 16  (40.0) | 30  | 12  (40.0) | 28  (60.0) |
| T2-T4                               | 52  | 46  (88.5) | 6   (11.5) | 9   | 2   (22.2) | 7   (77.8) | 68  | 33  (48.5) | 35  (51.5) |
| Lymph node regional metastasis      |     |     |     |     |     |     |     |
| No                                  | 27  | 25  (92.6) | 2   (7.4)  | 23  | 14  (60.9) | 9   (39.1) | 43  | 21  (48.8) | 22  (51.2) |
| Yes                                 | 36  | 31  (86.1) | 5   (13.9) | 25  | 11  (44.0) | 14  (56.0) | 51  | 24  (47.1) | 27  (52.9) |
| Grade                               |     |     |     |     |     |     |     |
| Well                                | 4   | 3   (75.0) | 1   (25.0) | 7   | 3   (42.9) | 4   (57.1) | 10  | 5   (50.0) | 5   (50.0) |
| Moderate                            | 27  | 22  (81.5) | 5   (18.5) | 17  | 9   (52.9) | 8   (47.1) | 40  | 17  (42.5) | 23  (57.5) |
| Poor                                | 29  | 27  (93.1) | 2   (6.9)  | 24  | 13  (54.2) | 11  (45.8) | 42  | 20  (47.6) | 22  (52.4) |
| ER                                  |     |     |     |     |     |     |     |
| Negative                            | 39  | 35  (89.7) | 4   (10.0) | 15  | 8   (53.3) | 7   (46.7) | 31  | 14  (45.2) | 17  (54.8) |
| Positive                            | 37  | 32  (86.5) | 5   (13.5) | 27  | 13  (48.1) | 14  (51.9) | 68  | 30  (44.1) | 38  (55.9) |
| PR                                  |     |     |     |     |     |     |     |
| Negative                            | 42  | 39  (92.9) | 3   (7.1)  | 16  | 9   (56.3) | 7   (43.8) | 40  | 16  (40.0) | 24  (60.0) |
| Positive                            | 34  | 28  (82.4) | 6   (17.6) | 26  | 12  (46.2) | 14  (53.8) | 59  | 28  (47.5) | 31  (52.5) |
| HER2                                |     |     |     |     |     |     |     |
| Negative                            | 42  | 51  (92.7) | 4   (7.3)^0.046 | 18  | 10  (55.6) | 8   (44.4) | 39  | 18  (46.2) | 21  (53.8) |
| Positive                            | 21  | 16  (76.2) | 5   (23.8) | 22  | 20  (45.5) | 3   (54.5) | 59  | 25  (42.4) | 34  (57.6) |
| TNBC                                |     |     |     |     |     |     |     |
| Yes                                 | 23  | 22  (95.7) | 1   (4.3)  | 10  | 5   (50.0) | 5   (50.0) | 15  | 6   (40.0) | 9   (60.0) |

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Table 2. TMEM240 mRNA expression and promoter hypermethylation in relation to the clinical parameters of breast cancer from TCGA.¹

|     | No | 53 | 45 | (84.9) | 8  | (15.1) | 32 | 16 | (50.0) | 16 | (50.0) | 84 | 38 | (45.2) | 46 | (54.8) |
|-----|----|----|----|--------|----|--------|----|----|--------|----|--------|----|----|--------|----|--------|

Ki-67

|     | <14% | 10  | 9   | (90.0) | 1  | (10.0) | 11 | 5  | (45.5) | 6  | (54.5) | 26 | 13 | (50.0) | 13 | (50.0) |
|-----|------|-----|-----|--------|----|--------|----|----|--------|----|--------|----|----|--------|----|--------|
|     | ≥14% | 16  | 11  | (68.8) | 5  | (31.3) | 23 | 10 | (43.5) | 13 | (56.5) | 66 | 27 | (40.9) | 39 | (59.1) |

p53

|     | Negative | 26  | 26  | (100.0) | 0  | (0.0)  |
|-----|----------|-----|-----|----------|----|--------|
|     | Positive  | 22  | 18  | (88.0)   | 3  | (12.0) |

1. These results were analyzed by the Pearson $\chi^2$ test. $P$ values with significance are shown as superscripts.
2. For some categories, the number of samples (n) was lower than the overall number analyzed because clinical data were unavailable for those samples.
3. When the TMEM240 expression level in breast tumors was less than half of the mean of TMEM240 expression levels in adjacent normal breast tissues was defined as low expression.
4. The TMEM240 promoter methylation level in breast tumors being 2-fold higher than in adjacent normal breast tissues was defined as hypermethylation.
| Characteristics    | Total | TMEM240 mRNA<sup>2</sup> |  | TMEM240 Methylation |  |
|--------------------|-------|--------------------------| |                      |  |
|                    |       | Low (n(%)) | High (n(%)) | Low (n(%)) | High (n(%)) |
| Overall            | 714   | 427 (59.8) | 287 (40.2) | 582        | 351 (60.3)  | 231 (39.7)  |
| Age                | 761   |             |             |            |             |
| < 65               | 551   | 331 (60.1) | 220 (39.9) | 453        | 264 (58.3)  | 189 (141.7) |
| ≥ 65               | 210   | 127 (60.5) | 83 (39.5)  | 170        | 108 (63.5)  | 251 (40.3)  |
| Race               | 525   |             |             |            |             |
| White              | 421   | 259 (61.5) | 162 (38.5) | 338        | 234 (69.2)  | 104 (30.8)  |
| Black/African      | 72    | 41 (56.9)  | 31 (43.1)  | 64         | 19 (29.7)   | 45 (70.3)   |
| American           |       |             |             |            |             |
| Asian              | 32    | 27 (84.4)  | 5 (15.6)<sup>0.022</sup> | 31 | 14 (45.2) | 17 (54.8)<sup>0.001</sup> |
| Menopause State    | 480   |             |             |            |             |
| Premenopause       | 123   | 76 (61.8)  | 47 (38.2)  | 103        | 69 (67.0)   | 34 (33.0)   |
| Perimenopause      | 16    | 9 (56.3)   | 7 (43.8)   | 14         | 6 (42.9)    | 8 (57.1)    |
| Postmenopause      | 341   | 218 (63.9) | 123 (36.1) | 287        | 174 (60.6)  | 113 (39.4)  |
| Histological Type  | 714   |             |             |            |             |
| ILC                | 170   | 65 (38.2)  | 105 (61.8) | 152        | 113 (74.3)  | 39 (25.7)   |
| IDC                | 504   | 341 (67.7) | 163 (32.3)<sup>0.001</sup> | 400 | 215 (58.8) | 185 (46.3)<sup>0.001</sup> |
| Mucinous Carcinoma | 15    | 8 (53.3)   | 7 (46.7)   | 14         | 11 (78.6)   | 3 (21.4)    |
| Mixed type         | 25    | 13 (52.0)  | 12 (48.0)  | 16         | 12 (75.0)   | 4 (25.0)    |
| Tumor Stage        | 535   |             |             |            |             |
| I and II           | 385   | 247 (64.2) | 138 (35.8) | 311        | 189 (60.8)  | 122 (39.2)  |
| III and IV         | 150   | 86 (57.3)  | 64 (42.7)  | 129        | 80 (62.0)   | 49 (38.0)   |
| Tumor Size         | 539   |             |             |            |             |
| T0-T1              | 137   | 93 (67.9)  | 44 (32.1)  | 114        | 80 (70.2)   | 34 (29.8)   |
| T2-T4              | 402   | 243 (60.4) | 159 (39.6) | 329        | 191 (58.1)  | 138 (41.9)<sup>0.022</sup> |
| ER                 | 535   |             |             |            |             |
| Negative           | 117   | 84 (71.8)  | 33 (28.2)<sup>0.023</sup> | 103 | 22 (21.4) | 81 (78.6)<sup>0.001</sup> |
| Positive           | 418   | 252 (60.3) | 166 (39.7) | 340        | 249 (73.2)  | 91 (26.8)   |
| Variable   | Univariate analysis | Multivariate analysis |
|------------|---------------------|-----------------------|
| Race       | 0.902               | 1.389                 |
| Age        | 2.055               | 1.516                 |
| Tumor type | 1.391               | 1.241                 |
| Stage      | 2.052               | 4.098                 |
| Menopause  | 1.256               | 1.498                 |
| TMEM240    | 2.747               | 6.172                 |

1. These results were analyzed by the Cox regression model.

2. The TMEM240 DNA methylation levels were derived from 640 breast cancer patients in TCGA data set.
| Characteristics | Total | Complete response | Progressive disease | P value |
|-----------------|-------|-------------------|---------------------|---------|
|                 | N (%) | N (%)             | N (%)              |         |
| **Chemotherapy** |       |                   |                     |         |
| Low methylation | 99    | 97 (98.0)         | 2 (2.0)            | 0.012   |
| High methylation | 158   | 142 (89.9)       | 16 (10.1)          |         |
| **Hormone therapy** |     |                   |                     |         |
| Low methylation | 24    | 21 (87.5)        | 3 (12.5)           | <0.001  |
| High methylation | 11    | 2 (18.2)         | 9 (81.8)           |         |
| **Targeted Molecular therapy** |   |                   |                     |         |
| Low methylation | 4     | 4 (100.0)        | 0 (0.0)            | 0.515   |
| High methylation | 8     | 6 (75.0)         | 2 (25.0)           |         |

1. These results were analyzed by the Fisher's exact test. The patients with a treatment duration of greater than 4 weeks were included in this analysis. When the β value of \( \text{TMEM240} \) methylation level in breast tumors was higher than 0.25 was defined as hypermethylation from TCGA data set using Infinium Human Methylation 450K BeadChip.

2. Chemotherapy drugs:
- antimetabolites drugs: 5-fluorouracil, capecitabine, gemcitabine, methotrexate.
- alkylating drugs: cyclophosphamide, cisplatin and carboplatin.
- topoisomerase inhibitors: doxorubicin, mitoxantrone and epirubicin.
- microtubule inhibitors: taxanes, vinca alkaloids, and epothilones.

1. Hormone therapy drug:
- Estrogen inhibitors: tamoxifen and fulvestrant.
- aromatase inhibitors: letrozole, anastrozole and exemestane.

1. Targeted Molecular therapy: Avastin and Herceptin.

Table 5. \( \text{TMEM240} \) promoter hypermethylation in relation to prognosis and drug treatment response in plasma of Taiwanese breast cancer patients.
| Characteristics                  | Total | Non-Progression N (%) | Progressive disease N (%) | P value |
|---------------------------------|-------|-----------------------|---------------------------|---------|
| **Overall**                     | 61    | 29 (47.5)             | 32 (52.5)                 |         |
| **TMEM240 in plasma**           |       |                       |                           |         |
| No methylation                  | 31    | 27 (87.1)             | 4 (12.9)                  | <0.001  |
| High methylation                | 30    | 2 (6.7)               | 28 (93.3)                 |         |
| **CA-153 in serum**             |       |                       |                           |         |
| Normal                          | 55    | 29 (52.7)             | 26 (47.3)                 | 0.237   |
| Abnormal (> 25 units/ml)        | 2     | 0 (33.3)              | 2 (100.0)                 |         |
| **CEA in serum**                |       |                       |                           |         |
| Normal                          | 49    | 26 (53.1)             | 23 (46.9)                 | 0.470   |
| Abnormal (> 5 ng/ml)            | 9     | 3 (33.3)              | 6 (66.7)                  |         |
| **Underwent hormone therapy**   | 36    | 23 (63.9)             | 13 (36.1)                 |         |
| **TMEM240 in plasma**           |       |                       |                           |         |
| Low methylation                 | 25    | 22 (88.0)             | 3 (12.0)                  | <0.001  |
| High methylation                | 11    | 1 (9.1)               | 10 (90.9)                 |         |
| **Ki-67 in breast tumors**      |       |                       |                           |         |
| Low expression                  | 16    | 10 (62.5)             | 6 (37.5)                  | 1.000   |
| High expression (> 15 %)        | 20    | 13 (65.0)             | 7 (35.0)                  |         |

1. These results were analyzed by the Fisher’s exact test. The patients with a treatment and monitoring duration of greater than one year were included in this analysis. When the circulating methylated TMEM240 levels normalized by circulating ACTB in plasma of breast cancer patients was higher than 0.002 was defined as abnormal.

2. For concentration of CA-153, CEA and Ki-67 expression, the number of samples (n) was lower than the overall number analyzed because clinical data were unavailable for those samples.

3. Non-Progression: Patients without Progression, Recurrence, Metastasis.

Progressive disease: Patients with Progression, Recurrence, Metastasis.

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**Figures**
Figure 1

Flowchart of gene selection and heatmap for TMEM240. (A) The criteria and step-by-step flowchart for gene selection. (B) Screening of intersecting genes by InteractiVenn. (C) Heatmap of the TMEM240 methylation pattern in breast cancer and endometrial and uterine cancer.
Figure 2

TMEM240 is localized in the cytoplasm and membrane and represses cancer cell growth and migration in breast cancer cells. A recombinant pMyc-DDK-hTMEM240 plasmid was transfected into MDA-MB-231 breast cancer cells (A) and T47D breast cancer cells (B) for 24 h, and the cells were then analyzed via immunofluorescence for TMEM240 protein (left, original magnification, ×200) and real-time RT–PCR for mRNA expression (middle). The proliferation of the MDA-MB-231 and T47D cells was analyzed using sulforhodamine B (SRB) assays (right). si-TMEM240 was transfected into MDA-MB-231 cells (C) and T47D cells (D). The cell morphology (left, original magnification, ×100), mRNA expression (middle), and rate of cell proliferation (right) of the breast cancer cells were analyzed. (E) The migratory ability of MDA-MB-231 cells after TMEM240 overexpression was measured via transwell assays. si-TMEM240 was transfected into MDA-MB-231 cells for 24 h, and the distribution of the cells was then analyzed using transwell assays (F) and wound healing assays (G). The data are presented as the mean ± SD; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001. A t-test was used to calculate group differences in all experiments. Experiments were performed using at least two biological duplicates and three technical replicates. Localization of the TMEM240 protein was determined by deconvolution and 3D reconstruction. (H) Recombinant pMyc-DDK vector control. (I) The recombinant pMyc-DDK-hTMEM240 plasmid was transfected into the cells for 24 h. Red, anti-Myc-hTMEM240 protein. Green, anti-DDK-hTMEM240 protein. Blue, DAPI staining.
Figure 3

Low expression of TMEM240 in breast cancer is mediated by promoter methylation. Representative figures showing TMEM240 protein expression as analyzed by IHC. (A) Normal mammary gland. (B) Breast cancer tissue with negative expression. (C) Breast cancer tissue with normal expression (original magnification, ×200). The scale bars indicate 200 µm. (D-E) Representative figure showing the TMEM240 mRNA expression level (D) and TMEM240 promoter hypermethylation as determined by RT–qPCR (E) in adjacent breast normal and tumor tissues. (F) Box plot of TMEM240 promoter hypermethylation levels in tissues. (G, H) DNA methylation and mRNA expression were measured after treatment with decitabine (DAC) in MDA-MB-231 (G) and T47D (H) breast cancer cells. The relative DNA methylation levels after treatment with DAC are shown in the left panel. The relative mRNA expression levels after treatment with DAC are shown in the right panel. The data are presented as the mean ± SD; ** p ≤ 0.01, *** p ≤ 0.001. A t-test was used to calculate group differences in all experiments. The experiments were performed using at least two biological duplicates and three technical replicates.
Figure 4

Hypermethylation of TMEM240 is associated with poor hormone therapy response and poor survival in breast cancer. (A) Kaplan–Meier survival curves were used to compare 10-year survival in breast cancer patients with low and high TMEM240 promoter hypermethylation. (B) Bar charts showing TMEM240 methylation levels in breast cancer patients. Orange bar: breast cancer patients with progressive disease after hormone therapy; blue bar, breast cancer patients with complete response after hormone therapy. (C, D) Box plots of TMEM240 methylation levels in patients with complete response or progressive disease after tamoxifen treatment (C) or aromatase inhibitor treatment (D). (E, F) Cell proliferation assays were performed in T47D cells treated with tamoxifen after si-TMEM240 transfection (E, right) or pMyc-DDK-hTMEM240 plasmid transfection (F, right). Brightfield views (E-F, left) are presented to illustrate the cell morphology.
Figure 5

Circulating methylated TMEM240 is increased in Taiwanese breast cancer patients with disease progression and poor hormone therapy response. The level of circulating methylated TMEM240 dramatically and gradually decreased in triple-negative breast cancer patients (A) and in breast cancer patients who received hormone therapy and did not experience recurrence or metastasis (B). (C) Recurrence and metastasis were detected in triple-negative breast cancer patients when circulating methylated TMEM240 was found in such patients after treatment, and it thereafter increased again. The concentrations of CEA and CA15-3 in serum remained normal. (D) The level of circulating methylated TMEM240 gradually increased in breast cancer patients with disease progression even after hormone therapy and chemotherapy. The concentrations of CEA and CA15-3 in serum are incapable of early monitoring of disease progression and poor treatment response. (E) Box plot showing the levels of circulating methylated TMEM240 in the plasma of 32 patients with recurrence/metastasis and in the plasma of 29 patients without recurrence/metastasis. (F, G) Box plots showing the concentrations of CEA and CA15-3 in the sera of 28 patients with recurrence/metastasis and in the sera of 29 patients without recurrence/metastasis. ***, P < 0.001. (H) ROC curves for disease progression prediction were calculated using the measured circulating methylated TMEM240 levels and the measured concentrations of CEA and CA15-3. (I) A box plot of the percentage of Ki-67 expression level in breast cancer tumors from 13 patients with recurrence/metastasis and in tumors from 23 patients without recurrence/metastasis is shown. (J) ROC curves for disease progression and hormone therapy response prediction were calculated using circulating methylated TMEM240 levels and Ki-67 expression levels.

Supplementary Files

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