Breast Cancer Risk-Associated SNPs in the mTOR Promoter Form De Novo KLF5- and ZEB1-Binding Sites that Influence the Cellular Response to Paclitaxel

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Abstract

ZEB1 (a positive enhancer) and KLF5 (a negative silencer) affect transcription factors and play inherently conserved roles in tumorigenesis and multidrug resistance. In humans, the rs2295080T-allele at the mTOR promoter locus has been associated with human cancer risk; however, the 63 bp spacing of another SNP rs2295079 has not been identified. Here, we discovered, for the first time, that rs2295079 (-78C/G) and rs2295080 (-141G/T) formed linkage haplotypes, with Ht1 (-78C/-141G) and Ht2 (-78C/-141T) being dominant, which were associated with distinct susceptibility to breast cancer, response to paclitaxel, and clinical outcomes in breast cancer. At the cellular level, compared with Ht1, Ht2 exhibits a much stronger effect on promoting mTOR expression, leading to enhanced tumor cell growth and strengthened resistance to PTX treatment. Mechanistically, the -141T allele of Ht2 creates a novel ZEB1-binding site; meanwhile, the -78C allele of Ht1 exists as an emerging KLF5-binding site, which synergistically induces promote/inhibit mTOR expression, cell proliferation, and excretion of cytotoxic drugs through the ZEB1/KLF5 axis. Our results suggest the existence of a ZEB1/KLF5-mTOR–CCND1/ABCB1 axis in human cells that could be involved in paclitaxel response pathways and functionally regulate interindividuated breast cancer susceptibility and prognosis.

Implications: This study highlights the function of haplotypes of mTOR -78C/-141G and -78C/-141T, in affecting breast cancer susceptibility and paclitaxel response regulated by ZEB1/KLF5-mTOR–CCND1/ABCB1 axis.

Introduction

Breast cancer is a leading cause of cancer-related death in women, with an estimate of more than two million new cases worldwide in 2018, and it accounts for 30% of all new cancer cases in women in the United States (1, 2). Accumulating evidence supported that the hereditary genetic variations of candidate genes, such as SNPs and their haplotypes, induced interindividualized cancer susceptibility and diverse responses to chemotherapeutic agents (3, 4). Compared with conventional analysis of individual single SNPs, recently developed multiallelic haplotype-based association analysis based on a cluster of two to three SNP markers significantly improved the power and robustness of detecting cancer susceptibility loci and responsiveness variability (5, 6). However, it is less well understood why SNPs form haplotypes, or “block-like” linkage disequilibrium (LD), and the functional advantages the linked SNPs have, and what the underlying molecular mechanisms are, need to be further examined.

The mTOR gene is located at chromosome 1q36.2 (7). mTOR acts as a central switch that determines cell fate in tumorigenesis, including cell proliferation (8–12), migration (13), and multidrug resistance (14, 15). The SNP rs2295080 (-141G/T), located in the promoter of the mTOR gene, has been found to be associated with an increased risk of various cancers, such as gastric cancer (16, 17), esophageal carcinoma (18), bladder cancer (19), etc. (20–22). However, previous studies have consistently focused on the SNP but have failed to study any molecules involved in tumorigenesis and progression. Given the functional importance of rs2295080, this SNP warrants further investigation to determine how it is controlled by key transcriptional factors, whether SNP rs2295080 gives rise to LD, and the potential functional impacts of the relevant

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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haplotypes on cell proliferation, progression, and drug response in breast cancer. ZEB1 and KLF5 are both prime elements of a network of transcription factors that play important roles in tumorigenesis and multidrug resistance (23–26). Recently, Liu and colleagues found that mTOR is positively regulated by ZEB1 because of multiple consensus E-box-binding sites for ZEB1 in mouse embryonic fibroblasts (27). KLF5, another key transcription factor, plays important roles in the regulation of cell cycle and protection against cell apoptosis (28). Furthermore, KLF5 is capable of protecting cells from a variety of harmful stimuli (29, 30).

In this study, a haplotype-based case-control study was performed on the basis of the HapMap Project Database (http://hapmap.ncbi.nlm.nih.gov/), and SNPs in the 5’ untranslated region (UTR) of mTOR gene were genotyped and sequenced. We discovered that rs2295080 (-141C/T) and rs2295079 (-78C/G) constitute linkage haplotypes, with H1t (-78C/-141G) and H2t (-78G/-141T) being dominant, which are associated with distinct susceptibility to breast cancer, response to paclitaxel, and clinical prognosis. We further found that H1t and H2t have silencer/enhancer properties, and a novel ZEB1-binding site created by the -141T allele of H2t and an emerging KLF5 binding site that exists on the -78C allele of H1t synergistically affect expression of mTOR in response to diverse paclitaxel and clinical outcomes. Molecular analyses were performed in cell lines, and xenograft mouse models revealed that ZEB1/KLF5 mediates the occurrence of functional interactions between the 5’ UTR of mTOR promoter haplotypes through the ZEB1/KLF5—mTOR—CCND1/ABCB1 axis, thereby affecting interindividualized breast cancer susceptibility, progression, and paclitaxel response.

Materials and Methods

Study population

The study was subject to approval by the Institutional Review Board of China Medical University, and all subjects gave their written informed consent prior to inclusion in the ongoing study. Briefly, 547 female patients with histopathologically confirmed breast cancer and 504 female healthy volunteers were consecutively recruited from First Hospital of China Medical University between October 2008 and June 2013. After an interview, 5 mL of peripheral venous blood was collected for genomic DNA isolation from a leukocyte cell pellet of each blood sample by using previously described methods (31). The TaqMan allelic discrimination method was used to genotype the selected SNPs. The SNP allele-specific probes were labeled with the fluorescent dyes HEX and FAM by using the TaqMan SNP Genotyping Assays of the ABI 7500 Fast Real-Time PCR platform (Applied Biosystems, Life Technologies Corporation). For quality control, 10% of the samples were confirmed by repeated DNA-sequencing analysis, and the genotypes were 100% concordant.

IHC

The samples were incubated overnight in 4°C with the primary antibody (p-mTOR 1:100, Cell Signaling Technology, #2976; mTOR 1:100, Cell Signaling Technology, #2983; ABCB1, 1:200, Boster, A00049; and CCND1, 1:200, Boster, BA0770). The immunostaining was examined under a light microscope by two pathologists blinded to the experimental conditions. The intensity of immunoreactivity was scored as follows: 0 for no staining, 1 for weak staining, 2 for moderate staining, and 3 for strong staining. The percentage of stained cells was scored by using 5% increments (0%, 5%, 10%, ... 100%). The final immunoreactive score was determined by multiplying the intensity score by the percentage of positively stained cells.

Cell culture

Human breast cancer MCF7 cell and 293T cell were purchased from Peking Union Medical College Cell Resource Center (PUMCCRC, Beijing, China). Cell lines were authenticated using short tandem repeat analysis by Shanghai GeneChem Co., Ltd. The cells were demonstrated to be free of contamination with mycoplasma by PCR. The MCF7 cells were cultured in DMEM culture medium (Invitrogen) containing 10% FBS (HyClone), 100 U/ml penicillin (Invitrogen), and 100 U/ml streptomycin (Invitrogen) with culture environment of 5% CO2 and 37°C. Cells between third and eighth passage were used for the experiments. The cells were maintained in a logarithmic growth phase by routine passages every 4 to 5 days at 1:3 split ratio. Paclitaxel-resistant MCF7 cell (MCF7/PTX) was induced by our laboratory. MCF7/PTX was added PTX on time to maintain the drug resistance phenotype.

Cell proliferation and viability assay

MCF7 or MCF7/PTX cells were seeded into 96-well plates (3,000 cells/well). Twenty-four hours after transfection, paclitaxel (0.03, 0.11, 0.46, 1.83, 7.3, 29, 117, and 468 nmol/L) was added into the culture medium. Forty-eight hours later, the cells were incubated with 10 μL of CCK-8 (Cell Counting Kit-8, Beyotime) at 37°C for 4 hours. The absorbance value was measured at 450 nm using an Anthos 2010 microplate reader (Anthos Labtec Instruments). The inhibition rate = (normal control – experiment)/ (normal control – negative control) × 100%. There was no paclitaxel in the normal control well, and there were no cells in the negative control well. The experiment was repeated three times.

Western blot analysis

The total protein of MCF7 or MCF7/paclitaxel cells was extracted and quantified. Sixty micrograms of proteins from each cell lysate were separated by 10% SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane. Primary antibodies and the final antibody concentrations are as follows: p-mTOR (1:1,000, 430.9 ng/ml, Cell Signaling Technology, #5536), mTOR (1:1,000, 89.0 ng/ml, Cell Signaling Technology, #2983), ABCB1 (1:1,000, 200 ng/ml, Boster, A00049), CCND1 (1:1,000, 200 ng/ml, Boster, BM0771), and GAPDH (1:1,000, 200 ng/ml, Boster, BM3876) were incubated overnight at 4°C. The secondary antibody (Boster, BA1054, 1:1,000, 1 μg/ml, BA1050, 1:1,000, 1 μg/ml) was incubated at room temperature for 1 hour. Chemiluminescence was detected by ECL (Amer sham). Data were statistically analyzed by FluorChem V2.0 (Alpha Innotech Corp).
Primary antibodies and the final antibody concentrations are as follows: p-mTOR (1:100, 4.309 μg/mL, Cell Signaling Technology, #5536) and mTOR (1:100, 0.890 μg/mL, Cell Signaling Technology, #2983) were incubated overnight at 4°C. The nuclei were located with DAPI (diamidino-2-phenylindole). Fluorescence was detected using a confocal laser scanning microscope (FV1000-SIM/IX81, Olympus).

Cell-cycle analysis
MCF7 and MCF7/paclitaxel cells were cultured in 6-well plates (5 × 10^4 cells well). After transfection, the cells were cultured with 7.3 nmol/L paclitaxel for another 24 hours. Then, cells were harvested, and cell density was adjusted to 10^7 cells/mL. The suspension (100 μL) was incubated in the dark at room temperature for 30 minutes with a solution of PI (propidium iodide). The cell cycle was analyzed by flow cytometry using a FACSCalibur instrument. The percentage of cells in S phase in the different groups was compared. The experiment was repeated at least three times.

In vivo xenograft experiments
The transplanted tumor model of BALB/c nude mice (female, 4-6-week-old) was prepared by subcutaneous injection at the scapula of MCF7 cells. The cells (8 × 10^6) were suspended in 200 μL PBS and Matrigel (1:1; BD Biosciences). Every 3 days, 10^4 cells were implanted subcutaneously and tumors were weighed and harvested for histologic examination. The detailed procedure was shown in the Supplementary Laboratory Animals. The assay was performed as in our previous publications, with some modifications (32, 33).

UPLC-MS/MS assay
Tissue from tumors was weighed and then homogenized with a high-speed homogenizer (Shanghai Kingdom Biochemical Instrument). Cells were cultured in 6-well plates (5 × 10^4 cells well), pcmTOR-H1, pcmTOR-H2, and NC were transfected into each group. Twenty-four hours later, the cells were cultured with 7.3 nmol/L paclitaxel for another 24 hours. Then, cells were lysed by repeated freezing and thawing. After centrifugation at 10,000 × g for 5 minutes, supernatant was collected for UPLC-MS/MS analysis. The detailed procedure was shown in the Supplementary Methods.

Luciferase reporter assay
The cells were transferred to 96-well plates (2 × 10^4/well). The pG3-H1, pGL3-H2, and pRL-TK plasmids were transfected into 293T or MCF7 cells. Fluorescence was measured by using Dual-Luciferase Reporter Assay System (Promega). The value of Fluc luciferase activity was corrected by RLuc luciferase activity. Relative luciferase activity was normalized to the negative control. Assays were conducted in triplicate in a single experiment and then as three independent experiments.

Fusion protein–NanoLUC luciferase reporter analysis
NLF1-C[CMV/Hygro] Vector was purchased from the Promega Corporation. The pNluc-ZEB1 and pNluc-KLF5 could generate an in-frame Nluc fusion protein. The pG3.H1, pG3.H2, pNluc-ZEB1, and pNluc-KLF5 plasmids were transfected at a concentration of 1 μg/mL. Cells were harvested at the following times: 4, 8, 12, 16, 20, and 24 hours, and then luciferase activity was detected by using Nano-Glo Dual-Luciferase Reporter (NanoDLR) Assay System (Promega).

Chromatin immunoprecipitation
The plasmids pcmTOR-H1 and pcmTOR-H2 were transfected to MCF7 cells. Samples were collected and subjected to immunoprecipitation with ZEB1 antibody (GENE Tex, GTX105278) and KLF5 antibody (MILLIPORE, 07-1580) overnight at 4°C. DNA fragments were purified by MiniBest Universal Genomic DNA Extraction Kit Ver.5.0 (Takara). A 2 μl of chromatin immunoprecipitation (ChIP) extraction solution was subjected to 40 cycles of amplification for qPCR assay. PCR products were sequenced by Sangon Biotech.

Statistical analysis
All data analysis was carried out using SPSS 16.0 software packages (SPSS Inc.). Associations between SNPs and breast cancer and clinical variables were analyzed by OR and 95% confidence intervals (CI) using unconditional logistic regression models. Overall survival (OS) was defined as the time between the surgery and death or last known follow-up. Disease-free survival (DFS) was measured from surgery until an occurrence of recurrence, death, or last known follow-up. The survival curves were generated by using the Kaplan–Meier method, and log-rank test was used to estimate the associations of the DFS or OS with SNPs. Multivariate Cox proportional hazards regression models were applied to obtain the adjusted HR and 95% CI for evaluating the independent prognostic value of genotypes and clinical variables. Data from qRT-PCR, reporter assays, cell proliferation assays, UPLC-MS/MS assays, and in vivo xenograft experiments were analyzed using two-tailed Student t test. All statistical tests were two-sided, and a P value of <0.05 was considered statistically significant.

Results
Chemotherapy response- and prognosis-associated polymorphism and haplotype identification in the mTOR promoter region
We explored the connection between breast cancer risk and genetic variations in the mTOR promoter region. On the basis of the HapMap Project Database (http://hapmap.ncbi.nlm.nih.gov/), -1,100 base pairs upstream from the transcription start site of mTOR gene were genotyped and sequenced in a relatively large
number of samples in a case–control study (Supplementary Table S1). Three SNPs at loci -78 (rs2295079, C/G), -141 (rs2295080, G/T), and -422 (rs17027478, C/T) were identified in the mTOR promoter region (the minor allele frequency >10%). Interestingly, SNPs at loci -78 (C/G) and -141 (G/T), but not at locus -422 (G/T), constitute a perfect LD block with \( r^2 = 0.860 \) (Fig. 1A). Ht1 (-78C/-141G) and Ht2 (-78G/-141T) account for more than 97.0% among the genotypes (Fig. 1B). The distribution frequency of Ht1 and Ht2 was 27% and 71% in controls, and 22% and 77% in breast cancer, respectively (Fig. 1C). Further cancer risk analysis revealed that Ht2 was significantly associated with an increased susceptibility of breast cancer [adjusted OR (95% CI): 1.339 (1.096–1.637), \( P = 0.004 \); Supplementary Table S2]. The distribution frequency of H2/H2 homozygous variation was a high-risk haplotype compared with those carrying Ht1/Ht1 (Fig. 1D and E).

The correlation of Ht1 and Ht2 with clinicopathologic parameters in patients with breast cancer was summarized in Supplementary Table S3. The distribution frequencies of Ht1 and Ht2 were significantly associated with tumor size (\( P = 0.019 \)), clinical stages (\( P = 0.004 \)), and lymph node metastasis status (\( P = 0.015 \)). For Ht2 carriers, a significantly increased frequency was observed in tumors of >3.0 cm [adjusted OR (95% CI): 1.438 (1.063–1.947)], clinical stage III or IV [adjusted OR (95% CI): 1.607 (1.159–2.228)], and higher in lymph node-positive patients [adjusted OR (95% CI): 1.431 (1.072–1.911)]. We therefore

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Figure 1. The association of Ht1 or Ht2 with susceptibility and clinical outcome in patients with breast cancer. A, Schematic graph representing the distribution of candidate SNPs and their haplotypes in the promoter region of mTOR gene. B, The percentage distribution of Ht1 and Ht2 in the healthy controls and the patients with breast cancer. C, Table listing the frequency distribution of haplotypes in the patients with breast cancer and controls. D and E, Pie chart illustrating and table listing mTOR haplotypes percentage distribution in both controls and patients. F, RECIST criteria and logistic regression analysis evaluated the response variation to paclitaxel-based NCT in patients with breast cancer with haplotypes of the mTOR gene. G, Kaplan–Meier plots of overall survival probability comparison between Ht1 and other haplotypes, between Ht2 and other haplotypes, and between Ht1 and Ht2 in the patients with breast cancer who received paclitaxel (PTX)-based chemotherapy. H, Comparison of the median survival time between Ht1 and other haplotypes, between Ht2 and other haplotypes, and between Ht1 and Ht2 in the patients with breast cancer who received paclitaxel-based chemotherapy.
modeled the associations with each SNP on cancer risk and clinicopathologic parameters. These conditional risk estimates and significance levels are presented in Supplementary Tables S3 and S4. These data suggest that Ht1 and Ht2 were the most predominant genotypes and potential biomarkers for evaluating individualized cancer susceptibility and progression.

For further analysis, we selected the direct haplotype of Ht1 and Ht2 to assess the response individuation of neoadjuvant chemotherapy (NCT). The response evaluation criteria in solid tumors (RECIST) were used to evaluate the response to paclitaxel-based chemotherapy. The response ratio in the patients carrying Ht2 (65.3%) was significantly lower ($P = 0.012$) than in the patients carrying Ht1 (81.8%), indicating a strong association between Ht2 and chemotherapy resistance in patients with breast cancer (Fig. 1F; Supplementary Table S5).

Next, we explored the effects of the two representative haplotypes on prognostic impacts using the Kaplan–Meier analysis and multivariate Cox regression analysis in patients with breast cancer. Specifically, with paclitaxel-based chemotherapy, the low-risk haplotype Ht1/Ht1 (-78CC/-141GG) carriers had significantly longer OS and median survival time than others (Fig. 1G and H). Multivariate Cox regression analysis repeated and verified that the haplotypes (Ht2 vs. Ht1) were independent prognostic indicators for both OS and DFS in patients with breast cancer, and Ht2 is associated with a significantly shorter OS ($P = 0.008$, adjusted HR [95% CI]: 2.353 [2.339–3.847]) and DFS ($P = 0.010$, 2.224 [2.072–3.695]) than Ht1 (Table 1). Notably, the prognostic impacts of each SNP (i.e., -78GG vs. -78CC, -144TT vs. -144GG) in the above patient cohorts were much more limited than the haplotypes (Ht2 vs. Ht1), suggesting that the linked SNPs contributed synergistically to the overall prognostic impacts of the corresponding haplotypes.

mTOR haplotypes are associated with the expression of mTOR in tissues of patient with breast cancer

Although a large number of SNPs of the mTOR gene have been found to be associated with an increased risk of various cancers, the effect of mTOR haplotypes has not yet been studied. To identify the potential haplotypes to be selected for functional analysis, we used different techniques to assess associations between these two haplotypes and local gene expression. (i) mRNA expression from the RNA-sequencing dataset platform (http://www.cbioportal.org/) of The Cancer Genome Atlas (TCGA; $n = 825$) was compared and showed elevated mRNA expression of mTOR, ABCB1, and CCND1 in breast tumors compared with adjacent normal breast tissue (Fig. 2A and B). (ii) Protein expression detected by IHC in breast cancer tissues based on 76 patients who received paclitaxel-based NCT identified a significant correlation of the risk haplotype Ht2/Ht2 carriers with elevated p-mTOR, mTOR, ABCB1, and CCND1 levels (Fig. 2C and D). (iii) Further protein correlation analysis revealed that the elevated mTOR protein expression showed a strong positive correlation with ABCB1 ($P < 0.0001$) and CCND1 ($P < 0.0001$; Fig. 2E). Consistent with the imbalance in protein expression, prognostic analysis revealed that Ht2/Ht2 carriers had shorter OS (Fig. 2F). Using our in-house data, we analyzed the association of mTOR expression with the prognosis of patients with breast cancer who received NCT ($n = 804$) in the Kaplan–Meier Plotter (http://kmplot.com/analysis/), and we found that high expression of mTOR was significantly associated with shorter

### Table 1. Multivariate COX regression analysis of mTOR genetic polymorphisms in association with DFS and OS in patients with breast cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>OS Adjusted HR (95% CI)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DFS Adjusted HR (95% CI)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
<td>1.00</td>
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<td>GC</td>
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<tr>
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<tr>
<td>TG</td>
<td>2.222 (0.970–5.102)</td>
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<td>0.896 (0.650–1.232)</td>
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<td>2.224 (2.072–3.695)</td>
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<td>&lt;0.001</td>
<td>2.566 (1.293–5.094)</td>
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<td>&gt;3.0</td>
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<td>2.942 (2.009–4.308)</td>
<td>&lt;0.001</td>
<td>2.326 (1.189–4.550)</td>
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<sup>a</sup>Adjusted HR (95% CI) were assessed using multivariate Cox regression analysis. $P < 0.05$ marked in bold.
DFS ($P = 0.013$) and OS ($P = 0.0033$; Fig. 2G). Such imbalanced expression and prognosis data indicate that Ht2 is associated with expression differences in local genes and exhibits a strong prognostic impact on survival in patients with breast cancer who received NCT, likely through altering the expression of mTOR.

**Prioritizing mTOR haplotypes for functional assays in human breast cancer cells**

To examine the haplotype-specific biological effects of the mTOR promoter, we utilized paclitaxel-sensitive MCF7 cells and PTX-resistant MCF7/paclitaxel cells transfected with pcmtOR-Ht1 (containing Ht1 genotypes of the mTOR promoter and mTOR-cDNA), pcmtOR-Ht2 (containing Ht2 genotypes of the mTOR promoter and mTOR-cDNA), and NC vector (Fig. 3A). Assays of cell viability in MCF7 cells showed that the proliferation of time course pcmtOR-Ht2 was much higher than pcmtOR-Ht1 in MCF7 but not in MCF7/PTX cells (Fig. 3B; Supplementary Fig. S1A). This finding was further confirmed at the mRNA and protein levels of p-mTOR, mTOR, ABCB1, and CCND1 by qRT-PCR, Western blot, and immunofluorescence assays (Fig. 3C–E; Supplementary Fig. S1B).

To further confirm the cellular mechanisms underlying haplotype-specific effects in breast cancer following paclitaxel treatment, we compared the effects between pcmtOR-Ht1 and
pcmTOR-Ht2-mediated mTOR overexpression. In paclitaxel-sensitive MCF7 cells, forced expression of pcmTOR-Ht2 substantially increased PTX IC50 from 4.17 nmol/L (control) to 61.61 nmol/L (Fig. 3F), but there was no significant difference in MCF7/paclitaxel cells (Supplementary Fig. S1C). Furthermore, the intracellular paclitaxel concentration was significantly reduced in MCF7 cells transfected with pcmTOR-Ht2 (Fig. 3G). However, the intracellular paclitaxel concentration was not significantly different in MCF7/paclitaxel cells transfected with different plasmids (Supplementary Fig. S1D). To further confirm these findings, we also employed cell-cycle assays. We observed that the percentage of cells in S-phase among MCF7 cells transfected with pcmTOR-Ht1, pcmTOR-Ht2, or empty vector combined with treatment with paclitaxel (Fig. 3H; Supplementary Fig. S1E). These data suggested that overexpression of Ht2...
promoted cell viability and chemoresistance to paclitaxel in MCF7 cells.

**In vivo** effects of mTOR haplotypes on tumor growth and chemoresistance of human breast cancer in xenograft mice

To investigate the **in vivo** effects of Ht1 and Ht2 on the chemosensitivity of paclitaxel, MCF7 cell lines transfected with pcmTOR-Ht1, pcmTOR-Ht2, or NC were subcutaneously implanted into nude mice, followed by paclitaxel treatment (Fig. 4A). The mice were sacrificed, and the tumors were removed during the seventh week after implantation. There were no significant differences in body weight of xenograft mice of different groups (Fig. 4B). Although paclitaxel treatment dramatically inhibited tumor growth, forced expression of pcmTOR-Ht2 almost completely reversed this inhibition. In contrast, forced expression of pcmTOR-Ht1 showed only a

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**Figure 4.**
The effect of Ht1 and Ht2 on tumor growth and chemosensitivity to paclitaxel in xenograft mice. **A,** Diagram showing the time of tumor formation and administration **in vivo.** **B,** The body weight of mice in each group (n = 5). **C,** Growth curves of tumor volumes in each group (n = 5). **D,** Tumor weight of xenograft mice at sacrifice (n = 5). **E,** Photographs of tumors of each group removed at day 35 after implantation. **F,** H&E staining showed the transplantation tumors after transfection with pcmTOR-Ht1 and pcmTOR-Ht2; IHC method showed p-mTOR and mTOR expression in the xenograft mice after transfection of pcmTOR-Ht1 and pcmTOR-Ht2. **G,** Diagram showing the time of tumor formation and blood collection **in vivo.** **H,** UPLC-MS/MS detection of docetaxel (internal standard) and paclitaxel in transplantation tumors. **I,** UPLC-MS/MS assays determined the concentration of paclitaxel in plasma and transplantation tumors at day 20, day 26, and day 32. ***, P < 0.001; **, P < 0.01; *, P < 0.05; /, P > 0.05.**
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(Fig. 5G). In contrast, pGL3-Ht2 with ZEB1 had relatively higher
respectively), day 26 (P = 0.0420 and P = 0.0434, respectively),
day 32 (P = 0.0070 and P = 0.0188, respectively). However,
there was no significant difference observed in the palcitaxel
concentrations of the plasma samples (Fig. 4H and I). These above
data indicate that, compared with Ht1, Ht2 exhibits a stronger
effect on promoting expression of mTOR, which in turn results in
a much more robust effect on promoting tumor growth and
confering resistance to palicitaxel treatment in vivo.

Transcription factor–binding analyses

We used both bioinformatic analyses and functional studies to
examine the transcription factor interactions in these two pri-
oritized haplotypes. The Jaspar (http://jaspar.binf.ku.dk/) and
h.html) prediction tools were utilized to predict the haplotypes
altering transcription factor binding (Fig. 5A and B). Subsequent-
ly, analysis of RNA fold secondary structure using RNAfold
(http://rna.tbi.univie.ac.at) showed evidence of distinct second-
ary RNA structures between Ht1 and Ht2 (Fig. 5C). Specifi-
cally, motif analysis showed that the -78C allele falls in the middle of
a consensus KLF5 motif (relative score of 86%), whereas the -78G
allele is predicted to have no favorable binding. In contrast, ZEB1
can specifically bind to the -141T allele of high-risk Ht2 (relative
score of 504) study, we showed that, compared with Ht1,
Ht2 is associated with increased risk of susceptibility of breast
cancer, as well as higher expression level of mTOR and poor
prognosis, suggesting that H2 may be a key genetic factor that
increases cancer risk and promotes progression. Notably, no
such large differences could be discovered if the analyses were
focused on the individual single SNPs (34–36). However, most previous reports have focused on the
study of single SNPs, ignoring the effect of potential haplotypes
of multiple SNPs (37, 38). Through a large-scale case (n = 547)-
control (n = 504) study, we showed that, compared with Ht1,
Ht2 is associated with increased risk of susceptibility of breast
cancer, as well as higher expression level of mTOR and poor
prognosis, suggesting that H2 may be a key genetic factor that
increases cancer risk and promotes progression. Notably, no
such large differences could be discovered if the analyses were
focused on the individual single SNPs (i.e., comparing -78G
with -78C or comparing -141G with -141T). Thus, two lineage
variations of each haplotype allele may function synergistically or
in combination to cause the overall effects or impacts of the
given haplotype.

To further verify whether the motifs display competitive bind-
ing to any prioritized haplotype sites, luciferase activities were
assessed in 293T cells and MCF7 cells transfected with promoter–
reporter constructs pGL3-Ht1 and pGL3-Ht2 (Fig. 5E and F).
Assays of luciferase activities showed that the transcriptional
activities of Ht2 sequence were much higher than those of Ht1
sequence in both 293T and MCF7 cell lines (P < 0.0001).

Then, we cloned pNluc-KLF5 constructs (that express Nluc and
KLF5 fusion proteins) and pNluc-ZEB1 constructs (that express
Nluc and ZEB1 fusion proteins), which were cotransfected with
pGL3-Ht1 or pGL3-Ht2 plasmids, respectively. We verified that
transcriptional activities of pGL3-Ht1 can be continuously and
time-dependently decreased by KLF5 (P < 0.0001), indicating that
KLF5 acts as an orientation-independent transcriptional silencer
(Fig. 5G). In contrast, pGL3-Ht2 with ZEB1 had relatively higher
target gene promoter activity than without ZEB1 (P < 0.0001),
suggesting that ZEB1 acts as an orientation-dependent enhancer
(Fig. 5H). To further assess the allelic binding specificity of the
mTOR locus, we performed the KLF5 and ZEB1 ChIP, followed by
qPCR and sequencing. ChIP assays confirmed enrichment of KLF5
binding to Ht1 and ZEB1 binding to Ht2 (Fig. 5I and J).

Prioritizing candidate haplotypes for bioinformatics and
functional assays

We considered two putative transcription factors that play a
role in mTOR regulation. This finding was further confirmed at
the cell viability, mRNA, and protein expression levels, as detected by
CCK8, qRT-PCR, and Western blot, respectively. The cell viability of
MCF7 cells transfected with pcmTOR-Ht1 and KLF5 was much
lower than that of cells transfected with pcmTOR-Ht2 and KLF5.
This finding is because KLF5 can inhibit the overexpression of
pcmTOR-Ht1 but cannot inhibit pcmTOR-Ht2. Remarkably, the
cell proliferation was strongly elevated in MCF7 cells transfected
with pcmTOR-Ht2 combined with ZEB1, as compared with cells
transfected with pcmTOR-Ht1 combined with ZEB1. This result is
because ZEB1 could elevate the overexpression of Ht2 but not Ht1
(Fig. 6A). Moreover, similar results were observed in mRNA and
protein expression data (Fig. 6B–D). Taken together, the compe-
tition with transcription factor–binding sites suggested that the
haplotypes synergistically contribute to substantially promoting
the expression of mTOR by facilitating the binding of ZEB1 (an
enhancer) and inhibiting the binding of KLF5 (a silencer; Fig. 6E).

Discussion

Genetic variation of SNPs constitutes the genetic basis for cancer susceptibility and different responses to chemotherapy
(34–36). However, most previous reports have focused on the
study of single SNPs, ignoring the effect of potential haplotypes
of multiple SNPs (37, 38). Through a large-scale case (n = 547)-
control (n = 504) study, we showed that, compared with Ht1,
Ht2 is associated with increased risk of susceptibility of breast
cancer, as well as higher expression level of mTOR and poor
prognosis, suggesting that H2 may be a key genetic factor that
increases cancer risk and promotes progression. Notably, no
such large differences could be discovered if the analyses were
focused on the individual single SNPs (i.e., comparing -78G
with -78C or comparing -141G with -141T). Thus, two lineage
variations of each haplotype allele may function synergistically or
in combination to cause the overall effects or impacts of the
given haplotype.

In addition, many previous studies have simply reported the
data of correlation analysis of the SNPs and cancer phenotypes,
with limited efforts to understand whether and how the SNPs
affect target gene expression, cancer pathogenesis, and drug
response (39, 40). Here, we show that, compared with Ht1, Ht2
weakens the binding of KLF5 and enhances the binding of ZEB1,
thereby resulting in a much stronger effect on promoting the
expression of mTOR. Thus, our studies provide an interesting
model showing how two linked SNPs in the promoter region of a
given gene coordinately fine-tune the expression of the target
gene, highlighting the functional importance and necessity of
forming haplotypes or LD.

mTOR protein, a serine/threonine protein kinase, can be phos-
phorylated by itself or by PKB, and thereby regulating the cell
growth, survival and autophagy, and so forth (41). PKB exerts its
function by phosphorylating Ser2448 of mTOR protein.
Figure 5.
Transcription factors KLF5 and ZEB1 binding analyses. A and B, Jaspar bioinformatics predicting allele-specific binding sites of Ht1 with KLF5 (left) and Ht2 with ZEB1 (right). C, RNAfolder bioinformatics predicting the diversity of secondary structure with the variations of Ht1 and Ht2. The module of specific binding affinity between KLF5 and -78C within Ht1 and ZEB1 and -141T within Ht2. D, TCGA dataset showed that elevated mTOR mRNA expression was positively correlated with ZEB1 and negatively correlated with KLF5 using RNA-sequencing. E and F, Schematic graph representing the construction of pGL3-Ht1 and pGL3-Ht2 recombinant plasmids; luciferase reporter gene assay depicting the relative mTOR gene transcription activity after transfection of pGL3-Ht1 and pGL3-Ht2 to 293T and MCF7 cells. G and H, Protein–luciferase fusion reporter assays depicting the transcriptional activities from 0 to 24 hours in each group. I and J, qPCR and ChIP assays using antibodies against KLF5 or ZEB1 in MCF7 cells. Normal rabbit IgG was used as a nonspecific antibody control. Graphs present the results of two biological replicates. ***, \( P < 0.0001 \).
However, amino acid starvation rapidly attenuated the reactivity of the Ser2448 phospho-specific antibody with mTOR (42). Our additional functional studies demonstrated that Ht2 exhibits a much stronger effect on promoting mTOR, CCND1, and ABCB1 expression, leading to enhanced tumor cell cycle, cell proliferation, and increased resistance to paclitaxel treatment, which is in agreement with the clinical data in patients with breast cancer.

The xenograft mice model of this study was performed by revised method as described in previous publication (43–45) and in this revised manuscript. The MCF7 cells were subcutaneously injected to nude mice with a Matrigel (1:1) mixture instead of estrogen. Previous studies supported that tumor cell lines that are difficult to form tumor in nude mice can easily grow if implanted with Matrigel, and the tumor formation rate is mainly

Figure 6.
Bioinformatics and functional assays of KLF5 and ZEB1. A, Cell proliferation and viability assay in MCF7 cells transfected with KLF5 or ZEB1 vectors with pcmTOR-Ht1 and pcmTOR-Ht2. B, qRT-PCR assays of mTOR mRNA expression. C and D, Western blot assays of p-mTOR and mTOR protein expression, accompanied with ABCB1 and CCND1. E, A graphical abstract of mTOR haplotypes regulated by KLF5 and ZEB1 in breast cells. KLF5 binds to the low-risk allele of Ht1 (-78C/-141G), whereas ZEB1 binds to the high-risk allele of Ht2 (-78G/-141T) to regulate gene transcription, expression, cell proliferation, and chemoresistance to paclitaxel. **, P < 0.01; *** , P < 0.001; ****, P < 0.0001.
dependent on the concentration of Matrigel and cell numbers (46, 47). Thus, Matrigel could assist tumor formation and then save study process and animal costs for less feeding time. Recent studies showed that the number of MCF7 cells injected into the mice was from 2 × 10^5 to 1 × 10^6 (48, 49). In our study, 8 × 10^6 MCF7 cells were injected per mice with Matrigel (1:1). More importantly, we searched the relevant articles and found that estrogen can upregulate mTOR and protein expression of ABCR1 and CCND1 (50, 51), which is not suitable for our study.

In conclusion, we have identified novel functional haplotypes in the mTOR promoter region in patients with breast cancer. To date, only very few published SNP-related studies have shown data from animal models that assess the biological functions of the SNPs or haplotypes, and our study will undeniably add an interesting new example (52, 53). These novel mTOR haplotypes could serve as potential biomarkers for evaluating cancer susceptibility and drug response. Patients carrying Ht2 should be treated differently from those carrying Ht1, as the former are often resistant to standard NCT (e.g., paclitaxel treatment) due to increased mTOR expression. Therefore, our studies not only provide an excellent model showing how haplotypes coordinate


to target gene expression and thereby affect cancer susceptibility and drug responses but also suggest that mTOR promoter haplotypes are important biomarkers for breast cancer risk stratifications and interindividualized treatment for patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Authors’ Contributions

Conception and design: Q. Chen, H. Wu
Development of methodology: Q. Chen, M. He, Y. Wang, H. Zhao, O.J. Olapade, H. Wu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Gaan, F. Jin, Y. Liu, H. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Hu, J. Zhang
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Wei, Y. Yao, H. Wu
Study supervision: J. Chen, M. Wei

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Breast Cancer Risk–Associated SNPs in the *mTOR* Promoter Form
*De Novo* KLF5- and ZEB1-Binding Sites that Influence the Cellular Response to Paclitaxel

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