Original Article ELOVL2: a novel tumor suppressor attenuating tamoxifen resistance in breast cancer

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Abstract: Epigenetic events have successfully explained the cause of various cancer types, but little is known about tamoxifen resistance (TamR) that induces cancer recurrence. In this study, *via* genome-wide methylation analysis in MCF-7/TamR cells we show that elongation of very-long chain fatty acid protein 2 (ELOVL2) was hypermethylated and downregulated in the samples from TamR breast cancer patients (n = 28) compared with those from Tam-sensitive (TamS) patients (n = 33) (P < 0.001). Strikingly, in addition to having tumor suppressor activity, ELOVL2 was shown to recover Tam sensitivity up to 70% in the MCF-7/TamR cells and in a xenograft mouse model. A group of genes in the AKT and ERa signaling pathways, e.g., *THEM4*, which play crucial roles in drug resistance, were found to be regulated by ELOVL2. This study implies that the deregulation of a gene in fatty acid metabolism can lead to drug resistance, giving insight into the development of a new therapeutic strategy for drug-resistant breast cancer.

Keywords: Breast cancer, epigenetics, methylation, tamoxifen resistance

Introduction

Tamoxifen is a non-steroidal antagonist of the estrogen receptor. It has been the first choice for adjuvant therapy in estrogen receptor-positive breast cancer as it reduces cancer recurrence and the annual mortality rate [1]. Despite the obvious benefits. 40% of breast cancer patient show cancer recurrence 5-10 years after initial therapy, which is one of the major setbacks for the clinicians [2]. This is partly because of the complexity of the signaling pathways that influence estrogen-mediated regulation in breast cancer [3, 4]. Thus, identifying the key molecular markers and elucidating the molecular mechanism of drug resistance are pivotal for offering appropriate treatment options to cancer patients. During the course of tamoxifen-resistance (TamR) acquisition, cancer cells undergo cellular as well as molecular changes. A key change in these cells is increased proliferation and decreased apoptosis via BAX and BCL2 regulation [5]. In addition, the TamR cells show greater stemness phenotype by over-expressing Nanog, Oct3/4, and Sox2 [6].

Complex factors/pathways are attributed to TamR cells, including the activation of estrogen receptor (ER) signaling, up-regulation of growth factors (HER2, EGFR, FGFR, and IGF1R), alterations in RTK, a crosstalk among them, and consequently, the deregulation of the PI3K/AKT/ mTOR pathway [7]. Cyclin D1/CDK4/6 complex is a target of the PI3K/AKT/mTOR pathway and has also been shown to crosstalk with the ER signaling pathway [8]. Previous studies have shown different Tam targets and their dysregulation from ER in TamR cancer, e.g., androgen receptor [9], Hedgehog signaling pathway [10], and non-coding RNAs [11], suggesting that the mechanism of TamR is far more complicated than just the modulation of ER-associated activity.

Therapeutic strategies for treating TamR cancer are under development, mainly targeting RTK pathways and PI3K/AKT/mTOR axis [12]. Further, cell cycle proteins can be targeted, for example, by using CDK4/6 inhibitors in combination with Tam [13]. Other approaches include targeting AKT pathway [14] and MYC [15] that are highly expressed in TamR cancer cells. However, clinical improvement has only been modest for these approaches till date; this may be because the affected pathways differ between patients. Thus, establishing accurate prognostic markers would hold the key to effective therapy for TamR breast cancer.

ELOVL2 is a member of the mammalian microsomal ELOVL fatty acid enzyme family, involved in the elongation of very long-chain fatty acids required for various cellular functions in mammals [16]. A study using ElovI2^{-/-} mice reaffirmed the importance of ELOVL2 for the elongation activity in rodents [17]. A prime characteristic of the gene is that the CpG near the gene exhibits consistent age-related changes in various tissues [18]. These strong associations have led to the development of a predictor that can accurately estimate the chronological age based on the methylation levels at the specific CpG site [19]. However, the relationship of ELOVL2 with cancer occurrence or development is unknown. Furthermore, no role of ELOVL2 in cancer drug resistance has been elucidated. In this study, MCF-7/TamR cells were developed in the aim of identifying epigenetically regulated genes that were crucially involved in the acquisition of TamR for cancer cells. Genome-wide methylation analysis represented ELOVL2 to be highly hypermethylated, which accompanied downregulation of the gene. The altered methylation and expression also appeared in tissues of TamR breast cancer patients. The involvement of ELOVL2 in the recovery of Tam sensitivity was suggested by presenting experimental evidence via in vitro as well as in vivo xenograft animal models. It was also suggested that ELOVL2 is a novel tumor suppressor by showing its lower expression in cancer and its inhibitory effect on cancer cell growth. Finally, the molecular mechanism of overcoming TamR by ELOVL2 was elucidated focusing on the signaling pathway, including THEM4 that was revealed as a target of ELOVL2.

Materials and methods

Cell culture and establishment of tamoxifenresistant MCF-7 (MCF-7/TamR) cells

The human epithelial breast cancer cell line MCF-7 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured under a humidified condition at 37°C, 5% CO₂ in RPMI 1640 medium (Gibco BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum (Capricorn, Germany) and 2% penicillin/streptomycin (Capricorn). All cells were used within 12 passages after resuscitation of stocks. The MCF-7/TamR cells were generated by culturing MCF-7 cells in the presence of 4-hydroxytamoxifen (Tam) (Sigma-Aldrich, St. Louis, MO, USA) in complete RPMI 1640 medium. The cells were continuously exposed to increasing concentrations of Tam up to 160 nM over a period of 22 weeks, during which the medium was changed twice a week.

Study subjects

Solid tissues and slide-mounted formalin-fixed paraffin-embedded (FFPE) tissue sections of tumor samples were obtained from patients who underwent surgery between 2012 and 2013 at the National Cancer Center (NCC) in Korea. TamS tissues were obtained from patients who showed a clinical response to Tam, *i.e.*, no tumor recurrence (n = 33). TamR tissues were obtained from patients who subsequently developed TamR (defined as disease recurrence while administering Tam; n = 28). Clinical details are presented in Table 1. All patients provided written informed consent to donate the removed tissues to NCC in Korea. and samples were obtained according to the protocols approved by the Research Ethics Board of NCC.

Generation of stable cell lines

Lentiviral particles with control clones and human ELOVL2 ORF clones containing C-terminal mGFP tag were purchased from OriGene (Rockville, MD, USA). MCF-7 and MCF-7/TamR cells were seeded at a density of 5×10^3 cells/ well in a 96-well plate 1 day before transduction. The next day, the cells were infected with lentivirus for 4 h in the presence of 8 µg/mL polybrene (Sigma-Aldrich), and then the medium was replaced with a fresh complete medi-

and the control group			
	Tamoxifen resistant (n = 28)	Control (n = 33)	P-value
Menopausal status			0.53
Pre	5	3	
Post	23	30	
Primary tumor size			0.33
≤ 2.5 cm	10	17	
> 2.5 cm	18	16	
Presence of node metastasis			0.51
Negative	12	18	
Positive	16	15	
Ki-67			0.15
≤ 20%	14	23	
> 20%	14	10	

 Table 1. Clinicopathologic features in the tamoxifen resistant

 and the control group

um. After 72 h, the cells were selected using 1 $\mu g/mL$ puromycin (Thermo Fisher Scientific, Waltham, MA, USA) for 10 days.

Cell transfection

siRNAs against ELOVL2 and THEM4 were purchased from Bioneer (Daejeon, Korea), and an ELOVL2-overexpressing vector was developed using the pEZ-MT02 plasmid vector (GeneCopoeia, Rockville, MD, USA) by CosmoGenetech (Seoul, Korea). All siRNAs were diluted in Opti-MEM Medium (Gibco BRL) with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), and the mixture was incubated for 5 min. The cells were transiently transfected at final concentrations of 20 nM or 40 nM with siRNA following the manufacturer's instructions. Overexpression vectors (2 µg) were transfected into the cells using Lipofectamine 3000 transfection reagent (Invitrogen). After 24 h of transfection, cells were harvested and used for the following experiments. All results for the optimization of transfection are demonstrated in Figure 1.

Cell proliferation assay

The cell growth rate was monitored by colony formation assay and colorimetric assay using CCK-8 reagent (Dojindo, Kumamoto, Japan). In all, 3×10^3 cells/well were seeded onto a 96-well plate and cultured up to 5-7 days. Following staining with CCK-8 solution according to the provided instructions, optical densi-

ties were measured on a microplate reader (Sunrise, Tecan, Switzerland) and OD₅₉₅ was eliminated from the OD_{450} . For colony formation assay, cells were seeded at a density of 3×10^3 cells/ dish on a 60-mm culture dish. After transfection and Tam treatment, cells were maintained in a 5% CO₂ incubator (37°C) for 14-20 days. Colonies were fixed with a 7:1 mixture of methanol and acetic acid, stained using 0.2% crystal violet (Gibco BRL), and counted with ImageJ software (NIH, MD, USA).

Flow cytometric analysis

Apoptosis was analyzed using an APC Annexin V Apoptosis Detection Kit with PI (BioLegend, San Diego, CA, USA). Annexin V staining was performed for cells diluted in Annexin V binding buffer for 8 min followed by propidium iodide (PI) reagent treatment for 10 min. Samples were measured using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) with 488-nm and 640-nm lasers. To monitor Tam uptake by cells, 1×10^6 cells seeded in a 60-mm dish were treated with FLTX1 (Aobious, Gloucester, MA, USA, AOB4054) for 2 h at final concentration of 10 µM. Cells were then harvested after washing with PBS, and a concentration of 1×10^6 cells/mL was prepared. FLTX1 fluorescence was detected with Becton Dickinson FACSAria III (BD Biosciences) and analyzed with the Flowing Software 2.5 (http:// flowingsoftware.btk.fi/).

Tam sensitivity assay

Alterations in sensitivity to Tam were measured by cytotoxicity assay. Briefly, 1×10^4 cells were seeded per well in a 96-well plate and transfected with recombinant cDNA-harboring plasmids and/or siRNAs. On the following day, Tam dissolved in sterile-filtered ethanol was added to cells at final concentrations of 0, 0.05, 0.1, 0.5, and 2 µM with a final ethanol concentration 0.1%. After 24 h, 10 µL of CCK-8 solution was added to each well and the plate was incubated for 90 min. Following this, the plate was read at 450 nm on a plate reader.



Figure 1. Optimization of plasmid and siRNA transfection into cultured cells. MCF-7/TamR cells are transiently transfected with an ORF to induce upregulation or with a siRNA to induce downregulation of the indicated gene. A. Overexpression of ELOVL2. Recombinant plasmid DNA (1-2 μ g/mL) is transfected, and 2 μ g/mL is used for further transfection. B. Expression of ELOVL2 is confirmed by qPCR and Western blot analysis. C. Downregulation of THEM4 using siRNA, as judged by qPCR. Two siRNAs targeting different sites of THEM4 were used in MCF-7/TamR cells (left) and MCF-7/TamR ELOVL2 ORF cells (right).

Methylation and expression microarray experiment

Genome-wide methylation analysis was performed with Macrogen (Seoul, Korea) on Illumina Infinium Human Methylation 450K and Illumina Infinium Methylation EPIC BeadChip (Illumina, San Diego, CA, USA) covering over 450,000 and 850,000 CpG sites, respectively, to compare the DNA methylation profiles between MCF-7 and MCF-7/TamR. All arrays were processed with Illumina GenomeStudio v2011.1. To identify global gene expression profiles, total RNA of MCF-7/TamR/ORF NC or MCF-7/TamR/ELOVL2 ORF cells was profiled using the SurePrint G3 Human Gene Expression 8x60K v3 microarray technology (Agilent, Santa Clara, CA, USA) containing 58,201 probes by Lugen Sci (Seoul, Korea). Agilent Feature Extraction software (v11.0.1.1) was used to extract and process raw data. The microarray data are deposited in the GEO database web-

site (http://www.ncbi.nlm.nih.gov/geo/) with the SuperSeries accession number GSE-132617: expression array, GSE132614; methylation array, GSE132615 and GSE132616.

Pathway and clustering analysis

Ingenuity Pathway Analysis tool (Ingenuity Systems, Redwood City, CA, USA) was used to generate significant networks and biological functions for the differentially methylated genes within the promoter ($|\Delta\beta| \ge 0.2$, *P*-value < 0.05) by acquisition of Tam resistance, and for the differentially expressed genes (|fold change| ≥ 2 , *P*-value < 0.05) by overexpressing ELOVL2 in MCF-7/TamR. Genes with significant changes were clustered using Clustering 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and the results were visualized using the TreeView v1.1.6 program (http:// jtreeview. sourceforge.net/).

Methylation-specific PCR (MSP) and quantitative real-time RT-PCR (qPCR)

MSP was performed to determine the methylation level of specific CpG sites, as previously described [20]. Briefly, DNA and RNA from FFPE sections were extracted using the RecoverAll Multi-Sample RNA/DNA Workflow (Invitrogen). Total DNA and RNA were prepared using ZR-Duet DNA/RNA MiniPrep kit (Zymo research, Irvine, CA, USA) from solid tissues and cultured cells. For preparing samples for methylation analysis, the genomic DNA was treated with bisulfite using a Zymo Research EZ DNA Methylation Kit (Zymo Research). Demethylation of the cytosine residues was achieved by exposing the cells to culture media containing a methyltransferase inhibitor, 5-Aza-2'-deoxycytidine (Aza) (Sigma-Aldrich), at a concentration of 5 µM for 72 h. PCR was conducted using 4-8 ng of DNA, and the yielded signals were calculated. To identify the transcript level of coding genes, cDNA was synthesized using a ReverTra Ace qPCR RT MasterMix with gDNA Remover kit (Toyobo, Osaka, Japan). gPCR analysis was conducted using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) on an ABI 7300 instrument (Applied Biosystems, Foster City, CA, USA). Oligonucleotide primers were purchased from Bionics (Daejeon, Korea) (Table 2).

Western blot analysis

Protein extraction from cultured cells and Western blot analysis were performed as previ-

ously described [21]. The following antibodies were used: anti-ELOVL2 (1:500, Bioss, Woburn, MA, USA, bs-7053R), anti-THEM4 (1:500, Abcam, Cambridge, MA, USA, ab106435), anti- β -Actin (1:1000, Bioss, bs-0061R), anti-phospho-AKT (1:300, Bioss, bs-5182R), anti-AKT (1:2500, Abcam, ab179463), and HRP-conjugated anti-rabbit IgG antibody (1:1000, GeneTex, Irvine, CA, USA, GTX213110-01). The bands on the membrane were detected using the ECL reagent (Abfrontier, Seoul, Korea) and analyzed with Image Lab software (Bio-Rad, Herculer, CA, USA).

Tumor xenograft experiments

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Dongguk University (No: IACUC-2017-010-1). We used 6- to 7-week-old female BALB/c nude mice (Orient Bio, Seongnam, Korea) for MCF-7 and MCF-7/TamR-derived xenograft models. The mice were anesthetized with a mixture of isoflurane (Piramal Critical Care, Mettawa, IL, USA) and oxygen, and administered 17β-estradiol pellets (0.72 mg/pellet total dose; Innovative Research of America, Sonnasota, FL, USA) subcutaneously in the lateral neck area. On the next day, subcutaneous injections of 1×10^7 breast cancer cells resuspended with 100 µL of 1:1 mixture of PBS (Gibco BRL) and Matrigel (BD Biosciences, Bedford, MA, USA) were administered to the mice. Tumor growth was monitored weekly, and tumor volumes were calculated based on the following formula: length \times width² \times 0.5. When tumor sizes reached approximately 100 mm³. the mice were randomized into two groups for Tam treatment (Sigma-Aldrich). One group received intraperitoneal administration of 100 μ L of 1 mg/kg Tam in corn oil (Sigma-Aldrich) and the other group was injected with a vehicle control for 5 days a week during the experiment. After 7 weeks of implantation, animals were sacrificed and tumors were harvested. The cancer tissues were fixed in 4% paraformaldehyde and embedded in paraffin blocks for histological analysis by Logone Bio (Seoul, Korea).

Immunohistochemical staining

Immunohistochemical analysis was performed using tumor tissues of xenograft mice. To do this, paraffin blocks were sectioned 10 μm thick, organized into slides, and rehydrated

Gene	Forward primer $(5' \rightarrow 3')$	Reverse primer (5'→3')	Supplier		
GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG	IDT		
SLC19A1	CATCGCCACCTTTCAGATTG	CGGAGTATAACTGGAACTGCTTG	Bionics		
SKAP1	TGCTAATGAGAAGAGCAAGCA	GCTGGACTAGTAGCTGTAAACTCA			
ELOVL2	ATGCTGGCAGAGCTCATTCT	ACCTTGGCTACCCGGATGT			
CD59	TGGGATTACAGGTTCTGTGGA	TGCAGGCTATGACCTGAATG			
MMP1	GACAGATTCTACATGCGCAC CCCTTTGAAAAACCGGACTTC				
THEM4	ACCCGAGCTGAGGTCATTTT				
EGFR	GCGTTCGGCACGGTGTATAA				
CCND1	TGTGCCACAGATGTGAAGTT CGGGTCACACTTGATCACTC				
CD146	CACCGTCCCTGTTTTCTACC	GTGCTGGGGTTCTGCTTG			
BCAR3	CCCGACAGGTGTCTGAAAAC	CGCTGAGTCGCAGAACTGT			
BAG1	CACACTGATCCTGCCAGAAA	GCCAGGGCAAAGTTTGTAGA			
IGF1	ACCTTGGGCAAGTCACTTCA	TGTTGCACCCTTACAGCAAC			
BMF	GAGCCATCTCAGTGTGTGGAG	GCCAGCATTGCCATAAAAGAGTC			
FTO	ATAGCTGTGAAGGCCCTGAA	TTGGTGGGTGGCATTGAGAT			
TGFB1	GTTGTGCGGCAGTGGTTG	GTGAACCCGTTGATGTCCAC			
ELOVL5	GGTGCCACACTTAATAGCTTCA	GGGACTGACGACAAACCATAGT			
ESR1	CTTGCTCTTGGACAGGAACC GACAGAAATGTGTACACTCCAGA				
HYOU1	GGCAAGAGGGGCACTATGG	CTTCATGGACTCACTGCCCA			
S100A9	GCTCCTCGGCTTTGACAGA	CACCAGCTCTTTGAATTCCCC			
CDC42EP2	AGCTCCTGAGACCTTGCTG AGGACGGCAAACTCTTCTGA				
AKT1	AAGGAAGTCATCGTGGCCAA AGTACTTCAGGGCTGTGAGG				
AKT2	ACGGGCTAAAGTGACCATGA TGTGTGAGCGACTTCATCCT				
AKT3	GATACTCCAGAGGAAAGGGAAGA GATGGGTTGTAGAGGCATCC				
mTOR	CCTCAGTGGTACAGGCACAC CTCAGACGCTCTCCCTCCAT				
PIK3CA	AGAGCCCCGAGCGTTTCT	CTAGGATTCTTGGGGGGCATC			
PDK1	TGCTAGGCGTCTGTGTGATT	TGGCTCTCATTGCATTCTTG			
CREB1	GAGGAGCTTGTACCACCGG	TGCTGGCATAGATACCTGGG			
ELOVL2 M	ATAATTAGGTAGATGGTAcg	TCCATATTCAATATTTACCTC	Bionics		
ELOVL2 U	ATAATTAGGTAGATGGTAtg	TCCATATTCAATATTTACCTC			
	Product name or sequence $(5' \rightarrow 3')$		Supplier		
THEM4 siRNA (1)	Sense: GAGGAAGUUUAGACAAUGAtt	Bioneer			
	Antisense: UCAUUGUCUAAACUUCCUCtt				
THEM4 siRNA (2)	Sense: GAGGAUUCAGUUUCCAUCUtt				
Control siRNA	trol siRNA Negative control siRNA (Cat. No. SN-1003)				
*ELOVL2 ORF vector	Cosmogenetech				
Negative control ORF	Genecopoeia				
ELOVL2 (NM_01777	Origene				
Lenti-ORF Control Pa					

 Table 2. Information of primers for qPCR and siRNAs employed in this study

*ELOVL2 ORF is subcloned into Genecopoeia ORF Vector (Cat. No. EX-M0461-M02).

through a graded ethanol series. Endogenous peroxidase activity in sections was ceased with 0.3% H_2O_2 treatment for 15 min and then rabbit anti-ELOVL2 (1:400, Bioss, bs-7053R) or rabbit anti-THEM4 (1:100, Abcam, ab106435) was

applied for 1 h at room temperature followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibodies (Dako, Glostrup, Denmark, K4003). Liquid diaminobenzidine tetrahydrochloride (DAB) (Dako, K3468) was used as a chromogen to detect horseradish peroxidase activity. After counterstaining with Mayer's hematoxylin, immunohistochemical images were generated using panoramic MIDI scanner (3Dhistech, Budapest, Hungary). The ImageJ program (NIH) was used to profile the DAB-positive areas of immunohistochemical images.

Statistical analysis

For microarray data, observations with adjusted *P*-values \geq 0.05 were removed and were excluded from further analysis. Adjustments were made to control for false discoveries. Following adjustments, the remaining genes were defined as differentially methylated if they displayed an increased or decreased methylation level which was equal to or higher than 0.2 compared with the control, or differentially expressed if they displayed at least a 2-fold difference compared with the control. Student's t-test was implemented to demonstrate statistical significance for all data from gPCR, MSP, ISH, and Western blot analysis comparing samples and control groups. Chi-squared test was used to analyze the differences in the rate of each variable for tumor tissues. Statistical analyses were conducted using SPSS for Windows, release 17.0 (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean ± standard error and considered statistically significant at P-value < 0.05.

Results

MCF/TamR cells acquired a faster growth rate

As a prerequisite to explore the molecular mechanism of TamR, a MCF-7/TamR cell line was established by exposing the MCF-7 cells to increasing concentrations of Tam up to 160 nM for 22 weeks (Figure 2A). The finally developed MCF-7/TamR cells showed a higher growth rate compared with their parental cells (Figure 2B) and also a large number of cells survived against high concentrations of Tam (0.1 and 0.5μ M) as judged by the colony formation assay (Figure 2C). The apoptosis rate was lower in TamR cells and less affected by Tam than in the MCF-7 cells (Figure 2D), possibly explaining the higher growth and survival rate in the presence of Tam. The acquisition of TamR was further monitored by examining a few marker genes, the expressions of which were previously known to be altered in the course of TamR acquisition. Thus, EGFR, CCND1, CD146, and BCAR3 were upregulated, whereas BAG1 and IGF1 were downregulated, as previously observed [22-25], confirming appropriate generation of MCF-7/TamR cells (**Figure 2E**). Notably, the level of Tam inside the TamR cells was not decreased compared with that in the parental MCF-7 cells after the two cell types were equally treated with Tam, indicating that the resistance is not caused by a net decrease of the drug transport across the plasma membrane (**Figure 2F**).

ELOVL2 is downregulated by DNA hypermethylation in TamR breast cancer

Genome-wide methylation analysis was performed in duplicates for each sample of MCF-7 and MCF-7/TamR cells. Comparison of the two cell types showed hyper- and hypo-methylation with $|\Delta B| \ge 0.2$ at 331 and 94 CpG sites. respectively, corresponding to 356 unique genes (Figure 3A). Among highly altered genes, ESR1 i($\Delta\beta$ = 0.35), MAGED1 ($\Delta\beta$ = 0.3), and RASAL1 ($\Delta\beta$ = 0.36) were listed, alteration in methylation of which in TamR cells has been previously known [26-28], indicating the reliability of MCF-7/TamR cells developed in the current study. The microarray data were also verified by examining the expression of five randomly selected genes from the highly altered genes via qPCR. Consequently, hypermethylated SCL19A1, SKAP1, and ELOVL2 were downregulated, whereas hypomethylated CD59 and MMP1 were upregulated (Figure 3B), supporting the close relationship between methylation and expression. Next, the 356 genes were examined for functional inter-relatedness using the IPA software tool. The top network with the highest confidence was "Skeletal and Muscular Disorders, Cellular Assembly and Organization, Connective Tissue Development and Function" (Figure 3C). Canonical pathway analysis identified "Neuroactive ligand-receptor interaction" as the predominant pathway (Figure 3D). Disease and function annotation analysis indicated that genes associated with "Cancer" and "Cell death and survival" are frequently included (Figure 3E).

To identify a novel and pivotal marker that contributes to the acquisition of TamR, ELOVL2 in **Table 3** was selected because the gene showed



Figure 2. Establishment of MCF-7/TamR cells. A. MCF-7 cells are treated with Tam by a step-wise increase up to 160 μ M for 8 weeks and further cultured until 22 weeks to finally obtain TamR cells. B. Comparison of growth rate between TamR and parental cells. TamR cells show a higher growth rate than MCF-7 cells. C. Drug resistance of MCF-7/TamR cells is confirmed by colony formation assay. TamR cells show a higher survival rate than the parental cells under Tam pressure. The bar graph shows the quantification of three independent experiments. D. Lower apoptosis of TamR cells. FACS analysis indicates that TamR cells became resistant to Tam for apoptosis, especially with regard to early apoptosis. E. Expression profiles of TamR marker genes. A few genes, the expressions of which

were previously revealed to be altered in the TamR cells, are examined by qPCR and the result confirm a similar expression alteration. F. The uptake rate of Tam into the cell. The uptake of fluorescent Tam is monitored by FACS and the result indicates no significant change with just a slight increase in TamR cells. All assays are performed in triplicates, and the results are depicted as mean \pm SE.



Figure 3. Highest confidence network and pathway of genes displaying altered methylation in MCF-7/TamR. (A) Clustering of genes in which methylation is affected in TamR. A heatmap is constructed with 405 CpG-containing genes showing significant methylation changes ($|\Delta\beta| \ge 0.2 P < 0.05$) from duplicated microarrays of MCF-7 vs. MCF-7/

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TamR. (B) Expression of genes showing highest methylation changes in the microarray assay. Five genes are selected from **Table 3** and their expression is examined by qPCR. (C) Genome-wide methylation analysis with 356 genes identifies "Skeletal and Muscular Disorders, Cellular Assembly and Organization, Connective Tissue Development and Function" pathway as the top network. Genes hypermethylated in MCF-7/TamR are shaded in red, whereas those hypomethylated are shaded in green, with the color intensity signifying the magnitude of methylation change. Solid lines represent direct interactions, and dashed lines represent indirect interactions. Top canonical pathways (D) and disease and biofunction (E) for the genes in which methylation is significantly altered in MCF-7/TamR.

Symbol	Accession	Accession Description		Fold change⁵
SLC19A1	NM_194255	solute carrier family 19 member 1		12.6487
ELOVL2	NM_017770	ELOVL fatty acid elongase 2	0.4891	4.0003
ARHGAP6	NM_013427	Rho GTPase activating protein 6	0.4738	3.9104
HES6	NM_018645	hes family bHLH transcription factor 6	0.4559	2.4814
ZCCHC5	NM_152694	zinc finger, CCHC domain containing 5	0.4464	2.6917
PLEKHG7	NM_001004330	pleckstrin homology domain containing, family G member 7	0.4338	2.6532
P2RY10	NM_014499	purinergic receptor P2Y 10	0.429	2.235
SKAP1	NM_003726	src kinase associated phosphoprotein 1	0.4168	2.7189
TRAPPC2	NM_014563	trafficking protein particle complex 2	0.4066	2.8467
OFD1	NM_003611	oral-facial-digital syndrome 1	0.4066	2.8467
SRPX2	NM_014467	sushi-repeat containing protein, X-linked 2	-0.3303	-1.7687
LNX1	NM_032622	ligand of numb-protein X 1	-0.3306	-1.8897
MMP1	NM_002421	matrix metallopeptidase 1	-0.3312	-1.9492
GPX5	NM_001509	glutathione peroxidase 5	-0.3323	-2.0417
GUCY1B3	NM_000857	guanylate cyclase 1, beta 3	-0.3624	-1.8898
CD59	NM_203329	CD59 molecule	-0.3951	-1.9305
RPS6KL1	NM_031464	ribosomal protein S6 kinase-like 1	-0.4119	-2.5277
TLR6	NM_006068	toll-like receptor 6	-0.42	-2.012
RASAL2	NM_170692	RAS protein activator like 2	-0.4394	-2.5287
LRP11	NM_032832	low density lipoprotein receptor-related protein 11	-0.5875	-3.2229

Table 3.	Top 20	genes of v	which	methylation	are highly	altered in	the N	ИCF-7/	TamR ce	ells
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^aThe values are obtained by subtracting the methylation level of MCF-7 cells from that of MCF-7/TamR cells. ^bThe values are obtained by dividing the expression level of MCF-7/TamR cells by that of MCF-7 cells.

significant hypermethylation ($\Delta\beta = 0.49$), but its role in cancer or drug resistance is not yet known. Both transcripts and proteins were downregulated in the MCF-7/TamR cells compared with those in MCF-7 cells (Figures 3B and **4A**). Induction of lower methylation by treating the MCF-7/TamR cells with Aza, a methyltransferase inhibitor, upregulated ELOV-L2, suggesting an epigenetic regulation of the gene (Figure 4B). Next, the methylation and expression of the gene were examined in breast cancer tissues obtained from patients showing resistance to clinical treatment with Tam. The result indicated that ELOVL2 was hypermethylated and downregulated in the TamR cancer tissues (n = 28) compared with the Tamoxifen sensitive (TamS) tissues (n = 33) (P < 0.001) (Figure 4C). Immunohistochemical analysis of tissues from cancer patients showed lower expression of ELOVL2 in the TamR tissues than in the TamS tissues (**Figure 4D**). The rate of distant metastasis-free survival (DMFS) for breast cancer patients, which was investigated through the GOBO database, indicates that lower rates of DMFS were observed in cancer patients with lower expression of ELOVL2, whereas higher rates of DMFS were observed in patients with a higher ELOVL2 expression (P< 0.05) (**Figure 4E**).

ELOVL2 inhibits MCF-7/TamR cell proliferation and recovers Tam sensitivity

To obtain information about the role of ELOVL2 in the acquisition of TamR, its effect on cell growth and drug sensitivity recovery was exam-



Figure 4. ELOVL2 is hypermethylated and downregulated in TamR breast cancer. A. Downregulation of ELOVL2 in MCF-7/TamR. Expression of ELOVL2 is examined by Western blot analysis. B. Demethylation of CpGs is induced by Aza in the MCF-7/TamR cells and ELOVL2 expression is analyzed by qPCR. C. Hypermethylation and downregulation of ELOVL2 in breast cancer tissues. MSP and qPCR are performed for breast tissues from Tam-sensitive and Tam-resistant cancer patients. N: number of samples. D. Immunohistochemical analysis of ELOVL2 in Tam-sensitive and Tam-resistant cancer tissues. Three tissue sets are analyzed and the protein expression is denoted by the bar graph. Images from two tissue sets are represented. Scale bar, 50 μ m. E. Kaplan-Meier survival analysis of ELOVL2 expression level. The log-rank test is performed in all tumor samples using distant metastasis-free survival (DMFS) as the endpoint. High ELOVL2 expression is significantly associated with higher DMFS in cancer patients (*P* < 0.005).

ined after overexpressing ELOVL2 in the MCF-7/ TamR cells. First, the recombinant vector (ELOVL2 ORF) was transfected to the MCF-7/ TamR cells and their growth rate was determined. As a result, ELOVL2 ORF induced retardation of the cell growth up to 18% compared with a negative control vector (**Figure 5A**). Next, the effect of ELOVL2 on tumor cell growth was



Figure 5. TamR cancer cells show a lowered ELOVL2 expression and grow faster in xenograft tumor tissue. (A) ELOVL2 is upregulated in MCF-7/TamR by transiently transfecting a recombinant plasmid vector. Effect of ELOVL2 on cell proliferation is examined by CCK assay. (B) MCF-7/TamR cells grow faster than MCF-7 in a xenograft animal model. MCF-7 and MCF-7/TamR cells are subcutaneously injected into nude mice and the tumor volume is measured for 7 weeks. n = 8. (C) Mice are sacrificed 8 weeks after transplantation to obtain the tumor tissues. Expression of ELOVL2 in the xenografted tumor is examined by Western blot analysis (D) and immunohistochemical analysis (E). Three tumor sets are analyzed and the average protein expression is denoted in a bar graph. Representative images are shown. Scale bar, 50 μ m.

monitored in tumor tissues after generating a mouse xenograft model. MCF-7/TamR cells grew faster than MCF-7 (**Figure 5B**) and showed

larger tumor volume in all the eight mice sacrificed 7 weeks after injection (Figure 5C). A decrease of ELOVL2 protein expression in the

tumor was verified by Western blot and immunohistochemical analysis (**Figure 5D** and **5E**).

The effect of ELOVL2 on the recovery of Tam sensitivity was examined in vitro and in vivo under the pressure of the drug. In the presence of Tam. the MCF-7/TamR cells transfected with ELOVL2 ORF showed a retarded growth, especially at Tam concentrations of $< 0.5 \,\mu$ M (Figure 6A). This observation was reproduced in the cell survival experiment of colony formation assay, where a lower number of colonies were observed in the ELOVL2 ORF-transfected MCF-7/TamR cells under Tam pressure (Figure 6B). The tumors implanted with the MCF-7/ TamR-ELOVL2 ORF cells that stably expressed ELOVL2 showed retarded growth compared with those implanted with the control MCF-7/ TamR cells (*n* = 6, *P* < 0.05) (**Figure 6C** and **6D**), indicating the tumor suppressive activity of ELOVL2. Furthermore, the tumors over-expressing ELOVL2 showed increased sensitivity to Tam by representing smaller tumor sizes (n = 4, n)*P* < 0.05) (**Figure 6C** and **6D**).

ELOVL2 resists Tam by suppressing the AKT pathway

To get an insight into the regulatory mechanism of ELOVL2 for drug resistance, a genome-wide expression assay was performed in duplicates for identifying target genes. A comparison of the expression profile between MCF-7/TamR and MCF-7/TamR-ELOVL2 ORF revealed 969 genes that were significantly altered (expression level change > 2) (Figure 7A). IPA analysis identified "Cardiovascular Disease, Cell-To-Cell Signaling and Interaction, Inflammatory Response" pathway and "Behavior, Reproductive System Development and Function, Cardiac Infarction" pathway as the top first and second networks, respectively (Figure 7B). In accordance with this, the canonical pathway analysis (Figure 7C) and the disease and function analysis (Figure 7D) predicted the immune-related pathway and cancer as the top categories, respectively. Notably, AKT and ERa are placed at the center of each network, interacting directly or indirectly with many genes in the pathways, such as THEM4, BMF, and FTO in the case of AKT pathway and HYOU1, CDC42EP2, and S100A9 in the case of ERa. The expression of the genes in the two pathways were further examined by gPCR and the result confirmed the same direction of expression alteration as observed in the expression array (**Figure 8**). The AKT pathway is a key pathway responsible for cell metabolism, growth and division, apoptosis suppression, and angiogenesis [29]. In particular, THEM4 is known to promote AKT phosphorylation and functions as an oncogenic molecule in breast cancer [30].

THEM4 is downregulated by ELOVL2 recovering Tam sensitivity

As THEM4, a key molecule in the AKT pathway, showed downregulation in the ELOVL2-overexpressing MCF-7/TamR cells via expression microarray analysis, we decided to elucidate the molecular mechanism of how THEM4 induces TamR in association with ELOVL2. THEM4 was upregulated in the MCF-7/TamR cells compared with MCF-7 cells at both RNA and protein levels (Figure 9A). Overexpression of ELOVL2 in the MCF-7/TamR cells set back the THEM4 expression to a lower level than that observed in the control MCF-7/TamR cells (Figure 9B). Western blot analysis (Figure 9C) and immunohistochemical analysis (Figure 9D, **9E**) also found a similar expression profile for THEM4 in the tumor tissues of the xenografted mice, which were generated from MCF-7/TamR and MCF-7/TamR-ELOVL2 ORF. Subsequently, the association of THEM4 with ELOVL2 was tested with regard to TamR, cell growth, and apoptosis. siRNA-induced downregulation of THEM4 contributed to the recovery of Tam sensitivity as shown by the colony formation assay (Figure 9F), and this effect was strengthened by ELOVL2 (Figure 9G). The dye-based CCK assay using the same THEM4-siRNA and ELOVL2-overexpression strategy confirmed the results of the colony formation assay (Figure 9H, 9I), suggesting an inhibitory and a stimulatory effect of ELOVL2 and THEM4, respectively, for the acquisition of TamR. However, the total apoptosis rate was not changed significantly. even though early and late apoptosis was decreased and increased, respectively, by the downregulation of THEM4 (Figure 10).

Considering all the experimental findings from this study and the previous literature, which indicate a close association of AKT and THEM4 in the signaling pathway, it is suggested that ELOVL2 contributes to the recovery of TamR by regulating pivotal genes such as THEM4 in the AKT pathway (**Figures 11**, **12**).



Figure 6. ELOVL2 suppresses tumor growth and recovers Tam sensitivity in *in vitro* and *in vivo* animal model. A. Effect of ELOVL2 on Tam sensitivity is examined after treating the ELOVL2 ORF-transfected MCF-7/TamR cells with Tam and then measuring the growth rate by CCK assay. B. Effect of ELOVL2 on Tam sensitivity is examined by colony formation assay. All the assays are performed in triplicates, and the result is depicted as mean \pm SE. Representative images are shown for the colony formation assay. NC, negative control vector. ORF, open reading frame. C. MCF-7/TamR cells that are stably transfected with ELOVL2-expressing cDNA or control DNA are subcutaneously injected into nude mice and Tam is administered 3 weeks after cell injection. The tumor volume is measured for 7 weeks. D. At week 8, mice are sacrificed to obtain the tumor tissues (n = 6 for corn oil-treated mice; n = 4 for Tam-treated mice).

Discussion

This study aimed at identifying epigenetically regulated marker genes responsible for TamR

in breast cancer patients and then elucidating the molecular mechanism to give an insight for the prevention and treatment of TamR recurrence in cancer patients. Occasionally, drug-



Figure 7. Highest confidence network of genes displaying altered ELOVL2 expression in MCF-7/TamR. ELOVL2 is overexpressed in MCF-7/TamR and a genome-wide expression analysis is performed. (A) Heatmap analysis of 969 genes that are significantly deregulated by ELOVL2. The data are from the microarray in duplicates. (B) Highest confidence network of genes displaying altered expression identifies "Cardiovascular Disease, Cell-To-Cell Signaling and Interaction, Inflammatory Response" pathway and "Behavior, Reproductive System Development and Function, Cardiac Infarction" pathway as the top networks. Genes that are upregulated are shaded in red, whereas those that are downregulated are shaded in green, with the color intensity signifying the magnitude of expression change. Solid lines representing direct interactions, and dashed lines representing indirect interactions. (C) Top 10 canonical pathways and (D) disease and function annotation of the genes of which expression is significantly altered by ELOVL2. The most significant canonical pathway is "Antigen Presentation Pathway" and disease and function annotation is "Solid tumor".





Figure 8. ELOVL2 regulates genes in AKT and ERa networks. A few genes in the top IPA network (Figure 7B) are selected and qPCR is performed to confirm the microarray expression data. All genes show the same expression trend with being up- or down-regulated in ELOVL2 overexpression cells compared with the MCF-7/TamR cells, although the alteration level is not the same.

resistant cancer cells have shown to actively pump the drug out of the cells, reducing the net amount of drug inside cells. This fact could partly explain drug resistance. For instance, a 10-fold lower Tam concentration was found in extracts from TamR tumors than that in TamS tumors in mice [31]. However, it is controversial whether all TamR cells acquire the high efflux rate, because the P-glycoprotein, an efflux pump that is known to bind to Tam, is not expressed in these tumors. Furthermore, the TamR cells generated and used in our study showed no significant change in Tam transport compared with the parental MCF-7 cells. Therefore, we speculated that genetic changes may have more strongly contributed to TamR than alterations in the drug efflux rate.

ELOVL2 has consistently shown hypermethylation and downregulation in TamR cancer, indicating its potential application as an epigenetic

marker for the diagnosis of TamR cancer. However, no significant difference of methylation or expression between normal and cancer tissues of breast was found. In normal tissue, ELOVL2 has been known to undergo hypermethylation on the promoter DNA as one age. The downregulation of ELOVL2 induced by the hypermethylation is not considered enough to drive cells into cancer, although suppression of ELOVL2 stimulated cell proliferation in our study. This may imply that the deregulation of ELOVL2 is crucial during the acquisition of TamR, rather than during the development of the primary cancer. Moreover, an association between ELOVL2 DNA methylation and future breast and colorectal cancer development has been observed [32].

ELOVL2 Expression has been previously known to be enhanced by $ER\alpha$ in breast cancer cells [33]. In this study, Tam exposure specifically



Figure 9. THEM4 is downregulated by ELOVL2 and increases Tam resistance. (A) Increased expression of THEM4 in MCF-7/TamR cells. qPCR (left) and Western blot analysis (right) are performed in MCF-7/TamR and MCF-7 cells. (B) Downregulation of THEM4 by ELOVL2 in MCF-7/TamR cells, determined by qPCR. ELOVL2 ORF: cells stably transfected with ELOVL2 cDNA; ORF NC; negative cDNA control. (C) Increased expression of THEM4 in the xenografted MCF-7/TamR but suppression by ELOVL2. Western blot analysis is performed for tumor tissues from ELOVL2 ORF and control. Immunohistochemical analysis of THEM4 in xenografted tumor tissue of MCF-7/TamR (D) and cells stably transfected with ELOVL2 cDNA (E) Scale bar, 50 µm. Effect of THEM4 on recovery of Tam sensitivity. THEM4 is downregulated *via* siRNA #2 at a final concentration of 40 nM in MCF-7/TamR (F) and ELOVL2-overexpressing MCF-7/TamR cells (G). Sensitivity to Tam is examined by colony formation assay. Representative images from three independent assays are shown. (H) Effect of THEM4 on cell proliferation is examined by a dye-based CCK assay. (I) Effect of THEM4 on TamR is examined by exposing the cells to Tam after downregulating the gene with siRNA. All the assays are performed in triplicates, and the result is depicted as mean ± SE.



Figure 10. Effect of THEM4 on apoptosis of MCF-7/TamR cells. FACS analysis is performed after downregulating THEM4 using siRNA #2 at a final concentration of 40 nM. Overall apoptosis rate is not changed significantly, but the early and late apoptosis is decreased and increased, respectively, by the downregulation of THEM4.

abolished ELOVL2 expression. Our microarray analysis also revealed upregulation of ER α by ELOVL2, suggesting a positive feedback mechanism for the regulation of the two genes. In addition, many ER α -regulated genes such as HYOU1 (3.8-fold decrease), S100A9 (1.2-fold decrease), and CDC42EP2 (2.4-fold decrease) were also deregulated by ELOVL2. HYOU1 is a hypoxia-induced protein and its upregulation suppresses programmed cell death, contributing to invasiveness in breast cancer [34]. S100A9 has been identified to be expressed by epithelial cells involved in malignancy and its expression levels are inversely correlated with ER α in breast cancer [35]. CDC42EP2 is a member of the binder of Rho GTPases (Borg) family and little is known about its role in the disease [36].

The AKT pathway is a pivotal one wherein a few TamR-related genes have been identified [23]. In accordance, previously identified AKT-regulated genes also appeared in the ELOVL2-overexpression network, such as THEM4, BMF, and FTO (**Figure 7B**). Furthermore, other genes involved in the AKT pathway, including mTOR,



Proliferation and Tam resistance

Figure 11. Schematic illustration of the regulatory pathway by ELOVL2. The uptake ratio of Tam across plasma membrane in MCF-7/TamR cells is similar to that in the parental MCF-7 cells. ERa and ELOVL2 crosstalk to regulate each other. ELOVL2 blocks the PI3K/AKT/mTOR pathway *via* inhibiting THEM4 and PI3K. In TamR cancer, ELOVL2 is downregulated by hypermethylation, resulting in loss of inactivation of AKT and also downstream genes such as CREB and mTOR, or activation of downstream genes such as BMF, eventually leading to Tam resistance and increased cell proliferation.

PIK3CA, and CREB1, were shown to be downregulated by ELOVL2 (Figure 12A). Notably, the expression of Akt as well as the phosphorylated form was downregulated in a similar ratio by ELOVL2 (Figure 12B). Therefore, the decrease of the phosphorylated form is caused by the lowered total AKT levels, suggesting that ELOVL2 regulates AKT and not p-AKT. The two networks closely communicating each other by sharing a few common genes. For example, NFAT5 is regulated by an estrogen-induced microRNA [37] and the regulation is mediated via PI3K/AKT-signaling pathways [38]. In addition, A100A9, a calcium-binding protein that is highly expressed in malignant breast cancer, induces a decrease of ERα in MCF-7 cells [35], and inhibits PI3K/AKT pathway in pancreatic adenocarcinoma cells [39]. Collectively, the current study proposes that ELOVL2 is an integral signaling molecule of the AKT axis in ER-positive breast cancer cells.

Further it is noteworthy that a representative aging marker gene is associated with drug resistance. ELOVL2 has shown to increase methylation at the promoter CpGs with aging, which accompanies a decreased expression with aging [18]. Considering the increased drug resistance and lower expression of ELOVL2 in cancer cells of patients, epigenetic aging could make cancer patients more vulnerable to acquisition of drug resistance. This gives us an insight on how to design the strategy for treating drug-resistant cancers. Meanwhile, it should be mentioned that ELOVL2 function is not limited to epigenetic aging and drug resistance. The gene showed tumor suppressor-like activity by inhibiting cancer cell growth in cultures cancer cells as well as in the xenografted mouse model. Therefore, ELOVL2 is considered to have a wide spectrum

of biological functions in addition to the fatty acid elongation activity.

Genome-wide methylation analysis has found that numerous genes were deregulated in addition to ELOVL2 in TamR cells, suggesting distortion of multiple pathways during the course of drug resistance acquisition. A genome-wide expression array also found > 1,200 genes clustered into ER α function, cell cycle regulation, transcription/translation, and mitochondrial dysfunction [40]. Therefore, to completely understand the molecular mechanisms and to conquer TamR in cancer, a further comprehensive approach is needed. In conclusion, ELOVL2 was identified as a marker that was hypermethylated and downregulated in TamR cancer com-

ELOVL2 attenuates tamoxifen resistance in breast cancer



Figure 12. ELOVL2 downregulates total amount of AKT and p-AKT. A. Downregulation of genes in the AKT pathway by ELOVL2. qPCR is performed for seven genes in the AKT pathway in the ELOVL2-overexpressing MCF-7/TamR cells. Samples are analyzed in triplicates, and the result is shown as mean ± SE. B. Protein level of AKT and p-AKT is analyzed in the MCF-7/TamR cells transiently transfected with a recombinant ELOVL2 cDNA plasmid. Western blot analysis is performed in triplicates, and a representative image is shown with a bar graph depicted as mean ± SE.

pared with TamS cancer. ELOVL2 is responsible for the recovery of TamS, which was shown in an *in vivo* animal xenograft model. AKT- and ERa-hubbed networks are pivotal in ELOVL2 signaling, where THEM4 contributes to the relaying ELOVL2 signaling. This study is the first to identify a linkage between drug resistance and a gene involved in fatty acid synthesis. Our data may give credence to elucidating the mechanism of TamR cancer and to developing its treatment strategy.

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Disclosure of conflict of interest

None.

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