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Metformin affects alternative splicing of the *estrogen receptor alpha* gene in breast cancer cells

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ABSTRACT

Metformin ameliorates insulin resistance and reported to have prophylactic potential in breast cancer. The *estrogen receptor-alpha* gene is known to be alternatively spliced to the ERα46 isoform with decreased expression in tamoxifen-resistant breast cancer cells. We show here that metformin had a differential effect on its alternative splicing in MCF-7 and its tamoxifen-resistant derivative, TAMR1 cells. Metformin altered the tamoxifen-induced expression of ERα46 and treatment of cell-transplanted nude mice showed decreased tumor weight only in TAMR1 cell tumors. This is the first report to show metformin specifically involved in alternative splicing of genes in breast cancer cells.

Keywords: Estrogen receptor-alpha (ERα), estrogen, tamoxifen, metformin

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INTRODUCTION

The *estrogen receptor-alpha* (*ERα*) gene is the key factor of breast cancer growth evidenced by the fact that about 75% patients benefit from ER-targeting drugs at early stages of treatment (1). However, nearly 20% of the patients develop resistance to these drugs (1, 2). Somatic mutations of the ligand binding domain of ERα (3) show constitutive activation that were found in nearly half of the patients after treatment by ER-targeting drugs (4-6). Besides these mutations, these drugs are known to modulate ER function through other pathways of other steroid receptors as well as growth factor and cytokine signaling pathways (7). Alternative splicing of the *ERα* gene has been studied for almost thirty years (8). Among them, ERα46 was discovered as the extra-mammary isoform transcribed from an upstream promoter along with the non-coding exon E/F and skipping of the entire exon 1 where the start codon of the full length lies in exon 2 (9). Thus, ERα46 protein lacks the N-terminus AF-1 function found in nuclear receptors but retains the DNA-binding domain, and thus would and was shown to compete with the AF-1 function of full length ERα (10). It is known to be expressed in ER-positive mammary carcinoma cells, especially when they are sensitive to ER-targeting drugs, and the expression is reduced in tamoxifen-resistant cell lines (11).

We have shown that HMGA1a, known as an oncogenic product and modified by various physiological stimuli (12), sequence specifically binds to an RNA-element in ERα exon 1. This HMGA1a-RNA-binding site in ERα exon 1 is adjacent to a pseudo-5’ splice site that anchors U1 snRNP to it and induces dysfunction of the authentic 5’ splice site leading to ERα exon 1 skipping and expression of ERα46 protein in MCF-7 breast cancer cells (13). HMGA1a-induced regulation of alternative splicing was originally found for aberrant splicing of *Presenilin-2* exon 5 found in sporadic Alzheimer’s disease where HMGA1a anchors U1 snRNP to the 5’ splice site and induces exon-skipping of exon 5(14). An RNA decoy of this HMGA1a would induce ERα exon 1 inclusion and improve tamoxifen resistance in TAMR1, the tamoxifen-resistant derivative of MCF-7 (13).

In this study, we found that the anti-diabetic drug, metformin, mimics the RNA decoy of HMGA1a(13, 15), in the sense that it regulated the ERα46 splicing event. In addition, we found that it oppositely regulated ERα46 splicing in MCF-7 breast cancer cells and its tamoxifen-resistant derivative, TAMR1. Moreover, this regulation was tamoxifen-dependent. We believe that metformin is the key compound in deciphering the mechanism of tamoxifen-resistance in estrogen receptor-positive breast cancer.

MATERIALS AND METHOD

Cell culture and compounds

MCF-7 breast cancer cells (ATCC: #HTB-22) were cultured in DMEM (Nacalai tesque, #08456-65) with 10% EquaFETAL (US Origin FBS substitute) (Atlas Biologicals, Inc., #EF-0500-A) with MEM Non-Essential amino acids and penicillin-streptomycin mixed solution. The tamoxifen-derivative of MCF-7 cells, MCF-7 Tam1 (ATCC: #CRL-3435) (renamed TAMR1), were cultured in DMEM/F12 Ham (Sigma, D6434) with 1% EquaFETAL (US Origin FBS substitute) (Atlas Biologicals, Inc., #EF-0500-A) and 2.5 mM L-Glutamine and 6 ng/ml insulin and penicillin-streptomycin mixed solution. TAMR1 cells were cultured in the presence of tamoxifen (1μM) but was removed where it is not indicated in the following experiments. Both cell types were cultured in 6-well plates at 5% CO2 and 37℃ at log phase throughout the experiments.

Tamoxifen (Caymen chemical Co, #13258) was dissolved in ethanol and metformin (1,1-dimethylbiguanide hydrochloride) (Tokyo Chemical Industry, Japan, M2009) in water and used at the concentrations indicated.

RT-PCR and primers

Total RNA of the cells from each well (6-well plate) was extracted by chloroform from PBS-washed cells ($1x10^6$) disrupted in TRIzol reagent (Invitrogen, #15596026) using two 3 mm stainless balls (AS ONE co, #2-9244-03) in a 1.5 ml Eppendorf tube. After precipitating the RNA from the aqueous phase of the TRIzol reagent treated sample, it was digested with RQ1 RNase-Free DNase (Promega, #M6101), and one microgram of total RNA was reverse transcribed using ReverTra Ace (Toyobo, 05038-64) and random primer (Toyobo, #FSK-301). PCR was conducted using Ex Taq (TaKaRa Bio, #RR001A). The primers used for amplifying ERα full length and ERα46 are E/F1f: 5'-AAGGAGTAAGCACAAAGATCTC-3', E/F1r: 5'-CTCACAGGACCAGACTCCATAATGG-3', E/F2f: 5'-CAGCACTTCTTCAAAAAGGATGTAGA-3', E/F2r: 5'-AGCATAGTCATTGCACACTGC-3' as we previously reported (13).

Western blot analyses and antibodies

The remainder of each TRIzol reagent-treated sample purified for total RNA (interphase and phenol phase) was protein precipitated by a acetone solution (acetone: methanol: ethanol: water =25:25:48:2). After sonication and protein measurement (Pierce 660 nm protein assay reagent (ThermoFisher, #22662)), 15-30 μg protein was subjected to sodium dodecyl sulfate-PAGE (SDS-PAGE), transferred to a PVDF membrane (BIO-RAD, #1620177) and subjected to western

blot analyses and revealed by SignalFire ECL reagent (Cell Signaling, 6883S). Primary antibodies used are: anti-ERα (HC-20) (Santa-Cruz, sc-543); and anti-HMGA1a (FL95; Santa Cruz, sc-8982).

Cell viability assay

Cells were cultured in 96-well plates with the appropriate compound. Cell viability was measured by crystal violet (Crystal violet Assay Kit (Cell viability); ABCAM LIMITED, ab232855) on day 1, day 3, and day 5. Medium with the compound was refreshed between day 3 and day 5.

Tumor growth in vivo

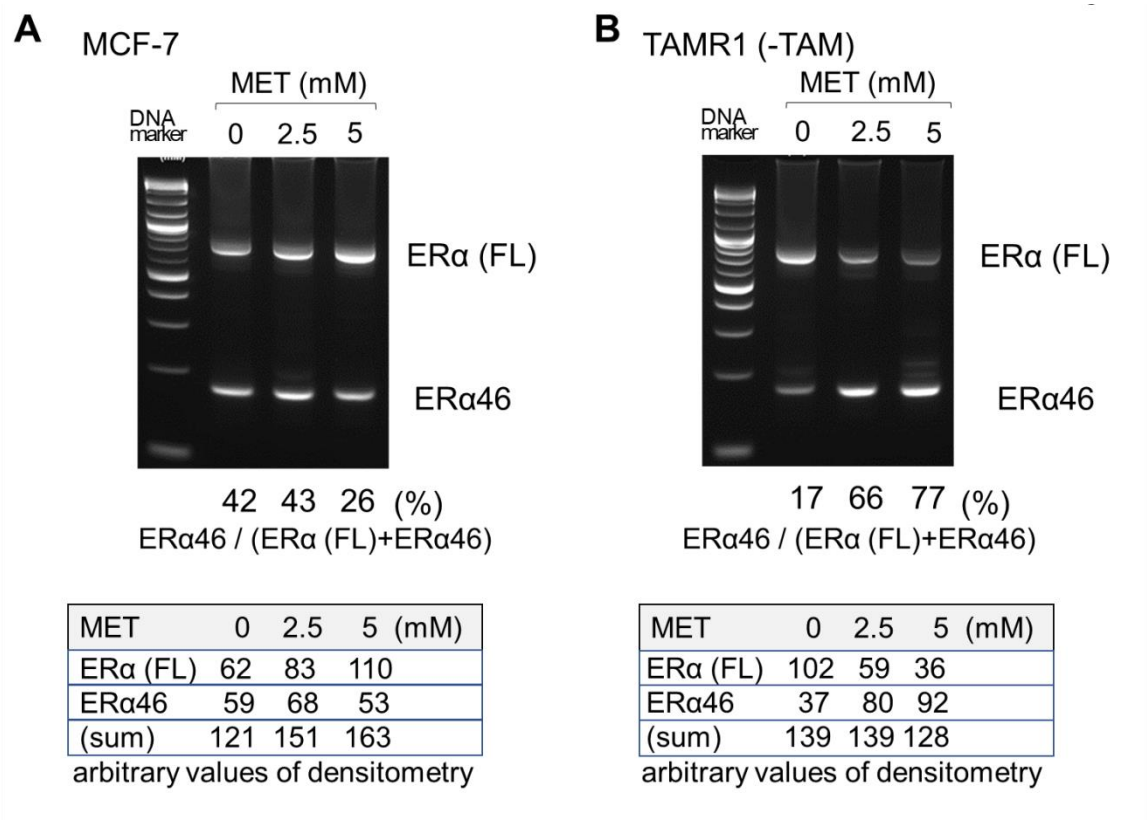
BALB/c nude mice (female, 4–8 W) were obtained from Charles River Laboratories Japan, Inc., and housed in specific pathogen-free barrier facilities at Fukuoka University. The mice were kept under conditions of a 12-h light, 12-h dark cycle, constant temperature (23–25 °C), and free access to food and water. The nude mice were implanted of 5×10^6 MCF-7 or TAMR1 breast cancer cells in Matrigel (Falcon, 354248) (0.1 ml). Metformin (Wako pure chemical industries, Ltd, Osaka, Japan) was delivered at 750 mg kg^{-1} day^{-1} by mixing it with the feed. Tamoxifen was delivered 20 mg/day per os (PO) once daily via gastric gavage.

Tumor and body weights were measured every week. After four weeks, animals were killed using an approved procedure. The use of animals was kept to the minimum necessary to validate the data, and all animal protocols were carried out according to the National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Fukuoka University Committee for Animal Care (Protocol No. 171116 accepted in April 2019).

RESULTS

High-dose metformin increased ERα46 mRNA expression in TAMR1 cells

First, we checked mRNA expression of ERα46 and ERα (FL) when MCF-7 cells and its tamoxifen-resistant derivative, TAMR1 cells, were treated with high-dose metformin (2.5, 5 mM). In MCF-7 cells, metformin decreased the ratio of ERα46 and (ERα (FL)+ERα46) mRNA expression (Fig. 1A). On the other hand, metformin induced a clear induction of ERα46 mRNA expression accompanied with a decrease of ERα (FL) mRNA of TAMR1 cells (Figure 1B) which had no tamoxifen in the medium for this particular experiment. This strongly suggested that metformin oppositely regulated alternative splicing of the *ERα* gene due to tamoxifen-sensitivity.



MET	0	2.5	5 (mM)
ERα (FL)	62	83	110
ERα46	59	68	53
(sum)	121	151	163

arbitrary values of densitometry

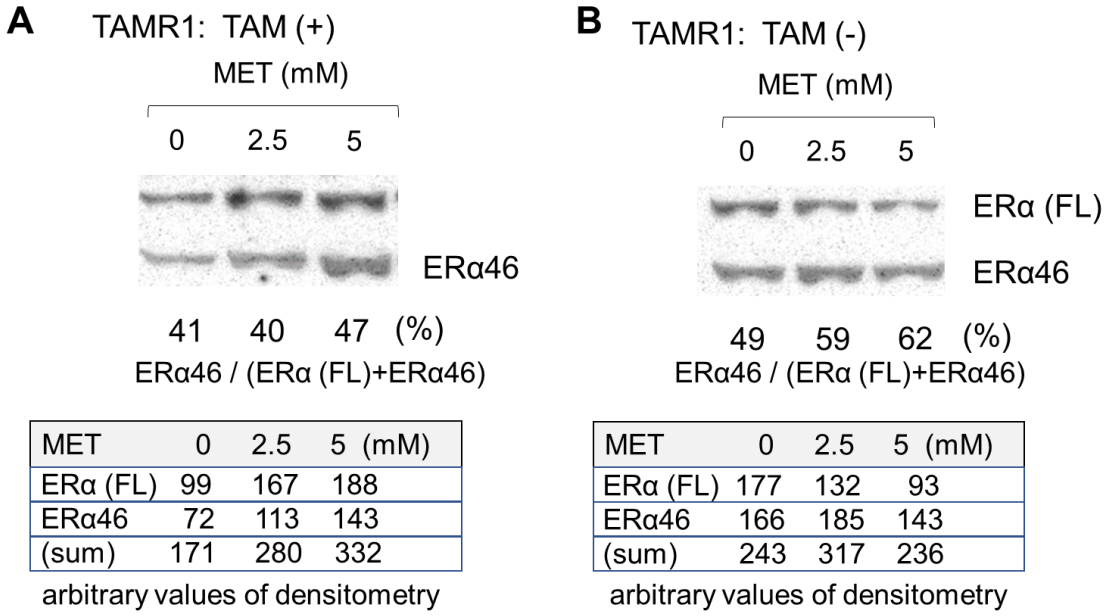
MET	0	2.5	5 (mM)
ERα (FL)	102	59	36
ERα46	37	80	92
(sum)	139	139	128

arbitrary values of densitometry

Figure 1: Differential induction of ERα46 mRNA in MCF-7 and its tamoxifen-derivative, TAMR1 cells. (A) Results of ERα (full length: FL) and ERα46 in high-dose metformin-treated MCF-7 cells. The table below shows arbitrary values of densitometry of the representative result of three separate experiments. (B) Results of ERα (full length: FL) and ERα46 in high-dose metformin-treated TAMR1 cells. The table below shows arbitrary values of densitometry of the representative result of three separate experiments.

High-dose metformin-induced ERα46 expression in TAMR1 cells was tamoxifen-independent

Since TAMR1 cells are a tamoxifen-resistant derivative of MCF-7 cells, we were prompted to check whether the high dose metformin-induced increase of ERα46 in TAMR1 cells (Figure 1) was tamoxifen-dependent. While metformin did not induce an increase of ERα46/(ERα (FL)+ ERα46) in the presence of tamoxifen (Figure 2A), an apparent increase was found in TAMR1 cells (Figure 2B). The increase of ERα (FL) and ERα46 was equal in proportion indicating that this increase in TAMR1 cells in the presence of tamoxifen is more likely to be transcriptional. Thus, the induction of ERα46 by alternative splicing was tamoxifen-independent. Next, we tested the cell viability of MCF-7 and TAMR1 cells by high-dose metformin in the presence and absence of tamoxifen.



MET	0	2.5	5 (mM)
ERα (FL)	99	167	188
ERα46	72	113	143
(sum)	171	280	332

arbitrary values of densitometry

MET	0	2.5	5 (mM)
ERα (FL)	177	132	93
ERα46	166	185	143
(sum)	243	317	236

arbitrary values of densitometry

Figure 2: ERα protein analyzed in metformin-treated TAMR1 cells. (A) TAMR1 cells were treated by metformin (MET: 5mM) in the presence of tamoxifen (TAM: 1μM) and analyzed by Western analysis using an anti-ERα antibody. ERα (FL) indicates full length ERα protein, and ERα46 is an isoform that lacks a portion of the N terminus of ERα protein. The table below shows arbitrary values of densitometry of the representative result of three separate experiments. (B) Metformin-treated TAMR1 cell were analyzed of ERα(FL) and ERα46 protein in the absence of tamoxifen.

High-dose metformin decreased cell viability of MCF-7 breast cancer cells as well as its tamoxifen-resistant derivative, TAMR1 cells

In order to investigate the role of metformin in tamoxifen-resistant breast cancer, we checked the cell viability of MCF-7 cells and its tamoxifen-resistant derivative, TAMR1 cells treated with metformin, tamoxifen, and combined. When treated by tamoxifen (1 μM), MCF-7 cells considerably lost cell viability (Figure 3A), while TAMR1 cells did not at day 3 and even increased growth at day 5 compared to solvent (ethanol) (Figure 3B). On the other hand, metformin (5 mM) showed loss of cell viability for both cell lines, as expected (Figure 3A, 3B). Combined treatment of tamoxifen and metformin in TAMR1 cells decreased tamoxifen-dependent growth (Figure 3B). Though high-dose metformin differentially affected ERα46 alternative splicing in MCF-7 and TAMR1 cells, it had an equal toxic effect on both cell lines. So, next we tested pharmacological level of metformin.

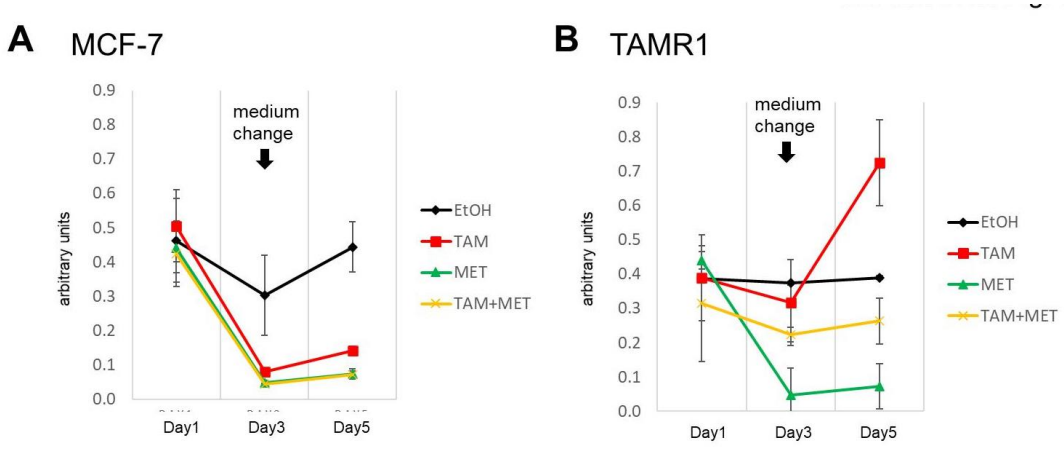
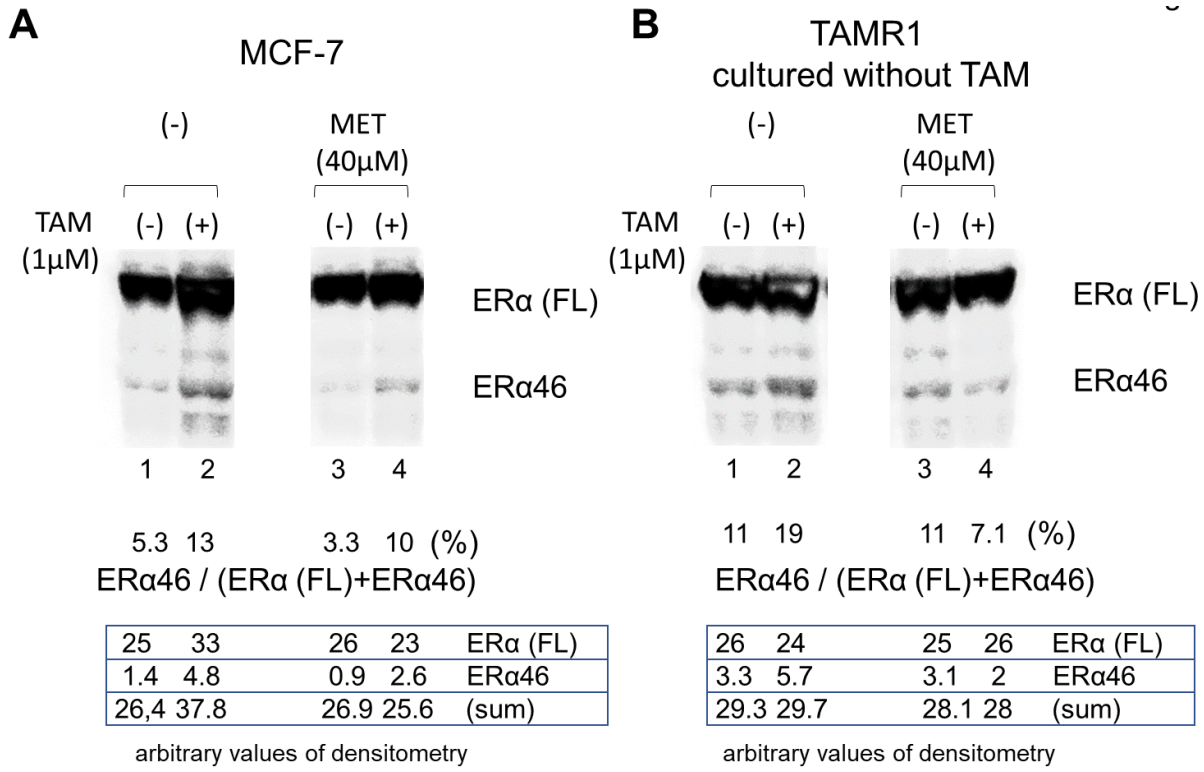


Figure 3:Cell viability assay of MCF-7 and TAMR1 cells treated by tamoxifen and metformin. (A) Cell viability assay of non-treated MCF-7 cells (black) and tamoxifen-treated (red), metformin-treated (green), and tamoxifen and metformin-treated MCF-7 cells of three independent experients. (B) Cell viability assay of non-treated TAMR1 cells (black) and tamoxifen-treated (red), metformin-treated (green), and tamoxifen and metformin-treatedTAMR1 cells of three independent experients. Medium was changed on day 3 as shown by black arrow.

Low-dose metformin attenuated ERα46 induction by tamoxifen

In order to check whether low-dose metformin (40 μM) had an effect on ERα46 alternative splicing, we pre-treated cells with low-dose metformin for one week prior to the experiments and added solvent or 1 μM tamoxifen. As shown in figure 4, tamoxifen induced ERα46 in MCF-7 cells (Figure 4A, compare lane 2 with lane 1) and TAMR1 cells (Figure 4B, compare lane 2 with lane 1). However, when the cells were pretreated with low dose metformin, tamoxifen-induced ERα46 expression was attenuated in TAMR1 cells (compare Figure 4B lane 3, 4 with lane 1,2), while the tamoxifen-induced ERα46 expression was comparable to the control (Figure 4A, compare lanes 3,4 with lane 1,2). Since ERα46 expression was induced differentially by low-dose metformin in MCF-7 and TAMR1 cells, we assumed low-dose metformin may have a TAMR1 cell specific effect in cell-transplanted nude mice.



25	33	26	23	ERα (FL)
1.4	4.8	0.9	2.6	ERα46
26,4	37.8	26.9	25.6	(sum)

arbitrary values of densitometry

26	24	25	26	ERα (FL)
3.3	5.7	3.1	2	ERα46
29.3	29.7	28.1	28	(sum)

arbitrary values of densitometry

Figure 4: Effect of low-dose metformin pretreatment on tamoxifen-treated MCF-7 and TAMR1 cells (A) Solvent (-) and 40 μM low dose metformin-treated MCF-7 cells non- and tamoxifen (1 μM)-treated MCF-7 cells analyzed by Western analysis using an anti-ERα antibody. The table below shows arbitrary values of densitometry of the representative result of three separate experiments. (B) Solvent (-) and 40 μM low dose metformin-treated TAMR1 cells non- and tamoxifen (1 μM)-treated TAMR1 cells analyzed by Western analysis using an anti-ERα antibody. The table below shows arbitrary values of densitometry of the representative result of three separate experiments.

Metformin decreased tumor growth of TAMR1 cell-transplanted nude mice

We tested metformin in MCF-7- and TAMR1-cell transplanted nude mice. The amount of metformin delivered to the mice (750 mg kg^{-1} day^{-1}) was roughly targeted achieve a concentration of 30 to 100 μM in serum and tumor of xenografted mice (16, 17), which is comparable to low-dose metformin level of figure 4. Both metformin and tamoxifen did not have a significant effect on MCF-7 transplanted nude mice but there a tendency of decreased MCF-7 tumor weight for tamoxifen-fed mice (Figure 5A, compare (-) with TAM) and an increase of it for metformin-fed mice (Figure 5A, compare (-) and MET). There was no effect of tamoxifen on this metformin-induced increase of MCF-7 tumor weight (Figure 5A, compare MET and TAM+MET). TAMR1 cells showed larger tumors compared to MCF-7 cells. TAMR1 tumors showed tamoxifen-dependent growth (Figure 5B, compare TAM with (-)). Metformin

showed significant decrease of TAMR1 tumor growth (Figure 5B, compare MET with (-)). Metformin did not attenuate tamoxifen-induced tumor growth (Figure 5B, compare TAM+MET with TAM). There was no significant body weight change with tamoxifen (Figure 5C, 5D, compare (-) and TAM) or metformin (Figure 5C, 5D, compare and MET) treatment but combination of the two induced significant body weight loss (Figure 4C, 4D, compare TAM+MET with (-) and TAM). Thus, metformin attenuated TAMR1 tumor weight but not those of MCF-7 tumors.

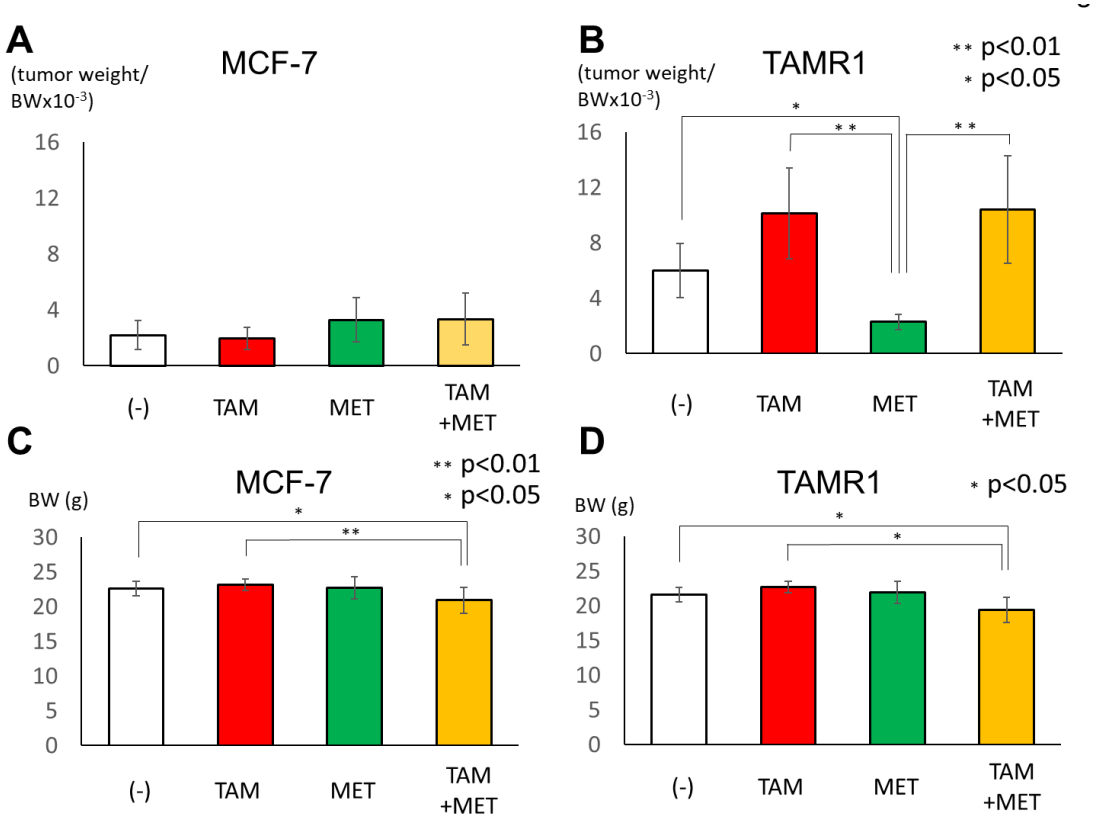


Figure 5: Tumor weight and body weight of nude mice transplanted of MCF-7 and TAMR1 cells treated with tamoxifen and metformin (A) Tumor weight of MCF-7 cell-transplanted nude mice treated with tamoxifen and metformin and combination of the two. (B) Tumor weight of TAMR1 cell-transplanted nude mice treated with tamoxifen and metformin and combination of the two. (C) Body weight of MCF-7 cell-transplanted nude mice treated with tamoxifen and metformin and combination of the two. (D) Body weight of TAMR1 cell-transplanted nude mice treated with tamoxifen and metformin and combination of the two.

DISCUSSION

Metformin has been implicated in cancer prevention by numerous cell line and mouse model studies (18-20). Several human clinical studies have been performed but most on therapy (19)

rather than cancer prevention (21). There are also controversial reports that claim metformin has no specific effect on cancer risk (22).

Concerning reports based on cell lines, one problem is that the usage of metformin at mM level, the concentration level that cannot be achieved in plasma (23). High doses of metformin (mM level) activate 5' adenosine monophosphate-activated protein (AMP)-kinase by interfering the mitochondrial respiratory complex 1(24, 25). At low μM concentration, metformin inhibits mitochondrial oxidation of glutamate + malate (25). As an anti-diabetic drug, metformin also is involved in down-regulation of circulating insulin and activation of the immune system(26). It also has been suggested in necroptosis, pyroptosis, ferroptosis, hypoxia (27). Another interesting aspect of metformin is its involvement in regulating alternative splicing at mM level by activating AMP-kinase (28) by phosphoryating the splicing factor serine/arginine-rich splicing factor 1 (SRSF1) (29).

Most of these studies are targeted to proliferation, therapy, and prevention of cancer with little known of its effect on tamoxifen-resistance. We showed here that metformin is involved in alternative splicing of the *ERα* gene at high-dose metformin. The effect of high-dose metformin (2.5, 5 mM) on ERα46 splicing in MCF-7 cells was reversed in its tamoxifen-resistant derivative, TAMR1 cells. Unfortunately, the effect of high-dose metformin showed an equal effect on cell viability of MCF-7 and TAMR1 cells.

In comparison to this high-dose metformin, low-dose metformin attenuated the tamoxifen-induced ERα46 expression in TAMR1 cells but not in MCF-7 cells. Lastly, metformin-fed cell-transplanted nude mice reduced tumor weight of TAMR1 but not MCF-7 cells. There are several limitations in this study, First, the expression of ERα46 is known to increase when MCF-7 cells become hyperconfluent (10). Though the cells in this study were cultured in log phase, there may be regions that were hyperconfluent. Thus, the differential expression of ERα46 in this study needs to be interpretated carefully. Second, combination of metformin and tamoxifen in nude mice showed a decrease in body weight by unknown toxicity of combined metformin and tamoxifen treatment.

Taken together, high-dose metformin was involved in alternative splicing of the *ERα* gene resulting in increased expression of ERα46 in TAMR1 cells but had comparable toxicity with MCF-7 cells. Low-dose metformin attenuated tamoxifen-induced increase of ERα46 expression in TAMR1 cells and attenuated growth of TAMR1 cell transplanted nude mice but not MCF-7 transplanted ones.

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AUTHOR CONTRIBUTIONS

Yo.H., Yur.H, and K.O. conducted the experiments with the assistance of T.T., Yut.H. K.O. wrote the manuscript. M.M., Y.M. helped and M.E. helped supervising the experiments.

Competing interests

All authors declare no conflicts of interest associated with this manuscript.

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