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MRTF-A transactivates COMT gene and decreases the anti-tumor effects of tamoxifen

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ABSTRACT

Myocardin-related transcription factors A (MRTF-A) is a myocardin-related transcription factor that have been found strongly activated CarG box–containing genes through its direct binding to serum response factor (SRF). In the present study, the MRTF-A expression vector was constructed. The MTT assay showed that transfection of MRTF-A could significantly decrease the anti-tumor effect of tamoxifen on MCF-7 breast cancer cells. The bioinformatics analysis found that the CarG element existed in the promoter region of COMT gene of many familiar vertebrates, including of human, rhesus macaque, chimpanzee, etc. The results of RT-PCR assay further showed that MRTF-A could enhance the transcription level of COMT. These results are the first to indicate that COMT might be a target gene which could be regulated by MRTF-A/SRF, and such transactivation event might be involved in the process of tamoxifen resistance.

Keywords: MRTF-A; Tamoxifen; COMT; Breast Cancer

1. INTRODUCTION

Breast cancer is the most common cancer diagnosed in women in the world. Seventy percent of diagnosed breast cancers express Estrogen Receptor alpha (ER*a*) and are likely to be hormone-responsive. The most common therapy for ER*a*–positive breast cancers has employed the use of selective estrogen receptor modulators (SERMs) such as tamoxifen. As an adjuvant therapy in breast cancer, tamoxifen improves overall survival, and its widespread use is thought to have made a significant contribution to the reduction in breast cancer mortality seen over the last decade. However, although many patients benefit from tamoxifen, the resistance is an important clinical problem [1,2].

Myocardin-related transcription factors (MRTFs); in-cluding myocardin, MRTF-A/MKL1/MAL, and MRTF-B/MKL2, comprise a family of related transcriptional coactivators. MRTFs drives transcription through inter-action with the ubiquitous transcription factor serum response factor (SRF), which acts on a responsive element $CC(A/T)_6GG$ (known as CarG box) that is commonly found in many gene promoters. Myocardin is expressed specifically in cardiac and smooth muscle cells and activates muscle genes associated with the differentiation of these cell types. MRTF-A and MRTF-B are expressed in a broad range of cell types [3,4].

Previous publications have suggested that MRTF-A might plays a role in development of mammary myoepithelial cells and breast cancer [5,6]. In the present paper, we further found that MRTF-A could decrease the anti-tumor effect of tamoxifen on MCF-7 human breast cancer cells, and the transcriptional activation of catechol-O-methyltransferase (COMT), a phase II metabolising enzyme of tamoxifen, might be involved in this process.

2. METERIALS AND METHODS

2.1. Regents and Cell Culcure

Tamoxifen (Wako Pure Chemicals, Osaka, Japan) was dissolved in Phosphate Buffered Saline (PBS). 1kb DNA ladder marker was purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). Human liver cell lines L02 and breast cancer cell line MCF-7 was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) containing 10% fetal bovine serum (FBS; Gibco).

2.2. Construction of MRTF-A Expression Vector

Total RNA of the LO2 cells was isolated using the method described in molecular cloning. First-strand cDNA was synthesized from the total RNA using M-MLV reverse transcriptase (Promega, Madison, USA) and oligo (dT)18 (Sangon, Shanghai, China). The full-ength MR-

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TF-A gene was amplified by polymerase chain reaction (PCR) using the cDNA and the following primer pair: 5'-CAAGGTACCATGCCGCCTTTGAAAAG -3' (forward) and 5'- CCCGAATTCAGCCAGAGAGCTA- CAAGC -3' (reverse). PCR was performed at 94°C for 5 min, then 28 cycles at 94°C for 45 s, at 60°C for 45 s, and at 72°C for 3 min; extension was carried out at 72°C for 10 min. The PCR product (2824 bp) was double- digested with kpnI and EcoRI (Takara, Kyoto) and inserted into the pcDNA3.1 (+) mammalian expression vector (Invitrogen, Carlsbad, Canada). The recombinant construction was analyzed by restriction-enzyme digestion and sequencing to determine reading frame orientation and confirm sequence fidelity, and the positive recombinant plasmid was named pcDNA-MRTF-A.

2.3. Cell Transfection

Transient transfection of pcDNA-MRTF-A plasmid into the MCF-7 cells using Lipofectamin^{TM} 2000 transfection reagents (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. As a negative control, the empty vector pcDNA3.1 (+) (mock) was transfected in parallel, and to assess the transfection efficiency, the pEGFP-C3 plasmid was also transfected as a positive control simultaneously.

2.4. Evaluation of the Inhibition Rate of Tamoxifen on MCF-7 Cells

The MCF-7 human breast cancer cells were plated in 96-well plates at a density of 8×10^3 cells/well and transfected with the pcDNA-MRTF-A plasmid or pcDNA3.1 (+). After 24h, the medium was removed and replaced with fresh medium containing tamoxifen at concentrations of 5, 10 and 20 μM. Forty-eight hours later, MTT assay was performed. In brief, MTT (5 mg/ml) was added to the wells (20 μl/well). The plate was incubated in a cell incubator for 4 h, then the supernatant was removed and 150 μl of dimethyl sulfoxide was added into each well. After incubation for 10 min, the absorbance of each well was measured using a microplate reader (Bio-Rad) with a wavelength of 570 nm, with the reference wavelength set at 630 nm. Absorbance (A) was directly proportional to the number of viable cells. All assays were performed using six replicates. The inhibition rate was calculated as follows:

$$\text{Inhibition rate}(\%) = (1 - \frac{A_{\text{experimental}}}{A_{\text{control}}}) \times 100$$

2.5. Bioinformatics Screen of the CarG Box in the Promoter of COMT Gene

The genomic sequence of COMT gene was extracted from the Database of GenBank. Their transcription start sites were then located by using the University of California at Santa Cruz (UCSC) genome brower, and the

Table 1. Primers used in RT-PCR analysis. (a. F: forward primer. R: reverse primer)

Gene	Primers^a	Product (bp)
GAPDH	F: 5'-ATTCAACGGCACAGTCAAGG-3' R: 5'-GCAGAAGGGGCGGAGATGA-3'	213
MRTFA	F: 5'-ACCGTGACCAATAAGAATGC-3' R: 5'-CCGCTCTGAATGAGAATGTC-3'	269
COMT	F: 5'-GGATTTCGCTGGCGTGAAG-3' R: 5'-TCCACCACCTCCCTGTATTCC-3'	306

sequence from 2000 bp upstream of the transcription sites to 200 bp downstream of the transcription start sites (–2000~+200) was extracted by using the UCSC database assemblage, and then the potential CarG box was analyzed using the primer premier 5.0 software [7].

2.6. RT-PCR Analysis

Total RNA of the transfected cells was extracted, and then the potential residual genomic DNA was eliminated with RNase-free Dnase I (Bio Basic Inc, Ontario, Canada). First-strand cDNA was synthesized as described above. For PCR amplification, primers specific for the cDNA of the MRTF-A, COMT gene and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used (**Table 1**). PCR was performed at 94°C for 5 min, then 25 cycles at 94°C for 30 s, at 54°C for 30 s, and at 72°C for 45 s; extension was carried out at 72°C for 10 min. PCR products were electrophoretically separated in 1.5% agarose gels and visualized by ethidium bromide staining. The densities (D) of the bands were analyzed with Quantity One software and relative mRNA levels were deduced from the ratio of the mean values of MRTF-A or COMT to that of GAPDH. Cells transfected with the empty plasmid pcDNA3.1 (+) was used as blank control. The assay was performed using three replicates. The relative mRNA level was defined as:

$$\text{Relative mRNA level} = \frac{D_{\text{target gene}}}{D_{\text{GAPDH}}}$$

2.7. Statistical Analysis

The data from the above mentioned experiments were expressed as mean ± SD. The statistical significance of differences was determined using Student's t test. The minimal level of significance was $P < 0.05$.

3. RESULTS

3.1. Construction of MRTF-A Expression Vector

The cDNA encoding human MRTF-A was obtained by RT-PCR from Human liver cell lines L02 and cloned into the pcDNA3.1 (+) vector. By the methods of restriction digestion and sequence analyses, we confirmed that the length, position and orientation of the inserted MRTF-A gene were all correct, suggesting that the re-

combinant pcDNA-MRTF-A plasmid was constructed successfully (**Figure 1**).

3.2. Cell Transfection

The endotoxin-free plasmids were extracted and transfected into the MCF-7 cells using Lipofectamin^{TM} 2000 transfection reagents. To assess the transfection efficiency, 0.5μg pEGFP-C3 plasmid was also transfected as a positive control simultaneously. As shown in **Figure 2**, the MCF-7 cell transfected with pEGFP-C3 exhibited bright fluorescence, indicating that the transfection efficiency in the experiment was high enough to perform the following experiments.

3.3. Effect of MRTF-A on the Anti-Tumor Activity of Tamoxifen

To investigate the effect of MRTF-A on the anti-tumor activity of tamoxifen, the MCF-7 breast cancer cells were transfected with the MRTF-A or the mock plasmid, and then treated with tamoxifen at different doses for 48 h. The inhibition rate was determined by MTT assay. As inhibition rates of cells transfected with MRTF-A were shown in **Figure 2**, at 5, 10 and 20 μM tamoxifen, the all significantly lower than the cells transfected with mock plasmid ($p<0.05$).

3.4. Bioinformatics Screen of the Potential Carg Box in the Promoter of COMT Gene

To investigate whether COMT was a potential target gene of MRTF-A, the sequence from 2000 bp upstream of the transcription sites to 200 bp downstream of the transcription start sites (–2000~+200) of COMT gene of familiar vertebrate was analyzed. As shown in **Table 2**, the CarG-like element existed in many vertebrates, including of human, rhesus macaque, chimpanzee, etc. This result indicated that COMT might be a target gene which could be regulated by MRTF-A/SRF.

3.5. Effect of MRTF-A on the Transcription Level of COMT

To further detect whether the transcription of COMT could be regulated by MRTF-A, the RT-PCR analysis was performed to detect the effect of MRTF-A on the
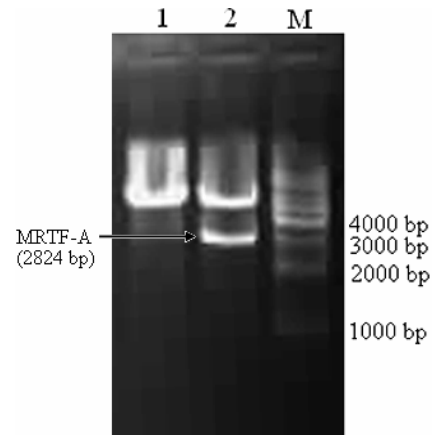

Figure 1. Identification of recombinant pcDNA-MRTF-A plasmid by restriction endonuclease. Lane 1: pcDNA3.1(+) plasmid double-digested with kpnI and EcoRI; Lane 2: pcDNA-MRTF-A double- digested with kpnI and EcoRI; Lane M: 1kb DNA ladder marker.
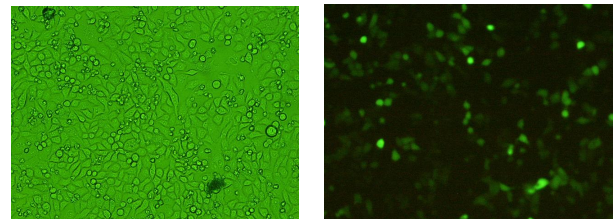

Figure 2. Cell image of MCF-7 transfected with PEGFP-C3-C3 plasmid. The left one was the normal micrograph and the right one was the fluorescence micrograph in the same visual field.

Table 2. The carg box in the promoter region of comt gene.

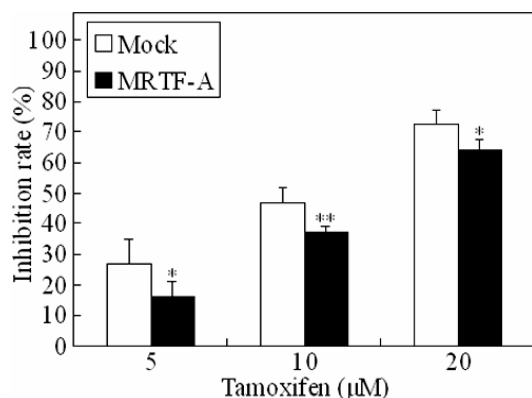| Organism		GenBank ID	CarG seq	Position
Sciencific name	Common name			
Homo sapiens	Human	NT_011519	CCTTTTATGG	-1105
Macaca mulatta	Rhesus macaque	NW_001095157	CCTTTATTGG	-933
Pan troglodytes	Chimpanzee	NW_001230944	CCTTTTATGG	-1187
Mus musculus	Laboratory mouse	NT_039624	CCTATTGGGG	-834
Rattus norvegicus	Rat	NW_047358	CCTGTTTTGG	-616
Bos taurus	Cattle	NW_001493554	CCAATACTGG	-9
			CCTAAAGTGG	-1175
Gallus gallus	Chicken	NW_001471459	CCTTATCAGG	-21
			CCTAAAAGGG	-1582
			CCCTAAAAGG	-1583
Monodelphis domestica (Opossum), Ornithorhynchus anatinus (duck-billed platypus), Bubalus bubalis (river buffalo), Canis lupus familiaris (Dog), Felis catus (Cat), Ovis aries (Sheep), Sus scrofa (Pig), Danio rerio (Zebrafish)		The typical CarG box was not found in the promoter of COMT gene. Alternatively, the COMT gene still remained unclear		

Figure 3. Decrease in anti-tumor of tamoxifen on MCF-7 breast cancer cells following transfection of MRTF-A. *p<0.05; **P<0.01 (compared with the mock).
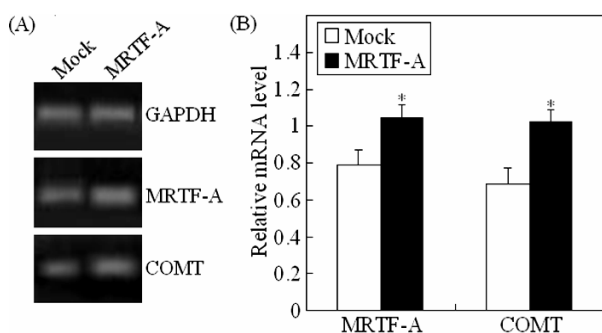

Figure 4. Promotion of COMT mRNA expression transfected with MRTF-A. (A) Electrophoretica graph of the RT-PCR assay; (B) The relative mRNA level determinded with the ratio of the band density of MRTF-A or COMT to that of GAPDH. *p<0.05; **P<0.01 (compared with the mock).

mRNA level of COMT. As shown in **Figure 3**, the relative mRNA level of MRTF-A and COMT of cells transfected with MRTF-A were both significantly higher than those of the mock cells, suggesting that MRTF-A could promote the transcription level of COMT.

4. DISCUSSION

In 1980s, tamoxifen was approved by the Food and Drug Administration (FDA) as an adjuvant therapy for the breast cancer. Despite the benefits of tamoxifen therapy, almost all tamoxifen-responsive breast cancer patients develop resistance to therapy. The drug resistance to tamoxifen therapy can take many forms, and one of the important possible mechanisms may be the metabolic activation [2,8,9].

The metabolic activation of tamoxifen involves the transformation of tamoxifen into the 4-OH-tamoxifen and 3, 4-di-OH-tamoxifen (catechol). It has been suggested that the anticancer activity of tamoxifen may be due to its 4-hydroxylated metabolite. However, because 4-OH-tamoxifen could be subsequently hydroxylated into the 3, 4-di-OH-tamoxifen, the 4-hydroxylated metabolite is usually at low observable levels in mammals.

The 3, 4-di-OH-tamoxifen is a suitable substrate of COMT, which always catalyzes the transfer of a methyl group from S-adenosyl-methionine (SAM) to one of the phenolic hydroxyl groups in a variety of catechols including catechol estrogens and catecholamine neurotransmitters. In the presence of SAM, the 3, 4-di-OH-tamoxifen would be catalyzed into monomethoxy 3, 4-di-OH-tamoxifen [10,11,12].

MRTF-A is a member of Myocardin-related transcription factors family, which transactivates the promoters containing consensus CarG box. Compared with myocardin, which is expressed specifically in cardiac and smooth muscle cells, MRTF-A is expressed in a broad range of cell types and has more extensive transactivating functions than myocardin [13]. Previous studies have showed that the metabolizing function of COMT within the coronary artery may be an important determinant of the cardiovascular protective effects of circulating estradiol, and the COMT expression may be regulated in the myometrium to control the local action of estrogen [14,15,16]. In the present study, the bioinformatics analysis found that the CarG element widely existed in the promoter region of COMT gene of many familiar vertebrates, especially in human, rhesus macaque and chimpanzee, and the results of RT-PCR further showed that MRTF-A could enhanced the transcription level of COMT. These results indicated that COMT might be a target gene which could be regulated by MRTF-A/SRF, and such transactivation might be involved in the process of tamoxifen resistance. Further studies addressing the detail relationship between MRTF-A, COMT and tamoxifen and the mechanism should provide fundamental insights into the function of MRTF-A in the development and overcome of the breast cancer.

5. ACKNOWLEDGMENTS

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