The Impact of TIMM17A on Aggressiveness of Human Breast Cancer Cells

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Abstract. *Background: The mitochondrial protein translocase of inner mitochondrial membrane 17 homolog A (TIMM17A) has been identified as a biomarker of breast cancer. The present study aimed to investigate the biological role of TIMM17A in human breast cancer cells. Materials and Methods: Anti-TIMM17A transgenes were stably transfected into MDA MB-231 and MCF-7 breast cancer cell lines. The impact of TIMM17A knock-down on cell migration and invasion were evaluated using the respective cell models. Results: Reducing the expression of TIMM17A in breast cancer cells resulted in reduction of cell migration using electric cell-substrate impedance sensing. It was also found that reduction of TIMM17A expression resulted in reduction of cell invasion compared to vector control. Conclusion: TIMM17A has a profound impact on the cellular function of breast cancer cells. A decrease of TIMM17A expression is associated with the reduction of the aggressiveness of breast cancer cells. TIMM17A, therefore, has potential in prognosis and treatment of breast cancer.*

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Mitochondrial dysfunction is found in many cancer types (1, 2). The mitochondrion is responsible for cell energy metabolism and is an important cellular organelle in apoptosis (3, 4). The mitochondrion has its own DNA which is different from that of other organelles in cells. Although a few mitochondrial proteins are encoded by its the mitochondrial genome, most mitochondrial proteins are encoded by genomic DNA in the nucleus. After translation, these proteins need the transported from the cytoplasm to the mitochondrial matrix through the outer and inner membranes of the mitochondrion. The inner membrane is denser than the outer and it is more difficult to transport material across it. The transporting channels in the mitochondrial inner membrane are referred to as translocase of inner mitochondrial membrane proteins (TIMM). Many proteins encoded by the nuclear genome are transported by TIMM23 complex (5-7). TIMM17A is an essential subunit participating in the membrane integrated part of this complex (8).

Mitochondrial protein TIMM17A was reported as being increased in fibrolamellar carcinomas (9), and as a biomarker which increased in breast cancer (10, 11). However, the effect and mechanism of action of TIMM17A in human cells are not clear. TIMM17A protein is encoded chromosome 1 (1q32.1) of the nuclear genome (12). This gene area has been reported to relate to carcinogenesis, such as bladder cancer, neuroblastoma, prostate cancer and cutaneous melanoma (13-16), and especially to poor outcome in breast cancer (17-19). Higher mRNA expression of *TIMM17A* has been found in human breast cancer tissues (11). Therefore, the mechanisms through which TIMM17A act in carcinogenesis and tumour progression need greater attention.

In this study, we researched data online and used bio-informatics to analyse them. Then we knocked down the expression of *TIMM17A* using stably transfected breast cancer cell lines, and carried out cellular function experiments to investigate the biological role of *TIMM17A* in human breast cancer cells.

Materials and Methods

Cell lines and culture. Human breast cell lines MDA-MB-231 and MCF-7 were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRC, Paisley, UK) at 37˚C, with 5% CO_2.

RNA preparation and reverse transcription reverse transcription polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from the cultured breast cancer cells using Total RNA Isolation Reagent (Abgene, Epsom, Surrey, UK). The concentration of RNA was determined by ultraviolet spectrophotometry (WPA UV 1101; Biotech Photometer, Cambridge, UK). Total cDNA was reverse transcripted using transcription kits (Sigma, Poole, Dorset, UK). RT-PCR reactions were run in a Gene Amp PCR System 2400 thermocycler (PerkinElmer, Fremont, CA, USA). The mRNA levels of *TIMM17A* were assessed using *TIMM17A* primers (forward primer: 5'-TTG TGG ATG ACT GTG GTG-3'; reverse primer: 5'-CCA AAA GGT GAG GAA GGT -3'). The quality of DNA was verified using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) primers (forward primer: 5'-AGC TTG TCA TCA ATG GAA AT-3';reverse primer: 5'-CTT CAC CAC CTT CTT GAT GT -3').

Real-time quantitative PCR. The mRNA level of gene expression was determined by the real-time quantitative PCR (qPCR) method using the quantitative cDNA as the template. Z Sequence (5'-ACT GAA CCT GAC CGT ACA-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added to the primer sequence for qPCR detection using Icycler IQ™ (Bio-Rad, Hemel Hemstead, UK). *GAPDH* expression was used as an internal control. The reaction conditions were: 94˚C for 5 min, then 40 cycles, each with 94˚C for 15 s, 55˚C for 35 s and 72˚C for 20 s.

Screening of breast cancer cell lines stably transfected with TIMM17A ribozymes. Human hammer-head ribozymes targeting *TIMM17A* were designed based on the secondary structure of the mRNA using the Zuker RNA mFold program. Primer sequences of the anti-*TIMM17A* ribozymes were: forward primer :5'-CTG CAGGCA CCA CTT GTG ATG GAG TTC CAG GCT GAT GAG TCC GTG AGG A-3'; reverse primer: 5'-ACT AGT ATG GTT CAA GTC AGA GGA AAG GAA GAT TTC GTC CTC ACG GAC T-3'. The ribozymes were accordingly synthesized and then cloned into pEF6/V5-His-Topo T/A vector (Invitrogen, Paisley, UK). The cloned vector was transfected into MDA-MB-231 and MCF-7 cells using an Easyjet Plus electroporator (EquiBio, Kent, UK). After selection with culture medium containing 5 μg/ml blasticidin, the verified transfectants were cultured in maintenance medium containing 0.5 μg/ml blasticidin.

Cell migration assay. 96W1E array (Applied Biophysics Inc., Troy, NJ, USA) (96 wells, one circular electrode in each well) was incubated with the medium with L-cysteine solution for 1 h. Then breast cancer cells were seeded at 300,000 per well in 200 μl medium. The 9600 model electric cell-substrate impedance sensing (ECIS) instrument (Applied Biophysics Inc., Troy, NJ, USA) was used for migration assay in this study (20). The impedance at 30 kHz was recorded for 11 h. The experimental data were analyzed using the ECIS-9600 system.

Cell invasion assay. Transwell inserts (Becton Dickinson, Labware, Oxford, UK) with 8 μm pores were pre-coated with 50 μg/insert of Matrigel (Collaborative Research Products, Bedford, MA, USA) before being rehydrated. Cells (30,000/insert) were seeded into each well. After 72 h, cells that had migrated through the matrix and adhered on the other side of the insert were fixed and stained with crystal violet. For analysis, the invaded cells were counted in 10 fields/insert (×40 magnification) to obtain the mean number of invading cells. The crystal violet was extracted with 10% (v/v) of acetic acid and the absorbance was obtained using a Bio-Tek ELx 800 multi-plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) to obtain a value reflecting the total number of stained cells (21).

Statistical analysis. Statistical analysis was performed using SPSS (IBM Corp, Armonk, NY, USA). Value of $p<0.05$ were considered statistically significant.

Results

The mRNA expression of TIMM17A is increased in human breast cancer tissues. We found the mRNA expression of *TIMM17A* significantly increased in human breast cancer tissues compared to normal breast tissue (n=1100) when using raw gene-expression data from the Cancer Genome Atlas (TCGA). mRNA expression of *TIMM17A* was significantly up-regulated in breast cancer compared to normal breast tissues (n=112) ($p<0.001$). Furthermore, the mRNA expression of TIMM17A was lower in stage I compared to stage II-V ($p=0.001$) breast cancer. The data are shown in Figure 1.

TIMM17A knock-down and establishment of stable cell lines. We next examined the expression level of *TIMM17A* in breast cancer cell lines. We also found high expression of *TIMM17A* in human MDA-MB-231 and MCF-7 wild-type cells (data not shown).

Plasmids containing ribozymes targeting *TIMM17A* or vector controls were stably transfected into MDA-MB-231 and MCF-7 wild-type cells. The expression of *TIMM17A* was found reduced in both MDA-MB-231 and MCF-7 cells with *TIMM17A* knock-down using RT-PCR (data not shown) and qPCR (Figure 2). The experimental results revealed a significant down-regulation of *TIMM17A* in MDA-MB-231 cells ($p=0.004$) and MCF-7 cells ($p=0.02$).

Reduction of TIMM17A expression reduced breast cancer cell migration and invasion. Our data showed that down-

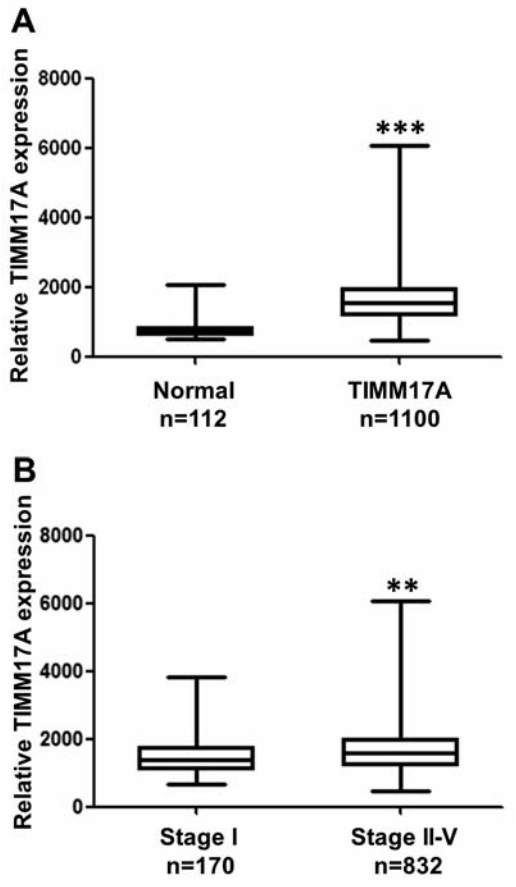

Figure 1. *The expression of translocase of inner mitochondrial membrane 17 homolog A (TIMM17A) was up-regulated in breast cancer according to the raw data deposited in the Cancer Genome Atlas (TCGA). The mRNA expression level of TIMM17A was up-regulated in breast cancer samples compared with normal tissues (A) and in stage II-V in breast cancer compared with stage I (B). The horizontal line indicates the median and the box the interquartile range; the whiskers represent the minimum and maximum values. Significance was assessed by the Mann–Whitney test (**p<0.01; ***p<0.001).*

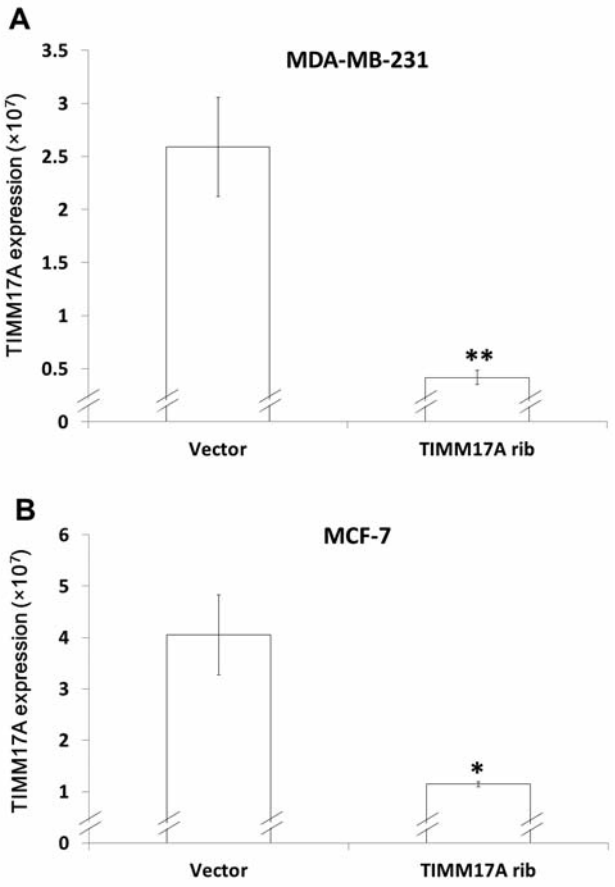

Figure 2. *Translocase of inner mitochondrial membrane 17 homolog A (TIMM17A) knock-down in human breast cancer cell lines. Ribozymes for TIMM17A were stably transfected into MDA-MB-231 and MCF-7 breast cancer cell lines and the mRNA expression of TIMM17A was then detected using the real-time quantitative PCR. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping control. All experiments were performed independently a minimum of three times (*p<0.05; **p<0.01).*

regulation of TIMM17A expression reduced migration in both MDA-MB-231and MCF-7 cells (Figure 3) to a significant extent (both at $p<0.001$).

The number of invaded cells was significantly reduced in *TIMM17A* knock-down cells compared to the pEF-transfected control cells. Tumour cell invasion was significantly suppressed through knock-down of *TIMM17A* in both MDA-MB-231 ($p=0.007$) and MCF-7 ($p=0.002$) cells (Figure 4).

Discussion

TIMM17A is critical for complex formation in mitochondrial function, that is closely related to human malignancies, including breast cancer. Mitochondrial dysfunction would have an influence on cancer diagnosis and therapy (22). At present, there is no report about the roles of TIMM17A in breast cancer or breast cancer cell lines.

In our study, TIMM17A, a mitochondrial protein was found to be highly expressed in breast cancer compared to normal tissues using data from TCGA, which has abundant clinical resources (23). TIMM17A was also expressed less in stage I than in other stages of breast cancer. Despite there being no direct evidence for altered mitochondrial activity in breast cancer cells, these data indicate that TIMM17A expression level is related to malignancy. Definitely, the higher the degree of malignancy, the higher the expression of TIMM17A as occurs by using data from the database. In keeping with our results, Salhab *et al*. (11) and Xu *et al*. (10)

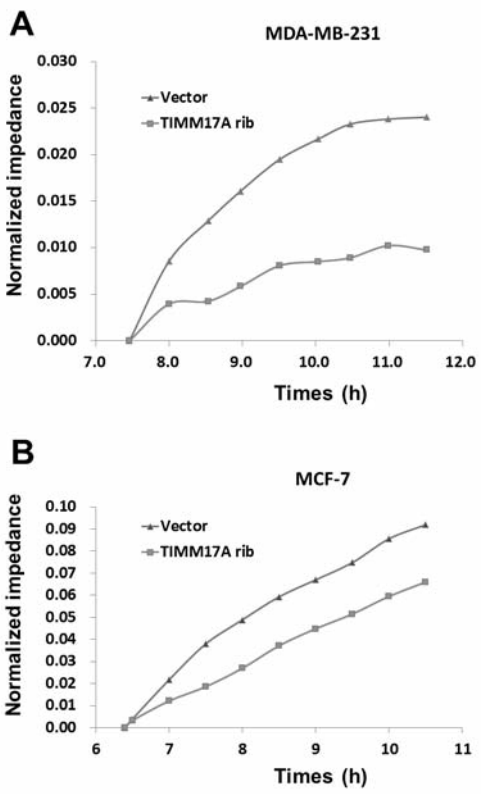

Figure 3. *Migration assay of translocase of inner mitochondrial membrane 17 homolog A (TIMM17A) knocked-down breast cancer cells and pEF vector control cells. The ECIS 9600 system (ECIS 1600R, 3000 Hz) was used in the migration assay. Knock-down of TIMM17A led to a significant decrease in cell migration when compared to control cells. All experiments were performed independently a minimum of three times (p<0.001).*

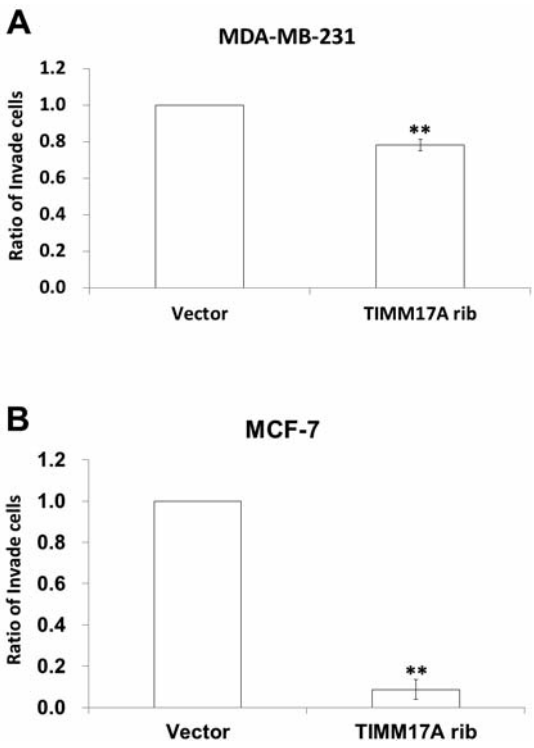

Figure 4. *Invasion assay of translocase of inner mitochondrial membrane 17 homolog A (TIMM17A) knocked-down breast cancer cells and pEF vector control cells. Knock-down of TIMM17A significantly suppressed invasion by MDA-MB-231 and MCF-7 cells compared to the pEF-transfected control cells. All experiments were performed independently a minimum of three times (**p<0.01).*

found higher TIMM17A expression was related to poorer disease-free and overall survival in breast cancer.

Mitochondrial dysfunction is related to nuclear genomic instability and apoptosis (24, 25). The function of mitochondria is closely related to the protein-transporting system of the inner mitochondrial membrane. TIMM23 complex is most important in this transport system. As a constituent subunit of TIMM23 complex, TIMM17A is critical for TIMM23 complex formation (26). Hence the TIMM17A level has an important role in mitochondrial dysfunction, possibly even in progression of tumor.

As we found a higher mRNA expression of *TIMM17A* in breast cancer tissues compared to normal breast tissue, we used ribozymes to knock-down the expression of *TIMM17A* in breast cancer cells. Using the ECIS system, we found that knocking-down TIMM17A expression reduced the migration and invasion of breast cancer cells. These results indicate that TIMM17A increased the breast cancer cell migration and invasion. Consistent with our findings, we have also found TIMM17A expression is inversely related to distant metastasis using breast cancer data from TCGA and gene sets that were expressed in primary Estrogen receptor (ER)-positive breast cancer, negatively correlate with developing distant metastases [(false discovery rate (FDR)=0.049, Normalized Enrichment Score (NES)=−1.488]. This indicates that TIMM17A may positively correlate to metastasis in ER-positive cases. Although a great amount of breast cancer expressed ER and received endocrine treatment, little is known about the impact of TIMM17A on metastasis of ER-positive breast cancer (27). This negative relationships indicates that higher *TIMM17A* expression would be related to malignant and metastatic competence.

In conclusion, TIMM17A was found be up-regulated in breast cancer tissues. The expression of TIMM17A appears to facilitate migration and invasion of breast cancer cells. Therefore, TIMM17A may have a potential role in prognosis of and be a relevant target in breast cancer.

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