MTERFD1 functions as an oncogene

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ABSTRACT

***MTERFD1*, also named MTERF3 (mitochondrial transcription termination factor 3), regulates transcription of the mitochondrial genome. MTERFD1 is a mitochondrial protein that represses mammalian mitochondrial DNA initiation *in vivo*. In this study, we found that *MTERFD1* gene amplification and high expression existed in many different types of cancer. Significantly, increased expression of *MTERFD1* gene was correlated with lower overall survival rate in clinical. Overexpression of *MTERFD1* gene promoted to tumor cell growth *in vivo* and *in vitro* and increased the percentage of cells in S phase. In conclusion, our data firstly indicated the *MTERFD1* was an oncogene in many types of cancer.**

INTRODUCTION

Recent advances in cancer genomics have led to the paradigm shifts in cancer research. Genomic studies of multiple tumor types have begun to reshape our understanding of cancer genomes and their complexity [1–3]. Emerging genomic data have clearly stablished that each tumor harbors a mixture of cancer-causing genomic aberrations and innocent bystander mutations with no oncogenic potential. Accordingly, distinguishing drivers from passengers in the noisy cancer genome is a crucial step [4].

The Cancer Genome Atlas (TCGA) research network has profiled and analyzed large numbers of human tumors to discover molecular aberrations at the DNA, RNA, protein, and epigenetic levels. The accumulated data provide a major opportunity to develop an integrated model of commonalities, differences and emergent themes across tumor lineages [5]. Data from the TCGA indicate that the human mitochondrial transcription termination factor domain 1 (*MTERFD1*) was mutated in many types of cancer.

MTERFD1 was involved in the regulation of transcription of the mitochondrial genome. *MTERFD1* belongs to the MTERF family which consists of four members including MTERF1, MTERF2, MTERF3 and MTERF4 [6]. Previous research showed that MTERFD1 is a mitochondrial protein that interacts with the mitochondrial DNA promoter region and decreases transcription initiation in mammalian mitochondria. This negative regulation is likely important for fine-tuning mitochondria transcription in response to physiological demands [7].

Here, we found the amplification of *MTERFD1* gene in many types of cancers. In addition, *in vivo* and *in vitro* data indicated that *MTERFD1* gene possesses oncogenic properties.

RESULTS

MTERFD1 amplification occurred in many different types of cancer

Initially, we aligned the human sequences of the four members of the MTERF family of human (Figure S1A), as well as the *MTERFD1* sequences of Homo sapiens, Rattus norvegicus and Mus musculus (Figure S1B). Next, we

found that there was a high amplification rate of *MTERFD1* in many types of cancer (Figure 1A). We then compared with the amplification rate of *MTERFD1* with those of confirmed oncogenes, including NRAS, HRAS, KRAS [8], TWIST1 [9], SNAI1, SNA2 [10, 11], ABL1 [12] and MDM2 [13]), and found that the amplification frequency of MTERFD1 was higher than these oncogenes (Figure 1B). Moreover, *MTERFD1* mRNA level was positively correlated with copy number of *MTERFD1* (Figure 1C).

MTERFD1 expression was positively correlated with carcinogenesis, cancer metastasis, estrogen or androgen independence, and cancer immune resistance

Next, we searched Gene Expression Omnibus (GEO) for data pertaining to the analysis of *MTERFD1* expression in different types of cancer. Importantly, we found that a positive correlation exists between *MTERFD1* expressions and the cancer clinical stage or subtype. In human colorectal cancer, pancreatic ductal adenocarcinoma, cervical cancer, nasopharynx cancer, rectal cancer, and breast cancer, MTERFD1 expression was higher in tumor tissues than in adjacent normal tissue (Figure 2A). Interestingly, in prostate cancer and melanoma, *MTERFD1* mRNA expression was higher in metastatic sites than in the primary tumor. In multistep pancreatic carcinogenesis, *MTERFD1* mRNA level was higher in intraductal papillary-mucinous neoplasm (IPMN) than in normal tissue, intraductal papillary-mucinous adenoma (IPMA), and intraductal papillary-mucinous carcinoma (IPMC). In metastatic prostate cancer, the metastatic tumor site tissues showed a higher *MTERFD1* mRNA level than the normal, tumor adjacent, and primary tumor site tissues. The breast cancer cell MCF7 showed a higher *MTERFD1* expression in tamoxifen resistance cells than in tamoxifen sensitive cells. In prostate cancer, *MTERFD1* expression was higher in androgen-dependent than in the androgen independent cells from microdissected primary tumors. In mouse immune resistant lung cancer cell lines, generated by subjecting immune resistant cells to three rounds of *in vivo* immune selection. We found that *MTERFD1* mRNA expression level was higher in immune resistant cell lines than in immune susceptible cell lines. Thus, the above data suggested that *MTERFD1* gene played important roles in carcinogenesis, metastasis, estrogen, or androgen related cancers. In addition, data from the mice cell line experiments suggested that MTERFD1 was involved in cancer immune resistance.

Higher MTERFD1 expression in lung cancer, hepatocellular carcinoma, breast cancer, and pancreatic cancer tissues

We also investigated the MTERFD1 protein expression in various tumor tissues, using MTERFD1 expression in matched adjacent tissues as control. In general, MTERFD1 protein expression was higher in lung cancer tumor tissues, hepatocellular carcinoma, breast cancer, and pancreatic cancer than in normal tissues. In lung cancer, there was only one pair where the adjacent normal tissue showed a higher MTERFD1 protein level. In hepatocellular carcinoma, there were 4 of 55 pairs where adjacent normal tissues showed a higher MTERFD1 protein level. For breast cancer and pancreatic cancer, the ratio of higher MTERFD1 in adjacent normal tissues was 2 of 30 pairs and 5 of 30 pairs, respectively. The representative histology data is shown in Figure 3B. In hepatocellular carcinoma, there were two cancer tissues that showed a lower MTERFD1 protein level (indicated by arrows), and one adjacent normal tissue showed a higher MTERFD1 protein level (indicated by an arrow).

Correlation between MTERFD1 expression and overall survival in different types cancers

To evaluate the clinical significance of MTERFD1, we investigated whether the alteration of *MTERFD1* mRNA expression was associated with overall survival in breast cancer. The Kaplan-Meier survival curves of tumor-free survival and overall survival in cohorts 1 and 2, according to the ratio of *MTERFD1* level in each tumor sample compared with its median *MTERFD1* level, were shown in Figure 4. In lung cancer, within 10 months, patients with lower *MTERFD1* level had a higher survival rate (Figure 4A). The overall survival rate of patients with hepatocellular carcinoma showed a similar trend within 60 months (Figure 4B). More significantly, the overall survival of breast cancer patients during the 200-months follow-up period revealed that low *MTERFD1* level favored patient's survival (Figure 4C).

MTERFD1 overexpression promoted tumor growth *in vitro*

We also over-expressed *MTERFD1* by plasmid transfection. Following transfection, the *MTERFD1* expression levels were increased in PANC1, MCF7, HepG2, and A549 cells (Figure 5A). The proliferation analysis with the MTT assay showed that up-regulation of *MTERFD1* in these four types of cell lines promoted cell proliferation (Figure 5B). In fact, *MTERFD1* overexpression could promote the tumor formation *in vitro*, especially in PANC1 and MCF7 (Figure 5C). BrdU assay revealed that *MTERFD1* overexpression promoted cellular proliferation in PANC1, MCF7, HepG2, and A549 cells (Figure 5D).

MTERFD1 overexpression promoted tumor growth *in vivo* and reduced the survival rate

We inoculated *MTERFD1*-transfected MCF7 cells into nude mice using cells transfected with a blank vector

as a control (Figure 6A). We found that after six weeks, not only the tumor incidence in *MTERFD1*-transfected mice was higher than in control, but the size of the formed tumors was larger (Figure 6B). In addition, the tumors derived after inoculation with *MTERFD1*-transfected MCF7 cells grew faster and were larger than the control *in vivo* (Figure 6C). The overall survival of tumor-inoculated mice was similar to that of cancer patients. In particular, mice that were inoculated with *MTERFD1*-transfected MCF7 cells showed a lower survival rate, whereas all mice in the control survived to the end of the observation period (Figure 6D).

DISCUSSION

In the study of the oncogenic function of *MTERFD1*, we found that the amplification of *MTERFD1* conferred a selective growth advantage to the cells. This is an important characteristic of mut-driver gene, which is meaningful in tumor molecular therapy. So far, the number of frequently altered mut-driver genes (mountains) is nearing saturation, and a plateau is being reached, because the same mut-driver genes keep being "rediscovered" in different tumor types [14–22].

Approximately 20,000 protein-coding genes have been evaluated in the genome-wide sequencing studies of the 294,881 mutation that have been reported to date. Only 138 mut-driver genes were defined by the 20:20 rules. The 20:20 rules are that, to be classified as an oncogene, > 20% of the recorded mutation in the gene are at recurrent positions and are missense [1].

Half of the newly found mut-driver genes encode proteins that directly regulate chromatin via the modification of histones or DNA. Examples include the histones HIST1H2B and H3F3A, as well as the proteins DNMT1 and TET1 [1, 23–27]. A previous study has shown that *MTERFD1* is a negative regulator of mitochondrial DNA transcription [7], and regulation of mammalian

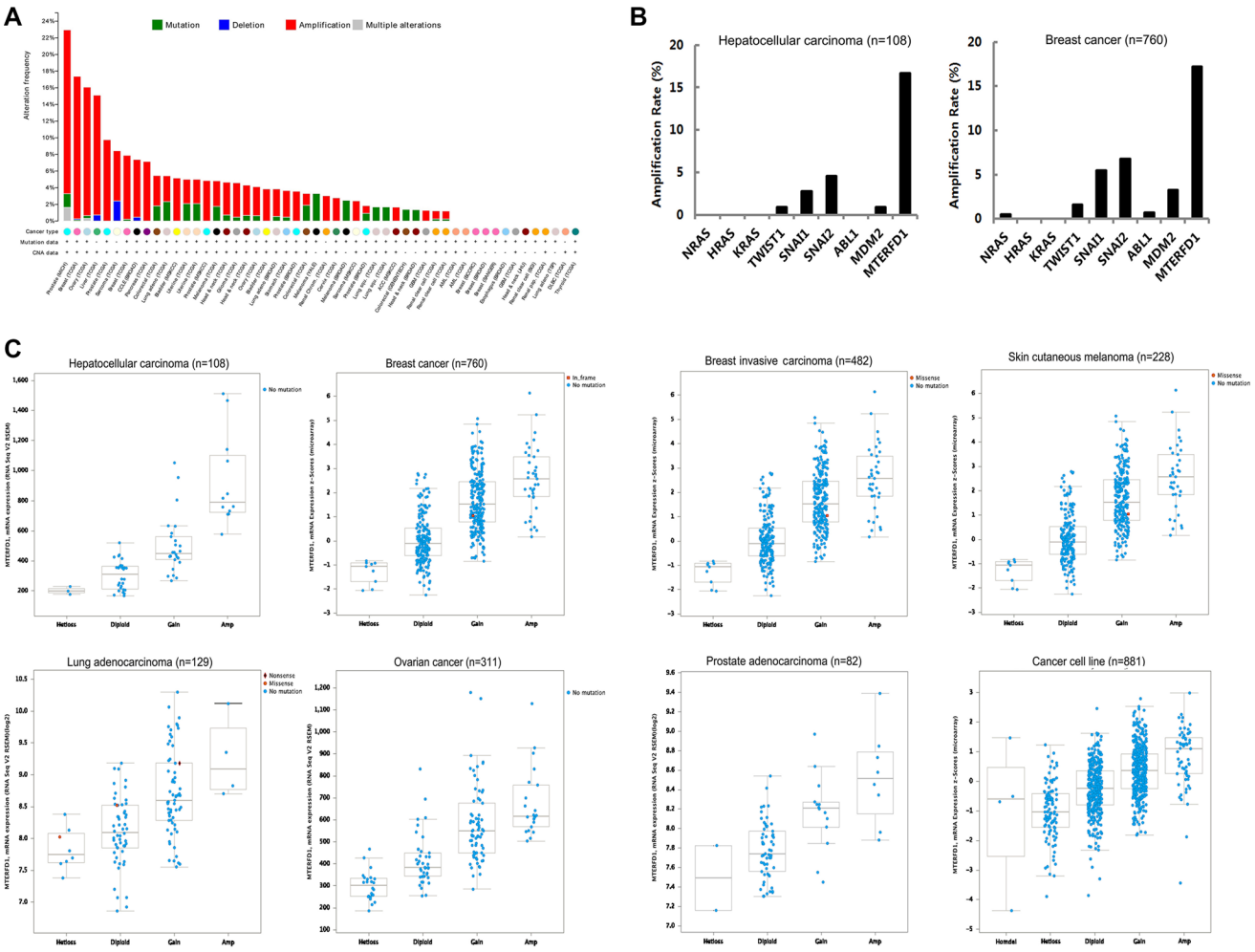

Figure 1: Frequency of alteration of *MTERFD1* in various types of cancer. Alteration of *MTERFD1* was visualized using the cBioPortal for Cancer Genomics. Mutation, deletion, amplification, and multiple alterations are shown in different colors. The main alteration of *MTERFD1* in different types of cancer is amplification (**A**). Amplification rate of *MTERFD1, NRAS, HRAS, KRAS, TWIST1, SNAI1, SNA2, ABL1* and *MDM2* (**B**). Comparison of MTERFD1 mRNA levels with copy number of *MTERFD1* in various types of cancer (**C**).

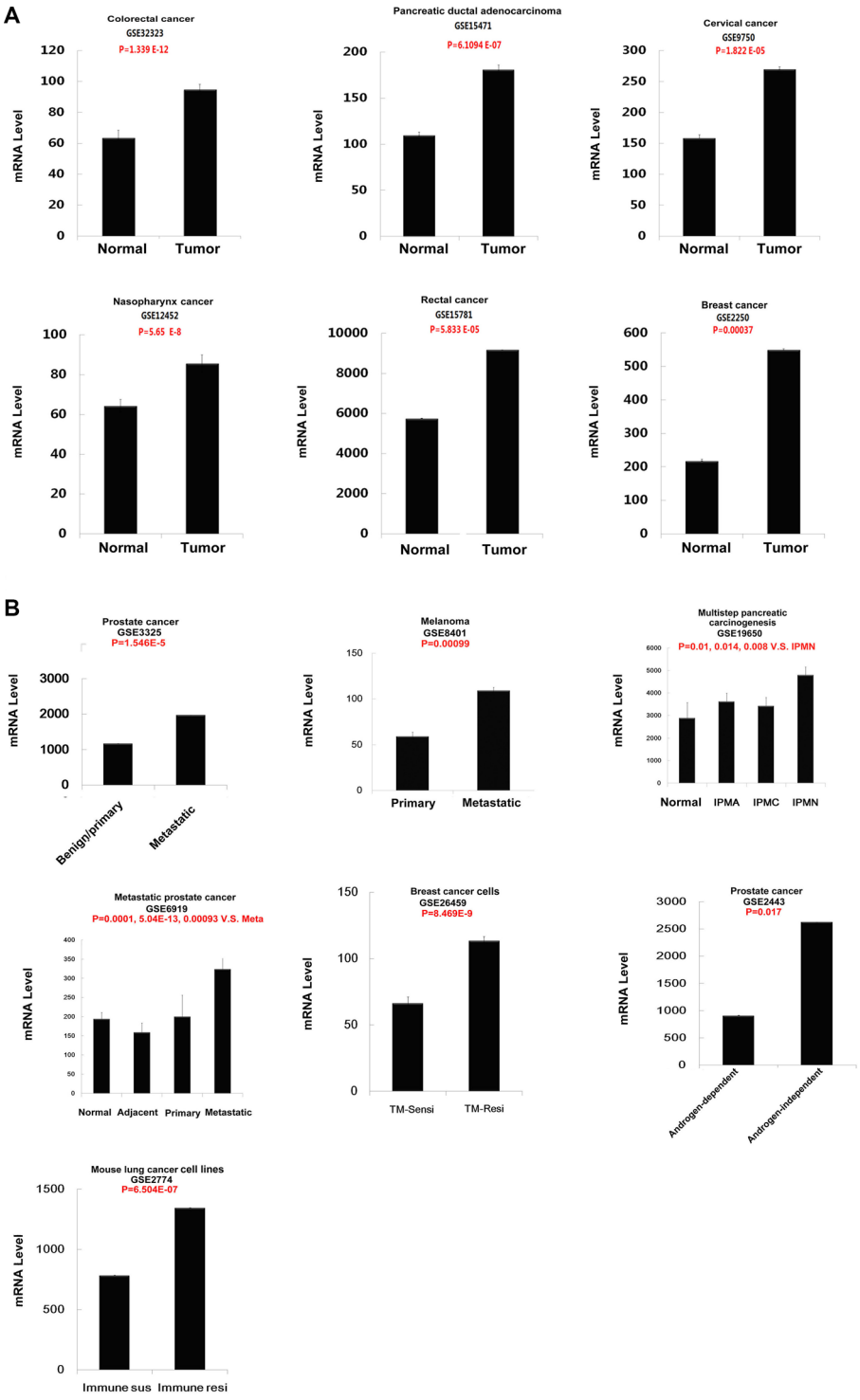

Figure 2: Correlation exists between *MTERFD1* expressions and the cancer clinical stage or subtype. *MTERFD1* mRNA expression level in tumors was compared with expression in the adjacent normal tissue in colorectal cancer, pancreatic ductal adenocarcinoma, cervical cancer, nasopharynx cancer, rectal cancer, breast cancer, prostate cancer, and melanoma. In multistep pancreatic carcinogenesis, *MTERFD1* mRNA expression level in papillary-mucinous neoplasm (PMN), intraductal papillary-mucinous adenoma (IPMA) and intraductal papillary-mucinous carcinoma (IPMC) were compared with expression in normal tissue. In metastatic prostate cancer, the *MTERFD1* mRNA level in primary tumor site and the metastatic tumor site were compared with the expression in the tumor adjacent tissue. In MCF7 breast cancer cell, the expression of *MTERFD1* in tamoxifen resistance cells and *MTERFD1* in tamoxifen sensitive cells were compared. In prostate cancer, expression of *MTERFD1* in androgen-dependent cells was higher than androgen-independent microdissected primary tumor cells. In mouse lung cancer cell lines, *MTERFD1* mRNA level in immune-resistant cell lines was compared with the expression of *MTERFD1* in immune susceptible cell lines. The GSE number was shown in the graphs, data was mean ± s.e.m. of MTERFD1 expression in different types of cancers $*P < 0.05$.

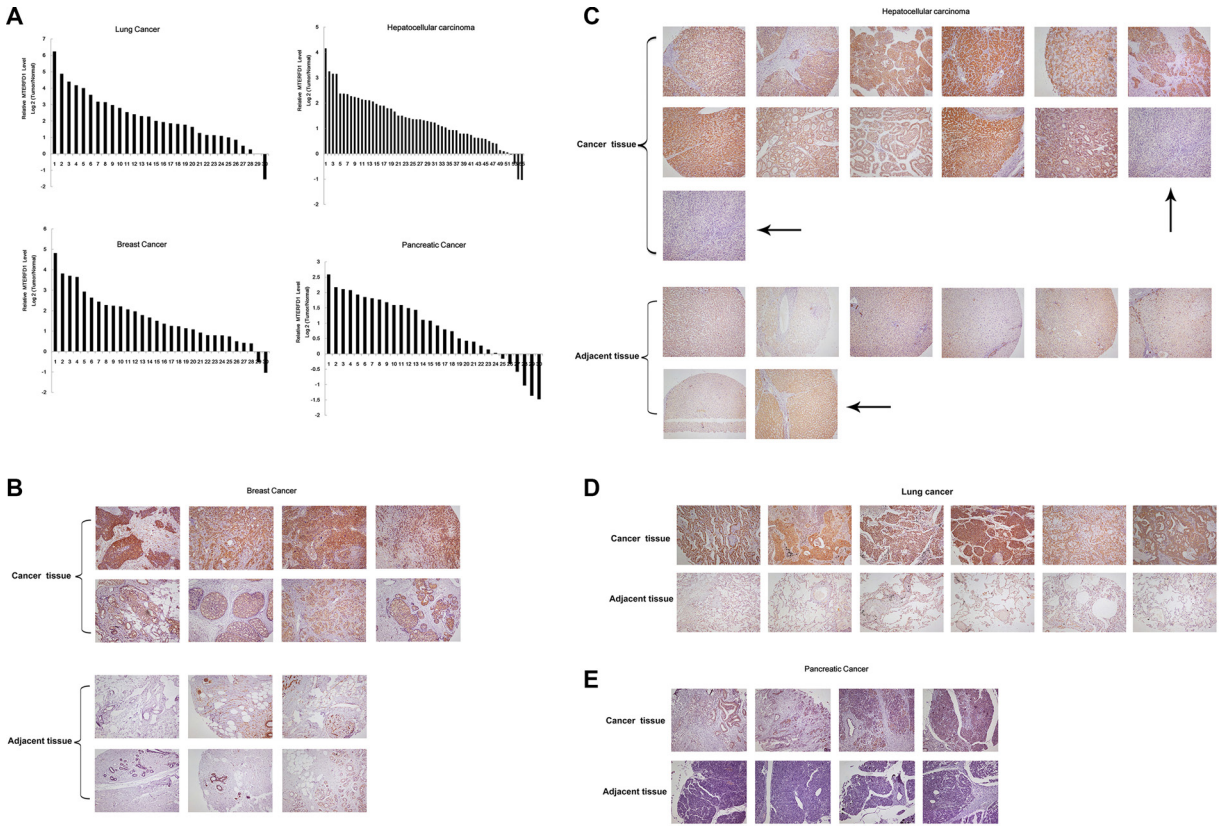

Figure 3: MTERFD1 expression in tumor tissues. The general expression of MTERFD1 in lung tumor tissues, 55 hepatocellular carcinomas, 30 breast cancers, and 30 pancreatic cancer tissues were compared with the matched normal adjacent tissue. The expression value in normal tissue was arbitrarily defined as 100% (**A**). Representative Immunohistochemistry analysis of MTERFD1 in breast cancer (**B**), hepatocellular carcinoma (**C**), lung cancer (**D**), pancreatic cancer (**E**). The arrow in the figures indicated that the cancer tissues have lower MTERFD1 expression, or the adjacent normal tissues have higher MTERFD1 expression.

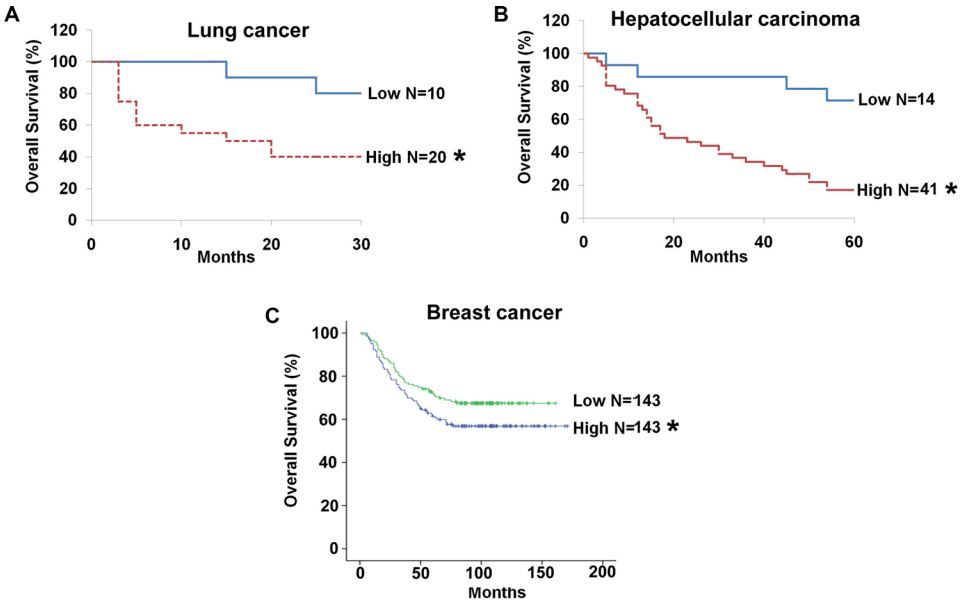

Figure 4: Correlation between *MTERFD1* and overall survival in three types of cancer. *MTERFD1* expression was divided into low and high expression groups by the corresponding median MTERFD1 level in lung cancers, hepatocellular carcinomas and breast cancers. Kaplan-Meier plots of overall survival in lung cancer patients (**A**), hepatocellular carcinoma patients (**B**) and breast cancer patients (**C**), post-operation according to the expression of *MTERFD1* (D). *$P < 0.05$.

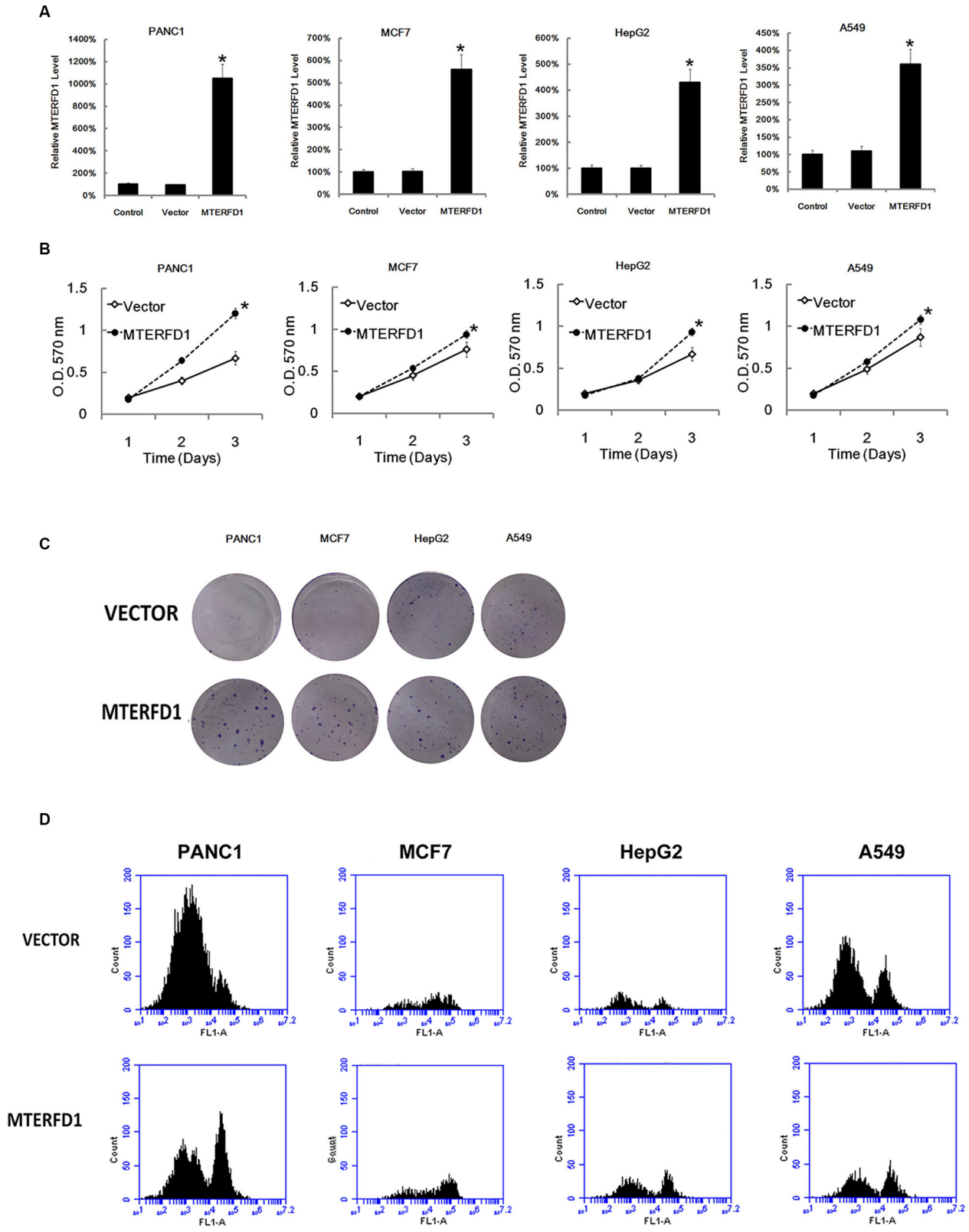

Figure 5: Transfection with an *MTERFD1* -overexpressing plasmid promoted tumor growth *in vitro*. Within 24 hours after transfection with the *MTERFD1*-overexpressing plasmid, the *MTERFD1* mRNA expression was assayed by qRT-PCR in PANC1, MCF7, HepG2 and A549 cell lines (**A**). Within 24 h after transfection with the *MTERFD1*-overexpressing plasmid, the cells growth was assayed by the MTT assay using transfection with an empty plasmid as a control. Data were presented as mean ± s.e.m. of three independent experiments (**B**). Within 24 h after transfection with the *MTERFD1*-overexpressing plasmid, cell clones in dishes are shown (**C**). After transfection, the cells were treated with 10 μM of BrdU for 1 hour, then were assayed by flow cytometry (**D**).

mitochondrial DNA gene expression is critical for altering oxidative phosphorylation to control the physiological capacity in response to physiological demands and disease processes. Accordingly, it seems that *MTERFD1* plays its oncogenic function via the regulation of mitochondrial DNA transcription. The precise mechanism however requires further investigation.

Survival analysis highlighted the importance of MTERFD1. Our study revealed a significant correlation between the expression of the MTERFD1 protein and overall survival in different types cancers. *MYC* is a classic oncogene. However, the *MYC* family members are not point-mutated, and recurrently amplified in cancers alongside MTERFD1. For example, in 760 cases of breast invasive carcinoma, there are 180 MYC amplification cases, and 130 MTERFD1 amplification cases.

In conclusion, our study demonstrated the oncogenic function of *MTERFD1*. Our data indicated that overexpression of *MTERFD1* decreased the survival by promoting tumor cells growth. Our study may provide a potential target for therapy.

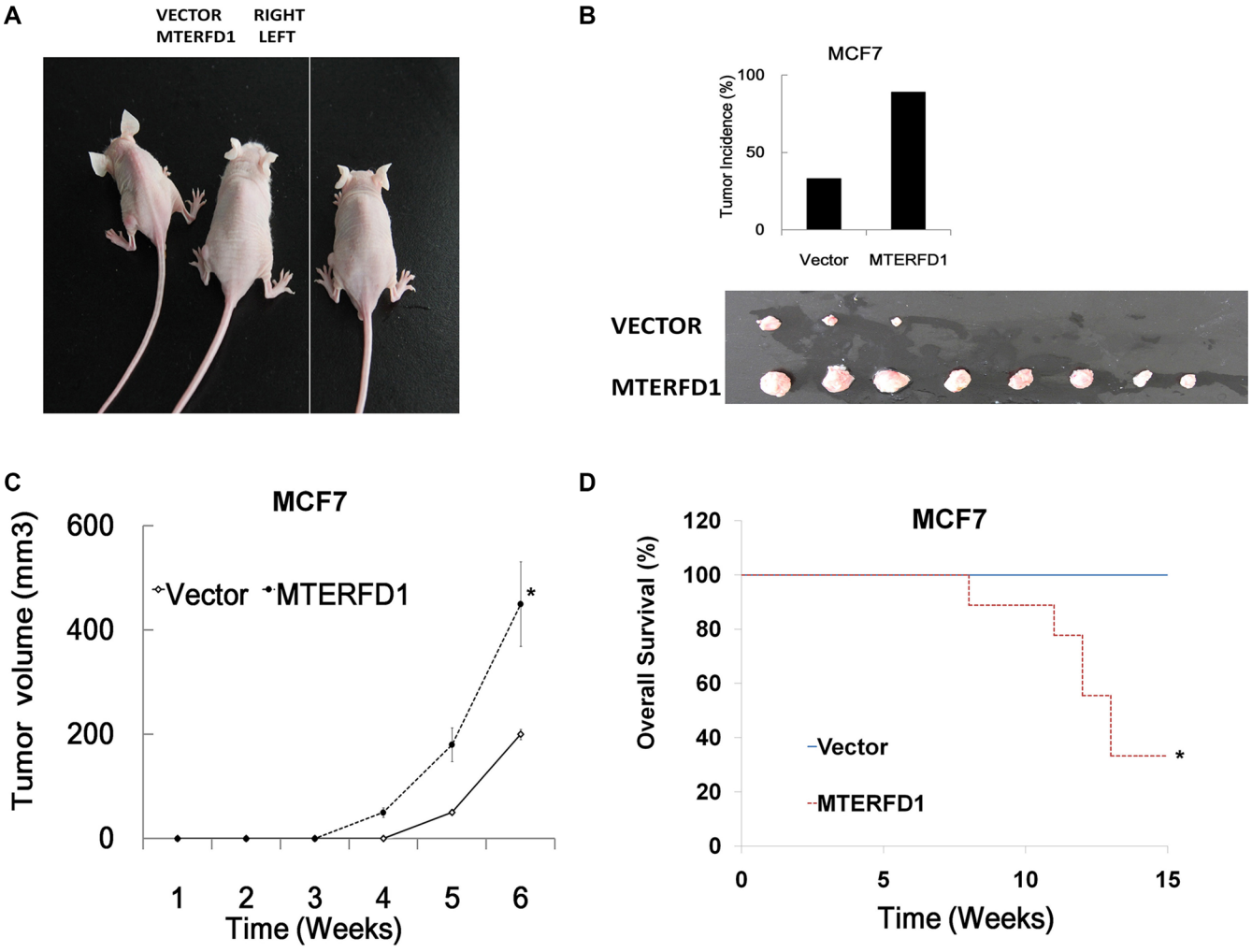

Figure 6: *MTERFD1* overexpression promoted tumor growth *in vivo* and reduced the survival rate. Nude mice were inoculated with cells overexpressing *MTERFD1* (MCF7 transfected with plasmid). The subcutaneous tumors formed after 6 weeks are shown (**A**). Ten nude mice were subcutaneously inoculated with MCF7 cells transfected with the *MTERFD1*-overexpressing plasmid. Ten nude mice, used as control, were subcutaneously inoculated with MCF7 cells transfected with an empty plasmid. The tumor incidence was calculated, and the subcutaneous tumors were isolated and measured (**B**). After the inoculation, the tumor volumes were measured every week. Data were presented as mean ± s.d. of the measurement of 10 mice (**C**). Kaplan-Meier plot of overall survival post-inoculation according to the expression of *MTERFD1* (**D**). **P* < 0.05.

MATERIALS AND METHODS

Gene and protein sequence alignment

MTERFD1 gene and protein sequences were aligned with those of the other three members of the MTERF family by using COBALT [28]. Additionally, *MTERFD1* of different species (Homo sapiens, Rattus norvegicus and Mus musculus) were also aligned using the same approach.

Gene alteration frequency analysis in cancer

The data of *MTERFD1* alteration frequency and mRNA expression level analyses were queried from TCGA via the cBioportal for Cancer Genomics (http://www.cbioportal.org/public-portal/index.do) [29, 30].

MTERFD1 mRNA expression level analysis

The data of *MTERFD1* mRNA expression in various types of cancer were queried from Gene Expression Omnibus.

Tissue microarray analysis and survival analysis

Lung cancer, hepatocellular carcinoma, breast cancer, and pancreatic cancer tissue microarrays were purchased from and analyzed by SHANGHAI OUTDO BIOTECH CO.,LTD (Shanghai, China). These tissues were obtained postoperatively from Changhai Hospital, Second Military Medical University (Shanghai, China). All patients gave signed, informed consent for their tissues to be used for scientific research. Ethical approval for the study was obtained from Changhai Hospital, Second Military Medical University. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. Tissues were obtained before chemotherapy and radiotherapy and were immediately frozen and stored, at the SHANGHAI OUTDO BIOTECH CO., LTD, at −80°C prior to qRT-PCR analysis. Corresponded patients were followed-up for the indicated number of years and all clinical data were electronically recorded.

Cell culture

Human pancreatic carcinoma cell line (PANC1), human breast cancer cell lines (MCF7), human hepatocarcinoma cell lines (HepG2) and human non-small cell lung cancer (A549) cell lines were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM or 1640 medium cultured in DMEM medium (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin (Bio Light, Shanghai, China) as described in our previous studies [31].

Plasmid transfection

MTERFD1 overexpression plasmid (pcDNA3.1-MTERFD1) was designed, constructed and confirmed by the SHANGHAI SHENGONG company (Shanghai, China). Plasmids were transfected into cells (6×10^4 cells per well) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were collected after 48 h for confirmation and further analysis.

Mice and treatment

Nude mice (6 weeks) were obtained from the Animal center of the Chinese Academy of Science (Shanghai, China), and maintained in the nude mice care center of the Second Military Medical University. MCF7 cells were subcutaneously inoculated into nude mice at the density of 1×10^7 cell/mL, in a 500 mL volume. After the MCF7 cells inoculation, mice were monitored and the tumor volumes were measured every week. During the 15-weeks follow-up period, the survival status of nude mice was recorded.

RNA extraction and real time q-PCR analysis

RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The cDNA synthesis and real-time qPCR were subsequently performed using the Qiagen system as described in detail in our previous studies [31]. Real-time quantitative PCR analysis was performed using standard protocols on an Applied Biosystem's 7500 Fast Real-Time PCR System (ABI, Foster City, CA, USA).

Cell growth assay

For cell growth assay, 500 cells per well were seeded in triplicate in a 96-well plate with complete growth medium. Cells were counted over 5 days using the MTT assay (Promega, Fitchburg, WI, USA) as previously described [31–34].

Edu cell proliferation assay

The cell proliferation were assayed by Edu (5-ethynyl-2'-dexoxyuridine) Flow Assay Kits (Promega, Fitchburg, WI, USA) Edu is a nucleoside analog to thymidine and is incorporated into DNA during DNA synthesis. Flow Cytometry assay was performed by using CellQuest Software (Becton Dickinson, Franklin Lakes, NJ, USA) as described previously [35].

Statistical analysis

Data, from at least three independent experiments, are presented as the mean ± s.e.m. The difference between groups was analyzed using a two-tailed Student′s *t* test when only two groups were compared. The difference between groups were analyzed using ANOVA when three or more groups were compared. Survival was evaluated by Kaplan-Meier analysis. Statistical analyses were performed using SPSS software version 17.0 (IBM, Armonk, NHY, USA). $P < 0.05$ was considered significantly different.

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CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

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