siRNA-mediated knockdown of hTDE2 retards cell cycle progression through transcriptional activation of p21

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Abstract. Carcinogenesis is a very complex process involving a series of changes of tumor-related genes. Therefore, genes differentially expressed in tumors have received significant attention. Among them is the tumor differentially expressed (TDE) protein family, which shows no homologue to any other protein families and is unique to eukaryotes. The members of the TDE (also known as Serinc) family are highly conserved, showing approximately 30-80% homologue of their amino acid sequences. Previous reports have shown that both human and mice TDE/Serinc proteins are always upregulated in carcinomatous tissues. However, their precise physiological roles remain unclear. The human TDE2/Serinc1 gene was cloned by our laboratory during the screening for differentially expressed genes in hepatocarcinoma. In the present study, we knocked down the expression of TDE2 with specific siRNA fragments in two human hepatocarcinoma cell lines, and this caused cell cycle arrest at G2. Cell cycle progression is monitored and regulated by several factors. p21, the cdk inhibitor, is a key player and could be transcriptionally activated by many factors including sterol regulatory element-binding proteins (SREBPs). Previous research demonstrated that rat TDE2 could facilitate the cellular sphingolipids biosynthesis in both yeast and mammalian cells. Therefore, we further analyzed the effect of TDE2 knockdown on p21 and SREBP, and found that endogenous p21 expression was upregulated, as was that of SREBPs (-1a and 2). In conclusion, our preliminary results indicated that TDE2 may have an effect on tumor cell growth by influencing the expression of SREBP and p21.

Introduction

The study of genes that are differentially expressed during tumor progression may identify key players in this complex and multistep biological process. Polyomavirus large-T antigen (PyLT-Ag) transgenic mice have been used as a model system for studying differentially expressed genes during tumorigenesis (1). With the pre- and post-adenomatous testis cell lines derived from the PyLT-Ag transgenic mice, as well as the differential display technique, a testicular tumor differentially expressed gene (TDE1) was identified to be upregulated during transformation (2-15 times compared to the control cell line), indicating a potential oncogenic property possessed by it (2-4). This gene was also independently cloned by other researchers, and was designated TMS-1, AIGP1 and/or Serinc3 (5-7). Subsequently, increasingly more homologous and orthologous genes of TDE1 were cloned and identified in species such as yeast, worm, fruit fly, zebrafish, mouse, rat and human, giving rise to a large TDE1/TMS1 protein family (also known as the Serinc family for serine incorporator) (5,7-9). This novel TDE1/TMS1/Serinc family appears in eukaryotic organisms and contains no amino acid sequence homology with other known protein families. At the same time, the TDE/Serinc proteins themselves are highly conservative, sharing between 30 and 80% homology (8). There is a total of five TDE/Serinc proteins (Serincl-5) identified in mammals while baking yeast harbors only one ortholog (TMS1). The TDE/Serinc proteins contain multiple transmembrane domains (up to 11) and often an N-terminal signal peptide.

The characteristic multiple transmembrane domains of TDE/Serinc proteins by peptide analysis suggested that they were most closely related to proteins involved in ion-channel formation or amino acid transportation. However, all attempts to detect amino acid transport with mouse Serinc1 and Serinc3 failed (5,7). In 2005, Inuzuka et al (7) studied the Serinc function by searching the yeast proteome database for its interacting partners. Two proteins involved in serine synthesis, SER3 and YGP1, were identified to interact with TMS1 (the only Serinc protein in yeast). Yeast dihybrid experiment further demonstrated that rat Serinc1 protein could also interact with...
SER3 and YPG1, indicating the possible conservation of these interactions. Moreover, it was also shown that Serinc proteins (Serinc1, 2 and 5) could additionally enhance incorporation of serine into the membrane lipid phosphatidylserine both in prokaryotes and eukaryotes. On the other hand, sphingolipids are another class of membrane lipids that could be synthesized by all eukaryotic cells from serine and palmitoyl-CoA. The condensation of these two molecules is catalyzed by serine palmitoyltransferase (SPT), the most key limiting factor in the sphingolipid de novo biosynthetic pathway. Inuzuka et al also showed that rat Serinc1 protein facilitated the cellular sphingo-
lipid biosynthesis in both yeast and mammalian cells, probably via interacting with and enhancing the activity of SPT. The same property was also demonstrated for Serinc2 and 5. Results of the biochemical assays performed by Inuzuka et al indicated that the Serinc family members might play pivotal roles in the biosynthesis of membrane lipids. Indeed, besides the upregulation of Serincs observed in carcinoma tissues, it was postulated that rat Serinc5/TPO1 might be involved in myelin biogenesis based on its expression pattern (9). Furthermore, in the hippocampus of rats with seizures induced by kainite, Serinc1, 2 and 5 were found to be differentially expressed compared to that of the control rats (7). These findings suggested that Serinc proteins might participate in the plasticity of the central nervous system, perhaps affecting membrane lipid biogenesis.

The conservative nature of the TDE/Serinc proteins suggests an important biological role for this family. Indeed, their overexpression was shown to correlate with carcinogenesis. In addition to the known overexpression of mouse TDE gene in testicular tumors from PyLT-Ag transgenic mice, human TDE1 (hTDE1) was also shown to be highly expressed in lung cancer tissues (10). Moreover, Player et al (11) cloned and localized human Serinc2 to chromosome 1 in 2003. Results of both in situ hybridization and real-time PCR showed that its mRNA was also upregulated in human non-small cell lung cancer tissue. However, besides the biochemical function of these proteins postulated by Inuzuka et al, the molecular mechanism underlying their oncogenic property remains to be elucidated.

Cell growth is regulated by several factors. In addition to duplication of DNA, protein, and other cellular components, recent studies have provided insight into the involvement of lipid metabolism, including membrane lipid biogenesis (therefore affecting plasma membrane, or, more importantly, secretory vesicles), in cell-cycle progression (12-16). On the one hand, Kurat et al (15) discovered that Tgl4, the yeast triacylglycerol lipase, was directly activated by Cdk1/Cdc28-dependent phosphorylation; on the other hand, sterol regulatory element-binding proteins (SREBPs), which are transcriptional factors involved in cholesterol and fatty acid synthesis, have also been shown to affect cell proliferation through accumulating cdk inhibitors such as p21 (17-19). hTDE2/Serinc1 located on chromosome 6, was first cloned by our group in 1998 during an investigation of genes differenti-
ally expressed in liver cancer tissues (GenBank access number AF087902; unpublished data). hTDE2/Serinc1 has a broad expression profile and is significantly upregulated in the hepatocarcinoma tissues examined, which is consistent with the oncogenic behaviors of other Serinc members such as Serinc2 and Serinc3. In the present study, we showed that knockdown of hTDE2/Serinc1 expression resulted in cell cycle arrest and cell growth inhibition. Moreover, downregulation of TDE2/Serinc1 upregulated SREBPs, and, eventually, expression of p21 was elevated causing cell cycle retardation.

Materials and methods

Cell lines, tissue samples and other materials. The human HCC cell lines L-02, YY-8103, SMMC-7721, QGY-7703 and Huh-7 were from Fudan University (Shanghai, China). FOCUS was from the Molecular Hepatology Laboratories, MGH Cancer Center. All other cell lines used in the present study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium or RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), accordingly. The cell cultures were incubated at 37˚C in a humidified incubator with 5% CO₂. The human multiple tissue cDNA panel was purchased from Clontech.

Paired hepatoma and non-hepatoma tissue samples were obtained from 32 hepatoma patients who had surgery at Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. The hospital's Ethics Committee had approved the specimen collection procedures, and written informed consent was obtained from each patient or their relatives.

To perform hTDE2 knockdown, three gene-specific siRNA fragments (460, 895 and 1394, targeting different regions of hTDE2) as well as one non-specific fragment (N.C.) were employed to transfec the HCC cell lines. The sequences were: 5’-CUGCAGCAUUUGCAAUUATT-3’ (sense) and 5’-AUAAUUGCAAUUGCGACATT-3’ (antisense) for fragment 460; 5’-GGUCAGCUAGCAUGATT-3’ (sense) and 5’-UCAUUGGCAUGCGACCTT-3’ (antisense) for fragment 895; 5’-GCCACACUUUGCUUCUACATT-3’ (sense) and 5’-UGUAGAGAAGUGGUGGCTT-3’ (antisense) for fragment 1394; and 5’-UUCUCGCAACCGUGUCACT-3’ (sense) and 5’-ACGAGCAGCUGUUGAGGATT-3’ (antisense) for fragment N.C. All fragments were synthesized at Shanghai GenePharma (Shanghai, China).

Real-time quantitative PCR and semi-quantitative PCR. First, total RNA was extracted from tissues or cells using TRIzol (Invitrogen) and was reverse transcribed into cDNA (Gibco-BRL) following the manufacturer's instructions. Then, real-time PCR was performed with an iCycler iQ (Bio-Rad Laboratories) to analyze the level of target mRNAs, using the dsDNA-specific binding dye SYBR-Green Premix (Toyobo). After normalizing to GAPDH, target mRNA levels were quantified with the ΔΔC τ method. Primer sets used were: 5’-AGA TAATGAAAGGGATGGTGTC-3’ (sense) and 5’-ACAGC ACGATGCCAATCCAAT-3’ (antisense) for hTDE2; 5’-CG GCCGGGGAACCACTT-3’ (sense) and 5’-CGCAGCGC CCTCC-3’ (antisense) for hSREBP1a; 5’-GAAGGCTGAG ACCAGAAGA-3’ (sense) and 5’-CGTCCACCCACGACA GATGA-3’ (antisense) for hSREBP2; 5’-TGGAGACTCTCAG GGTCCGAAA-3’ (sense) and 5’-AGAAGTCGCAGCATCA TTG-3’ (antisense) for p21; and 5’-AGGCTGCTTTTTA ACTCTGTG-3’ (sense) and 5’-CCCCACTGTATTGGGAG GGA-3’ (antisense) for GAPDH.
To compare the expression of hTDE2 in hepatocarcinoma and pericancerous tissue samples, semi-quantitative PCR was performed. Total RNA was extracted and reverse transcribed into cDNA as described above. According to the PCR signal generated from the internal standard β2-MG, the template amount of each cDNA pool was adjusted to give the same exponential phase signal strength after 24 cycles. PCR reactions were then performed with appropriate conditions. The final PCR samples were then subjected to electrophoresis on 1.5% agarose gels and ethidium bromide staining. After capturing digital images under UV, the densitometry value of each PCR-generated DNA fragment was measured with UVP Gelworks ID Advanced software (Version 2.51). DR (dosage ratio) was used to describe the relative expression difference between the tumor and its pericancerous tissues. DR is calculated with the following formula: 

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DR = \frac{\text{Density}_{\text{tumor-hTDE2}}}{\text{Density}_{\text{tumor-β2-MG}}} \times \frac{\text{Density}_{\text{pericancerous-hTDE2}}}{\text{Density}_{\text{pericancerous-β2-MG}}}
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For semi-quantitative PCR, primer set 5'-TCTCTTCCAGTTGGATTTGCACT-3' (sense) and 5'-CCATAACCTACTACTTGTCCAC-3' (antisense) was used for hTDE2, while primer set 5'-ATGAGTATGCTTGCCGTGTAGAC-3' (sense) and 5'-TGTTGACCACTCTGTCAGATA-3' (antisense) was used for β2-MG.

Cell cycle, growth curve and colony formation assay. Cells were transfected with appropriate siRNA fragments and were collected 48 h after transfection. Prior to flow cytometric analysis, cells were fixed with pre-chilled (at -20°C) 75% ethanol for 2 h at 4°C, followed by washing with PBS. PI (final concentration, 50 µg/ml) and RNase (final concentration, 100 µg/ml) were then added to the cell suspension and the mixture was kept for 20 min in the dark. Analysis was completed within 3 h after PI staining.

MTS assay was performed according to the manufacturer's instructions. The experiments were carried out in triplicate and the average absorbance was calculated to generate the growth curve.

For the colony formation assay, cells were seeded in 6-well plates as 400 cells/well, followed by culturing at 37°C with proper medium for 6-10 days. Every other day, old medium was replaced with fresh one. At the end of the incubation, cells were washed and fixed with 4% paraformaldehyde for 15 min. The fixed cells were washed again and subjected to Giemsa (Sigma) staining at room temperature for 2 h. Images of the colonies were captured after the plates were washed and dried.

Western blot analysis. Cells seeded in 6-well plates were transfected with appropriate siRNA fragments (50 pmole/well) using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed and processed for SDS-PAGE electrophoresis. Proteins were then transferred to nitrocellular membranes (Bio-Rad Laboratories). After blocking with 5% milk in TBST buffer, the membranes were subjected to primary and secondary antibody incubation with washing in between and after. Membranes were then incubated in enhanced chemiluminescence (ECL) solution (Amersham) for 1 min followed by exposure to Hyperfilm. The primary antibodies used were: anti-human SREBP-1 polyclonal antibody (C-20, sc-366, 1:200; Santa Cruz Biotechnology), anti-human SREBP-2 polyclonal antibody (H-164, sc-5603, 1:200; Santa Cruz Biotechnology), anti human p21 monoclonal antibody (1:1,000; Abcam), anti-human p53 monoclonal antibody (1:200; Sigma) and anti-human β-actin monoclonal antibody (1:5,000; Sigma). The corresponding secondary antibodies used were all diluted as 1:3,000.

Luciferase constructs and luciferase assay. p21-2400-Luc and p21-221-Luc reporter plasmids were kind gifts from Dr Huang Haojie from Mayo Clinics (Rochester, MN, USA) with a pGL3-Basic backbone (Promega). In the reporter plasmids, nucleotide sequences from +73 to -221 and/or -2400 of the p21 gene were cloned in front of the luciferase reporter gene. p21-2400-Luc, but not p21-221-Luc, contains two p53-binding sites. Both plasmids harbor the SRE element which is located between positions -90 and -98 of the p21 promoter. The other two luciferase constructs, p21-2400-Luc-Mut and p21-221-Luc-Mut, were generated by PCR with a site-directed mutagenesis kit (Toryo). As a result, the wild-type SRE sequence, TGGGCCGAG, was replaced by TACAAATGTG (20).

To perform the luciferase assay with the dual-luciferase reporter system (Promega), cells were seeded on 24-well plates one day before transfection. Luciferase reporter plasmid (0.1 µg) and pRL-SV40, the Renilla luciferase internal control plasmid (0.01 µg; Promega), were co-transfected together with appropriate siRNA fragments into the cells using Lipofectamine 2000 kit (Invitrogen). Luciferase activity in each transfected sample was examined and normalized to that of the Renilla luciferase activity.

Statistical analysis. The values are expressed as means ± SD. The Student's t-test was used to assess the differences. P<0.05 was considered to indicate a statistically significant result.

Results

hTDE2 gene is upregulated in hepatocarcinoma tissues. hTDE2 (hSerinc1) belongs to the Serinc protein family which was highly conserved from fungi to vertebrates. Previous research indicated that high expression of these family members was correlated with carcinogenesis (4,10,11). However, the physiological function of this family remains unclear. In the present study, we first assessed the expression profile of hTDE2 in different human tissues with a human multiple tissue cDNA panel. The quantitative PCR results indicated that the tissue expression profile of hTDE2 is broad, with the highest expression in the brain and the lowest in the thymus (Fig. 1A).

To further analyze whether hTDE2 was also correlated with hepatocarcinogenesis, we measured hTDE2 expression in 32 paired HCC and corresponding non-HCC neighboring tissue samples with semi-quantitative PCR. In 6/32 paired samples, hTDE2 was downregulated in the HCC tumor tissues; in 23 paired samples (23/32 = 71.9%), clear upregulation of hTDE2 was observed in the tumor tissues. The remaining 3 paired samples showed a similar expression of hTDE2 in the tumor and normal tissues (Fig. 1B). This result is consistent with the previous findings that a correlation exists between upregulation of TDE proteins and carcinogenesis.
We also measured the hTDE2 mRNA level in different liver cell lines commonly used in our laboratory, and found that all the cell lines expressed this gene and its expression was lower in L-02 compared to other cell lines (hepatocarcinoma lines) examined here (Fig. 1C). Since L-02 was generally considered to be a normal liver cell line, this result was consistent with previous ones with human tissue cDNA panel and HCC samples. We selected two of the hepatocarcinoma cell lines, QGY-7703 and SMMC-7721, to carry out the following study.

**Knockdown of hTDE2 hinders the growth of hepatocarcinoma cells and arrests the cell cycle at G2.** Since hTDE2 was significantly upregulated in the HCC specimens, we investigated whether knocking down its expression could have any effect on tumor cell growth. Therefore, we designed three siRNA fragments specifically targeting hTDE2 expression (siRNA 460, 895 and 1394) and analyzed their efficacy with quantitative PCR (Fig. 2A). All three siRNA fragments reduced hTDE2 mRNA levels in both QGY and SMMC cells with siRNA 895...
being relatively less efficient. Then, we utilized FACS assay to detect the cell cycle progression of both HCC cell lines. We found that, in both QGY and SMMC, the number of cells in G1 phase decreased and that of G2 cells increased upon hTDE2 knockdown (Fig. 2B). This verified our postulation that reduction of hTDE2 expression may affect HCC tumor cell proliferation. Furthermore, our cell growth curve by MTT assay also confirmed that downregulation of hTDE2 indeed hindered the growth of tumor cells (Fig. 3A). Three days after siRNA fragment transfection, growth curve of the testing cells started to deviate from that of the controls.

We also performed a colony formation test to further determine the effect of hTDE2 knockdown. With QGY, we found that the colony numbers decreased significantly when the expression of hTDE2 was reduced. This effect was not statistically significant in SMMC cells (Fig. 3B). Our unpublished data further demonstrated that knockdown of hTDE2...
increased apoptosis in QGY, but not in SMMC, which may explain the above inconsistency within the two lines with the colony formation test.

In conclusion, we showed that downregulation of hTDE2 hampered the growth of HCC cells QGY and SMMC, at least partly, via cell cycle retardation.

Knockdown of hTDE2 upregulates p21 and SREBP. We then investigated through which signaling pathway knockdown of TDE2 caused cell cycle arrest. Studies by Inuzuka et al indicated that the transmembrane Serinc/TDE proteins might be involved in the biosynthesis of membrane lipids by facilitating SPT, a rate-limiting enzyme involved in the very early step of multiple membrane lipid biogenesis (7). Therefore, it is reasonable to postulate that reduction of TDE2 expression may affect membrane lipid biogenesis, and this would probably hinder cell cycle progression. p21 functions as a regulator of cell cycle progression. p21 was indeed upregulated upon TDE2 knockdown. p21 was upregulated upon TDE2 knockdown. We also performed western blot analysis to check p21 expression at the protein level, and similar results were reached (Fig. 4B). As mentioned above, we hypothesized the transactivation of p21 here may be through the SRE element. Therefore, expression of the SRE binding factors, SREBP1 and SREBP2, was also detected with real-time PCR and western blot analysis. In humans, there are total three SREBP proteins encoded by two genes, SREBP1 for SREBP-1a and -1c (resulting from alternative splicing) and SREBP2 for SREBP2 protein. In our experiments, the expression of SREBP-1c (mRNA and protein) in both QGY and SMMC was very low and undetectable. Therefore, only results for SREBP-1a and SREBP2 are shown. We found that SREBPs (-1a and 2) were upregulated at both the mRNA and the protein level upon TDE2 knockdown, which is consistent with our speculation.

Moreover, the activation of p21 transcription here was p53-independent, with p53 protein level in both QGY and SMMC being unaffected (data not shown). Consistent with this, we also knocked down TDE2 in H2199, a lung cancer cell line with p53 deficiency, and upregulation of p21 was detected as expected (data not shown).

Knockdown of TDE2 transcriptionally activates p21 promoter via SRE element. To further test whether the transcriptional activation of p21 is mediated via the SRE element located in its promoter, we constructed several luciferase reporter plasmids with p21 promoter sequences (full or partial) inserted in front of the luciferase gene (as described in Materials and methods). As shown in Fig. 5A, the promoter sequence of luciferase
gene in p21-2400-Luc harbors both p53 binding site and SRE element, while that in p21-221-Luc contains only SRE but not the p53-binding site. For both reporter plasmids, we also further mutated its SRE element specifically.

With the dual luciferase report assay, we found that specific knockdown of TDE2 indeed activated p21 promoter. Consistent with previous results, this activation was independent of the p53-binding site. Mutation of SRE sequence completely abolished this transcriptional activation (Fig. 5B and C). We performed the experiments in both QGY and SMMC cells, and obtained similar results.

Discussion

Serinc/TDE proteins belong to a new transmembrane protein family that is generally tumor differentially expressed. We found that Serinc1/TDE2 was significantly upregulated in hepatocarcinoma tissues and cell lines. However, its precise physiological function remains to be elucidated.

In the present study, we showed that p21 was upregulated upon knockdown of Serinc1/TDE2 expression, and this was likely due to SREBP, but not p53. We knocked down Serinc1/TDE2 expression in H1299 cells (p53-deficient), and upregulation of p21 was still detected at both the mRNA and protein levels (unpublished data). Our conclusion was further supported by results of the dual luciferase report assay. Moreover, the independence of p53 and SREBP on p21 activation was previously also shown in the p53-deficient Saos-2 cells (20).

In humans, three isoforms of SREBP (-1a, -1c and 2) exist. SREBP2 plays a vital role in the regulation of cholesterol synthesis. While SREBP-1a is involved in the transcription of
a wide scope of genes involved in cholesterol, fatty acids, and phospholipid synthesis, SREBP-1c has a strong transcriptional activity for enzyme genes involved in fatty acids and triglycerides in lipogenic organs (17, 18). Other studies showed that both SREBP-1a and 2 could activate p21 transcription and cause cell growth inhibition. In addition, SREBP-1a could regulate p21 transcription by directly binding to SRE identified in its promoter (20). Although the physiological significance of SREBPs to p21 activation still needs to be clarified, it is postulated that fast growing cells, which require active (membrane) lipid synthesis, may briefly hold cell growth via activating p21 by SREBPs in case of lipid deficiency. Results from the biochemical study of Serinc1/TDE2 (7) and from the present study support the above postulations.

Furthermore, we exogenously expressed hTDE2 in both QGY and SMMC and examined its effect on cell cycle progression. However, compared to the control groups, the experimental groups did not show significant differences in the cell cycle analysis. This may be due to the fact that, under normal conditions, cell cycle progression is limited by factors other than lipid synthesis. Therefore, the overexpression of Serinc1/TDE2 protein will not show considerable effects on cell cycle progression. If we culture the cells in lipid-deficient medium or add drugs to the medium (give cells pressure), the cells may benefit from over-supply of TDE2. Indeed, Boscolasco et al (8) showed that cell apoptosis induced by starvation or drug treatment could be partially rescued by TDE1 overexpression.

In conclusion, the present study showed that in both hepatocarcinoma cell lines, downregulation of hSerinc1/hTDE2 clearly arrested cell cycle progression. We also observed an increase of both SREBPs and p21 expression upon Serinc1/TDE2 knockdown. However, the molecular mechanism underlying this observation still requires detailed investigation. Moreover, although we speculated that the cell cycle arrest observed in the present study might be caused by p21 upregulation via SREBP, we could not exclude other signaling pathways.

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References


