RESEARCH ARTICLE

Ectopic Overexpression of COTE1 Promotes Cellular Invasion of Hepatocellular Carcinoma

Hai Zhang[1&], Chang-Jun Huang[1&], Yuan Tian[1&], Yu-Ping Wang[2], Ze-Guang Han[2], Xiang-Cheng Li[1*]

Abstract

Family with sequence similarity 189, member B (FAM189B), alias COTE1, a putative oncogene selected by microarray, for the first time was here found to be significantly up-regulated in hepatocellular carcinoma (HCC) specimens and HCC cell lines. mRNA expression of COTE1 in HCC samples and cell lines was detected by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR, while protein expression of COTE1 in HCC tissues was assessed by immunohistochemistry. In addition, invasion of HCC cells was observed after overexpressing or silencing COTE1. In the total of 48 paired HCC specimens, compared with the adjacent non-cancer tissues, the expression of COTE1 was up-regulated in 31 ($p<0.01$). In HCC cell lines, COTE1 expression was significantly higher than in normal human adult liver ($p<0.01$). Overexpression of COTE1 enhanced HCC-derived LM6 and MHCC-L cellular invasion in vitro. In contrast, COTE1 knockdown via RNAi markedly suppressed these phenotypes, as documented in LM3 and MHCC-H HCC cells. Mechanistic analyses indicated that COTE1 could physically associate with WW domain oxidoreductase (WWOX), a tumor suppressor. COTE1 may be closely correlated with invasion of hepatocellular carcinoma (HCC) cells and thus may serve as an effective target for gene therapy.

Keywords: COTE1 - cellular Invasion - hepatocellular carcinoma - WWOX

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Introduction

Hepatocellular carcinoma (HCC) is one of the most fatal tumor worldwide, particularly in Sub-Sahara Africa and South-eastern Asia (Parkin et al., 2005). In recent years, the incidence rate of HCC has increased in China (Wang et al., 2001). The major risk factors for the development of HCC commonly include infection by hepatitis B and C viruses, exposure to aflatoxin B1, and cirrhosis of any etiology (Coleman, 2003). Up to date, liver resection and transplantation are regarded as effective treatment. However, the postoperative survival rate is only 30–40% at 5 years (Aravalli et al., 2008), because of high rate of recurrence and metastasis. Therefore, it is urgently to find new clues to understand HCC metastasis and to explore effective therapeutic strategies.

Family with sequence similarity 189, member B (FAM189B), alias as COTE1, mapped onto chromosome 1q 21, is widely expressed in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Yu et al., 1997). The COTE1 gene was originally identified by Winfield, it is found that COTE1 located near the gene for the lysosomal enzyme glucosylceramidase: a deficiency in this enzyme is associated with Gaucher disease (Winfield et al., 1997). Alternative splicing of COTE1 results in multiple transcript variants: transcript variant 1, 2, and 3. The variant 1 represents the longest transcript and encodes the longest protein (isoform a). The variant 2 and 3 lack an in-frame portion of the 5' coding region compared to variant 1, the resulting proteins (isoform b and c) are shorter than isoform a (NCBI Reference Sequences). The protein COTE1 includes 669 amino acids with two potential N-glycosylationsites, a leucine zipper, and multiple potential phosphorylation sites and N-myristoylationsites (Winfield et al., 1997). The recent data showed COTE1 contains a predicted four-transmembrane domains, making it possible that it would reside in a membrane-bound subcellular organelle such as the Golgi (Ludes-Meyers et al., 2004). In addition, Anders Kallin found that the expression of COTE1 was correlated with endogenous SREBP-1 (sterol-regulatory element binding proteins) activation in vitro, it was speculated to play a role in lipid metabolism (Kallin et al., 2007). Moreover, the protein has been identified as a potential binding partner of a WW domain-containing protein which is involved in tumor suppression (Ludes-Meyers et al., 2004; Abdeen et al., 2011).

In the present study, based on a previous genome-

*[1]Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Key Laboratory of Living Donor Liver Transplantation, Ministry of Public Health, [2]Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, China [&]Equal contributors *For correspondence: drxcli@njmu.edu.cn*

wide approach, for the first time, we found COTE1 was markblely regulated in HCC clinical specimens, as compared to adjacent non-cancerous livers (data not shown). To confirm that, we verified upregulation of COTE1 in 31 of 48 paired HCC specimens and 4 invasive HCC cell lines. These indicated it may contribute to cellular invasion of HCC as a new potential oncogene. Followed experiments showed that overexpression of COTE1 enhaced HCC-derived LM6 and MHCC-L cellular invasion in vitro. By contrast, COTE1 knockdown via RNAi markedly suppressed these phenotypes, as documented in LM3 and MHCC-H HCC cells. Furthermore, the mechanistic analyses indicated that COTE1 could physically interact with WW domain oxidoreductase (WWOX), a tumor suppressor.

Materials and Methods

Tissue specimens

Total of 48 pairs of clinical specimens were obtained from patients suffered from HCC hospitalized in the First Affiliated Hospital of Nanjing Medical University with informed consent. Adjacent non-tumor tissues were cut off 2 cm away from the edge of primary focus. Both HCC specimens and adjacent non-tumor tissues, immediately stored in liquid nitrogen after excision, were proved by pathological examination. The project and protocol for the investigation involving human and animals were approved by the Institutional Animal Care and Use Committee at Nanjing Medical University.

Liver cancer cell lines

Human hepatocellular carcinoma cell lines (SK-hep-s, LM6, MHCC-L, LM3 and MHCC-H) and normal human adualt liver tissue used in this study were obtained from Chinese National Human Genome Center at Shanghai. All of these cell lines propagated in a 5% CO_2, 37°C-humidified incubator in following media: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50U/ml), and streptomycin (50 μg/ml) .

Semiquantitative RT–PCR and quantitative real-time PCR

Total RNA was extracted from clinical samples or cell lines using TRIZOL solution (Invitrogen) according to the manufacturer's protocol and then reverse transcribed into cDNA using M-MLV reverse transcriptase kit (Promega). Primers used in Semiquantitative RT–PCR and quantitative real-time PCR follows as: COTE1-203bp: 5'-GGGCTCTGACCTAGGCTTCT-3' (forward); 5'-ACAGAAGCTCTCCCAGTCCA-3' (reverse); COTE1-534bp: 5'-CACCACCGAGAGCATCAAGAG-3'(forward); 5'-TCCAGCATGGTGTGTAGGAG-3'(reverse); beta-actin-230bp (loading control): 5'-AGAGCCTCGCCTTTGCCGATCC-3' (forward); 5'-CTGGGCCTCGTCGCCCACATA-3' (reverse). All primers were synthetized by (Shanghai Biosune Co. Ltd.).

Immunohistochemical staining

Tissues of HCC specimens were used to detect COTE1 expression by immunohistochemistry. Briefly, formalin-fixed samples were paraffin embedded and cut into 4-μm sections. The slides were incubated with goat anti-COTE1 polyclonal antibody (1:50; Santa) at 4°C overnight, where normal goat IgG was used as negative control. MaxVision TM HRP-Polymer anti-Goat IHC Kit (Maixin.Bio. Ltd. China) were used in the following procedures according to the manufacture's protocol. Stained slides were observed under light microscopy.

siRNA preparation

Two siRNAs against COTE1 were designed according to the web server of Invitrogen Co. and chemically synthesized by (Shanghai GenePharma Co.). Commonly utilized Negative Control (NC) siRNA, supplyed by Qiagen Co., was used as control. The sense and antisense strands of human COTE1 as follows: siRNA-2852 sequence: 5'-GUAUGUAAGCCUUCAAU AAdTdT-3' (sense) and 5'-UUAUUGAAGGCUUACAUACdTdT -3' (antisense); siRNA-3129 sequence: 5'-AGCUCUUAACAGUAUGUAAdTdT-3' (sense) and 5'-UUACAUACUGUUAAGAGCUdTdT-3' (antisense).

Construction of COTE1 expression vector

For the construction of COTE1 recombinant plasmid, pcDNA3.1B-FLAG-GFP, obtained from Chinese National Human Genome Center at Shanghai, was used in this expriment. COTE1 open reading frame (ORF) was amplified from human liver cDNA library (Genbank: NM_006589.2) using nest PCR, and then inserted it into pcDNA3.1B-FLAG-GFP.

Cell transfection

Both RNAi and plasmid transfection were performed by Lipofectamine2000 (Invitrogen) according to the manufacturee's instructions. Cell density were 30%-50% and 80%-90%, respectively.

Cell invasion assay

The 24-well transwells (8-μ m pore size; BD Biosciences), coated with Matrigel (Falcon 354480; BD Biosciences) were used for cell invasion assay. After starving overnight in DMEM free medium, total of 1×10^5 cells suspended in 500μl DMEM containing 10% FBS was added to the upper chamber, while 750μl DMEM containing 10% FBS and 10 μg/ml fibronectin (catalog no. 356008, BD Biosciences) was placed in the lower chamber. For the control, medium containing 10% FBS was added to the lower chamber. After 48 hours' incubation, Matrigel and cells remaining in the upper chamber were removed by cotton swabs. Cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in at least 6 random microscopic fields (magnification, ×200) were counted and photographed. All experiments were performed in duplicate and repeated 3 times (Huang et al., 2010).

Cell viability measurement

For the cell proliferation assay, transfected cells were plated into 96-well to make sure cell density at 30%-50%.

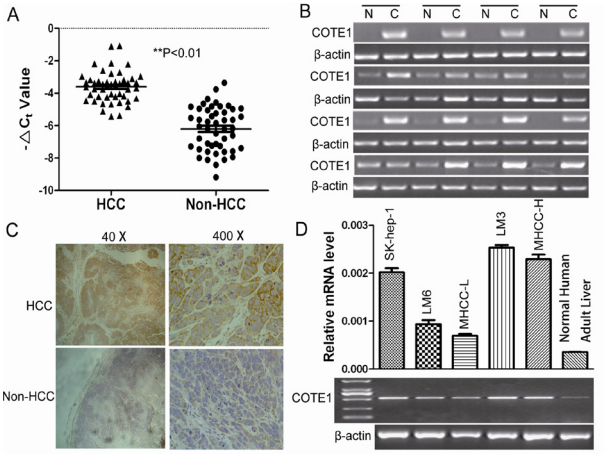

Figure 1. The Upregulation of COTE1 in Clinical Specimens and Cell Lines of HCC. N, non-HCC; C, HCC; A: The expression of COTE1 in 48 paired HCC specimens by real-time PCR; B: Representative results of upregulation of COTE1 in HCC specimens by semi-quantitative RT-PCR; C: Representative results of upregulation of COTE1 in HCC specimens by immunohistochemistry; and D: COTE1 was up-regulated in 5 HCC cells

The Cell Counting Kit-8 (CCK-8; Dojindo Labs) was used to measure cell viability according to the instructions of the manufacturer: 90μl of DMEM free medium plus 10μl of CCK-8 solution added to 96-well plate. After incubated at 37°C for 1 hour, the plate was read at 450 nm. Three replicate wells were tested per assay condition, and each experiment was repeated at least 3 times.

Western blot analysis

Cell lysates were prepared by cold lysis buffer containing 25 mmol/L Tris-Cl (pH 7.5), 5 mmol/L EDTA 1% SDS, and protease inhibitor cocktail (Sigma). After 5 minutes' boiling, samples were subjected to electrophoresis in SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane, which was blocked in 5% blocking buffer for 2 hours at room temperature. Further more, the membrane was incubated with the primary antibody in PBST (0.1% Tween-20 in PBS) at 4°C overnight and then incubated with the secondary antibody at room temperature for 1 hour. Antibodies used in this study include: goat anti-COTE1(Santa Cruz Biotechnology), mouse anti-WWOX (Santa Cruz Biotechnology), mouse anti-flag (Santa Cruz Biotechnology), and anti-β-actin (Santa Cruz Biotechnology). Proteins were detectd by Odyssey Infared Imaging System (Li-COR).

Co-Immunoprecipitation (Co-IP)

Cells transfected with pcDNA3.1-COTE1-Flag were resuspended in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 1 mM EDTA and protease inhibitor cocktail). Immunoprecipitation of lysates was conducted using the anti-Flag antibody (1:100; Santa Cruz Biotechnology Inc.), followed by immunoblotting with antibodies against WWOX (1:1,000; Santa Cruz Biotech-nology Inc.) or COTE1 (1:100; Santa Cruz Biotechnology Inc). The cell lysates transfected with empty vector (pcDNA3.1) served as a control (Huang et al., 2010).

Table 1. The expression of COTE1 versus clinical features

HCC parameters	Numble of patients	COTE1(+)	COTE1(-)	χ^2	p
gender					
male	38	23	15		
female	10	6	4	0.001	0.976
subtotal	48	29	19		
age					
≥45 y.o.	33	17	16		
<45 y.o.	15	9	6	0.299	0.584
subtotal	48	26	22		
etiology					
HBV +	35	20	15		
HBV -	13	6	7	0.461	0.497
subtotal	48	26	22		
tumor size (cm)					
≥3cm	27	15	12		
<3cm	21	13	8	0.426	0.514
subtotal	48	28	20		
Invasion					
YES	29	18	11		
NO	19	12	7	0.021	0.884
subtotal	48	30	18		
Child-Pugh stage					
A	31	14	17		
B	17	8	9	0.016	0.9
subtotal	48	22	26		
Edmondson grade					
I-II	39	28	11		
III-IV	9	4	5	2.339	0.126
subtotal	48	32	16		
AFP level (ng/ml)					
≥100	36	21	15		
<100	12	2	10	6.743	0.009
subtotal	48	23	25		

COTE(+) indicated upregulation of COTE1 gene (fold more than 0.5); – indicated no upregulation of COTE1

Statistical analysis

All quantitative data were recorded as mean ± S.D. Differences between two groups were assessed by Student's t-test (two-tailed) using GraphPad Prism 5 software. Comparisons among multiple groups were performed by one-way ANOVA analysis, LSD-t test. Categorical data was evaluated by cross χ^2 test. In all tests, $P<0.05$ were considered to be statistically significant.

Results

Overexpression of COTE1 in HCC

RT-PCR and quantitative PCR was firstly performed to measure mRNA expression level of COTE1 in 48 paired HCC clinical specimens relative to the levels in corresponding adjacent non-cancer livers. The results indicate that mRNA of COTE1 was obviously upregulated in HCC specimens (Figure 1A and B). To evaluate the protein level of COTE1 in HCC, immunochemical staining with a specific antibody against COTE1 was performed in the same 48 matched samples. Of the specimens, 17/48 (35.4%) showed no or weak (+/-) possitive staining in non-HCC tissues, whereas, 31/48 (64.6%) was possitive in cancerous specimens, including 5 mild possitive, 14

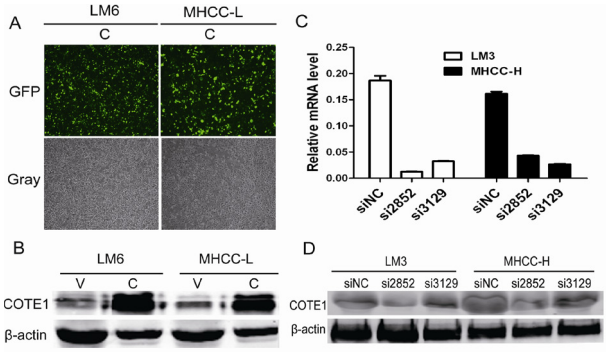

Figure 2. The Transfection Efficacy and the Expression of COTE1 Post-transfection. V, vector; C, vector-COTE1-GFP. A: The transfection efficacy of pcDNA3.1B-COTE1-GFP in LM6 and MHCC-L. Observation under a fluorescence microscope and a regular microscope (× 200); B: The expression of COTE1 in LM6 and MHCC-L post-transfection was detected by western blot; C: Relative mRNA level of COTE1 in LM3 and MHCC-H with siRNA transfection; and D: Western blot analysis of COTE1 expression in transfected LM3 and MHCC-H cell

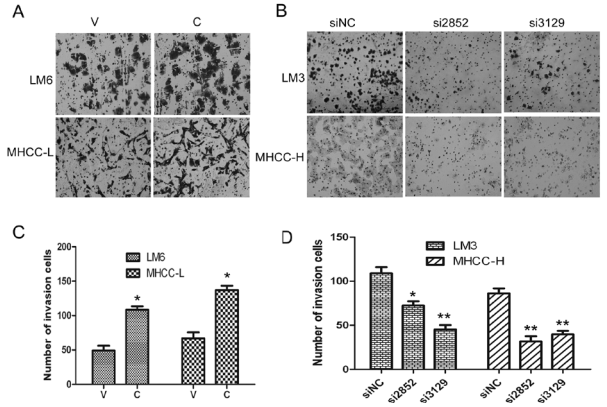

Figure 3. COTE1 Contributes to HCC Cellular Invasion. V, vector; C, vector-COTE1; A and B: Cell invasion of LM6, MHCC-L, LM3, and MHCC-H cells was evaluated with Matrigel assay. Observation under a fluorescence microscope and a inverted microscope (× 200); C and D: The number of invasive cells were counted in 5 random microscopic fields (× 200)

moderate possitive and 12 strong possitive (Figure 1C). Furthermore, we evaluated the expression of COTE1 in 5 HCC cell lines by RT-PCR. The result showed that COTE1 was highly expressed in the 5 HCC cells compared to normal human adult liver tissue (Figure 1D). To sum up, the collective data showed that COTE1 is upregulated in HCC, which is consistent with the observation in the gene microarray analysis.

COTE1 and AFP (Alpha Fetal Protein) were complementary

To further evaluate the significance of COTE1 in clinical HCC samples, the results of real time RT-PCR in the 48 HCC specimens was analysed. The clinical characteristics of the patients and tumor are summarized in Supplementary Table 1. The prepared specimens were grouped by gender (male or female), age (≥45 y.o. or <45 y.o.), etiology (HBV+ or HBV-), pathology size (≥ or <3cm), metastasis (YES or NO), Child-Pugh stage (A or B), Edmmdson grade (I-II or III-IV) and AFP level (≥100ng/ml or <100ng/ml). The resulting data showed

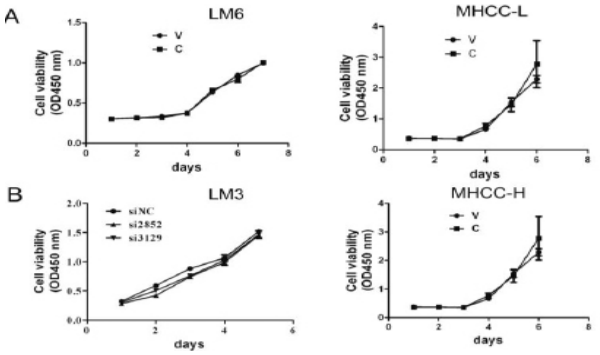

Figure 4. The Effect of COTE1 on Proliferation of LM6, MHCC-L, LM3 and MHCC-H. V, vector; C, vector-COTE1. A: Enforced overexpression of COTE1 showed no effect on proliferation of LM6 and MHCC-L; B: Gene silencing of COTE1 via siRNA interference in LM3 and MHCC-H showed no cell viability inhibition

that in the total of 36 AFP (+) specimens, 58.3% (21/36) was COTE1 (+), while 83.3% (10/12) samples showed COTE1 (-) in AFP (-) ones. The overexpression of COTE1 was significantly correlated with the expression of AFP (Table 1, $p<0.05$). However, upregulation of COTE1 did not appear to be correlated with the gender, age, hepatitis B virus infection, tumor size, metastasis, Child-Pugh stage or Edmmdson grade ($p>0.05$).

The transfection efficacy and the expression of COTE1 post-transfection

To overexpress COTE1, the recombinant pcDNA3.1-COTE1-GFP was firstly transfected into LM6 and MHCC-L cells. It was shown that nearly 100% transfection was obtained 48 hrs post-transfection (Figure 2A). The protein level of COTE1 was markedly enhanced in LM6 and MHCC-L cells (Figure 2C). In order to knock down endogenous COTE1 in LM3 and MHCC-H, two chemically synthesized siRNAs and shRNA derived from recombinant pSUPER were used. After evaluating the efficiency of RNAi, both of them were considered to be appropriate for COTE1 knockdown (Figure 2B and D).

COTE1 contributes to HCC cellular invasion

We performed Matrigel experiment to evaluate the effect of COTE1 on cell invasion. Based on the expression pattern of COTE1 on HCC cell lines, recombinant pcDNA3.1-COTE1-Flag was transiently transfected into LM6 and MHCC-L, both of which express relative low level of COTE1 (Figure 1D), where the empty vector pcDNA3.1-Flag was used as control. When fibronectin used as an attractant, large density of invasion cells could be seen in LM6 and MHCC-L cells (Figure 3A and C), while the opposite phenomenon appeared in LM3 and MHCC-H when transfected with siRNAs (Figure 3B and D). Importantly, as no influence of COTE1 on proliferation could be seen in LM6, MHCC-L, LM3 and MHCC-H (Figure 4A and B), we consider the results described above about metastasis were independent. These collective data suggested that COTE1 take part in cellular invasion.

Potential COTE1-WWOX mediated cellular invasion regulation of HCC

The data above suggested that COTE1 present as an

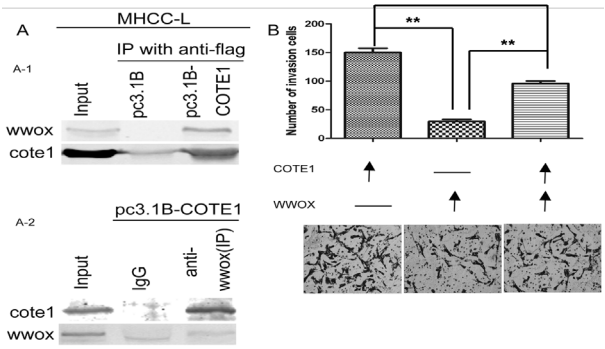

Figure 5. Potential COTE1-WWOX Mediated Cellular Invasion Regulation of HCC. A: Co-immunoprecipitation of wwox and exogenous cote1 in MHCC-L cells; B: The invasion efficiency of MHCC-L was measured with COTE1, WWOX, or COTE1/WWOX transfection. Black arrow, overexpression; Leading dash, no overexpression

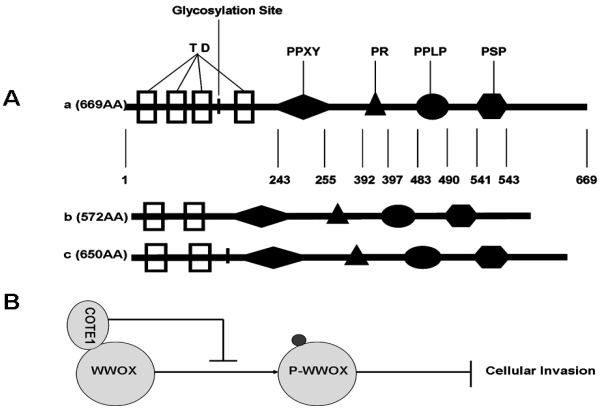

Figure 6. Schematic of the COTE1 Proteins and the Possible Involvement of COTE1 and WWOX in Cellular Invasion Signaling Pathways. A: Model of protein COTE1 (a, b and c) with its putative functional sites and domains. AA, Amino Acid; TD, Transmembrane Domain; PPXY, Pro-Pro-X-Tyr motif; PR, Pro-Arg motif; PPLP, Pro-Pro-Leu-Pro motif; PSP, Pro-Ser-Pro motif. B: A hypothetical schematic of the contribution of COTE1 to cell invasion via inactivation of the WWOX signaling pathway. The COTE1 protein bind to WW domain of WWOX via its PPXY, PR, PPLP or PSP motif, inhibit phosphorylation of WWOX and which in turn inactivate the cellular invasion signaling pathway of WWOX

novel oncogene in HCC, Consider previous reports about COTE1 binding with the WW domain of WWOX in vitro, we attempted to investigate whether the effect of COTE1 on invasion was mediated by WWOX. We performed co-immunoprecipitation (Co-IP) assays to determine wether COTE1 and WWOX physically interact in MHCC-L transfected with pcDNA3.1-COTE1-Flag. The mutal Co-IP data indicated that COTE1 physically associates with WWOX (Figure 5A). In addition, recombinant plasmids of pcDNA3.1B-COTE1-GFP and pcDNA3.1B-WWOX were cotransfected into MHCC-L. The number of invasive cells with COTE1/WWOX cotransfection was statistically less than that with COTE1 overexpression, however, significantly more than that with WWOX overexpression (Figure 5B). The data above suggest that the inhibition of cellular invasion by WWOX may be blocked via COTE1 up-regulation. To explore the internal effect of COTE1 on WWOX, we measured the expression of WWOX in transfected cells, including LM6, MHCC-L, LM3 and MHCC-H. However, no change appears in the protein level of WWOX.

Discussion

In most cancer cells, chromosomes are broken, truncated, deleted, amplified or translocated to other chromosomes. Chromosomal abnormalities may lead to the inactivation of tumor suppressor genes (TSGs) or activation of oncogenes via amplification (Kim et al., 2008). According to previous study, high incidence of C1q copy number gain was found in HCC (60 to 80%) (Nathalie et al., 2001). Many up-regulated cancer-related genes, such as JTB, SHC1, CCT3, and COPA, were observed in HCC located at 1q12-q22 (Wong et al., 2003). COTE1, a novel potential oncogene, identified by our lab, located at chromosome 1q 21. Thus, we hypothesized that the COTE1 gene could be a candidate HCC-specific molecular marker.

The biological functions of COTE1, especially in cancers, remain unclear. In the present study, as compared with adjacent liver tissue, COTE1 was up-regulated. Besides, high expression of COTE1 was found in HCC cell lines. These implied that COTE1 could function as an oncogene on HCC. Herein, we showed that overexpression of COTE1 promoted cellular invasion of LM6 and MHCC-L in vitro. By the contrast, gene silencing of COTE1 inhibited cellular invasion potential of LM3 and MHCC-H in vitro. Together, COTE1 indeed played important role in HCC neoplasia. The results of our study show that COTE1 appears to physically associate directly with WWOX, a tumor suppressor. The WWOX protein induces two WW domains and a short-chain dehydrogenase/reductase 3, which may be involved in sexsteroid metabolism (Salah et al., 2010). With WW domains that interact with a growing list of interesting proteins containing PPXY, PR, PPLP and PSP motifs (Aqeilan et al., 2007; Del et al., 2009; Kurek et al., 2010), WWOX participated in TRADD (TNF receptor-associated death domain protein)-mediated cell death and mitochondrial apoptosis (Aqeilan et al., 2005; Aderca et al., 2008; Hong et al., 2009; Kurek et al.,2010). It is inactivation in a range of tumor cells, and its decreased activity correlates with the invasiveness of human tumors (Gulnur et al., 2003; Teng et al., 2012). WWOX overexpression in SKOV3 ovarian cancer cells, result in reduced attachment and migration on fibronectin, an extracellular matrix component linked to peritoneal metastasis (Charlie et al., 2009).

Suprisingly, our work indicates that co-Immunoprecipitation of COTE1 and WWOX exist in HCC cell, and the inhibition of cellular invasion by WWOX may be blocked via COTE1 up-regulation. However, the expression level of WWOX showed no siginificant difference in transfected HCC cells. Take the WWOX could be activated via phosphorylation, including Tyr-33, Tyr-287, Tyr-61, Tyr-293 and Ser-14 residues (Chang, et al., 2003; Chen et al., 2005; Charlie, 2009; Chang et al., 2010), into consideration, we speculated that phosphorylation of WWOX may contribute to COTE1 cell invasion progression: the binding of COTE1 and WWOX

may inhibit phosphrylation of WWOX, which result in reduced p-WWOX, and the suppression of p-WWOX on cellular invasion was in turn weakened.

In conclusion, COTE1 contributes to cellular invasion of HCC and may present a new target for HCC gene therapy.

Acknowledgements

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