

The role of the TOB1 gene in growth suppression of hepatocellular carcinoma

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Abstract. The TOB1 gene, mapped on 17q21, is a member of the BTG/Tob family. In breast cancer it has been identified as a candidate tumor suppressor gene. However, whether TOB1 is a bona fide tumor suppressor and downregulated in hepatocellular carcinoma (HCC) remains unclear. In addition, whether its expression is regulated through methylation requires investigation. In the present study, we therefore analyzed the expression of TOB1 in HCC and its methylation levels in human HCC and breast cancer. No significant difference in the expression levels of TOB1 was observed between tumor tissues and adjacent normal tissues in HCC. Quantitative methylation analysis by MassArray revealed no significant differences at single CpG sites or in the global promoter region, and all these CpG sites shared a similar methylation pattern in HCC and breast cancer. Moreover, 5-aza-2'-deoxycytidine treatment of three tumor cell lines did not cause elevation of TOB1 mRNA in HepG2 cell lines. Based on these data, we speculate that TOB1 may be a candidate non-tumor suppressor gene in HCC. Furthermore, the clinical outcome was not correlated with TOB1 expression or expression rate. In addition, TOB1 expression or expression rate was not correlated with the overall survival (OS) rates or cumulative recurrence rates. Taken together, we suggest that TOB1 does not act as a tumor suppressor in HCC.

Introduction

The BTG/Tob family comprises at least six distinct members in vertebrates, namely BTG1, BTG2/TIS21/PC3, BTG3/ANA, PC3B, TOB2 and TOB. The family may be divided into two subgroups, the BTG family and the TOB family (1-3). Both families have been reported to suppress cell proliferation when expressed exogenously in cultured cells (4-7). They commonly share a conserved amino-terminal region known as the BTG homology domain which is responsible for their antiproliferative function (8-10).

TOB, a transducer of ErbB-2, also known as TOB1, is ubiquitously expressed in human adult tissues and was first identified by screening an expression library that detected protein-protein interactions with an ErbB2 probe (11). It is located on chromosome 17q21 and codes for a 45-kDa protein (12). Its function involves many aspects of the biological process. First, cell growth suppression is related to gene TOB1 and it is hampered by the presence of kinase-active p185erbB2. Co-immunoprecipitate assay showed that p185erbB2 could directly interact with the carboxyl-terminal half of TOB and negatively regulated TOB-mediated cell growth suppression (11). Unphosphorylated TOB, the active form of TOB, is necessary in exerting its antiproliferative effect and elevated TOB1 phosphorylation abrogates the antiproliferative effect in lung cancer (13). Second, previous studies have shown that TOB binds Smad1, Smad5 and Smad8, and negatively regulates BMP2-dependent bone formation by inhibiting the transcriptional activity of Smad (14,15). Third, TOB1 is required for correct dorsoventral patterning through inhibiting β -catenin transcriptional activity and preventing the formation of β -catenin/LEF1 complexes (16). Finally, TOB also enhances mRNA deadenylation in the mRNA decay process by simultaneous interaction with the poly(A) nuclease complex CCR4-CAF1 and the cytoplasmic poly(A)-binding protein, PABPC1 (17). In addition, Yoshida *et al* reported that mice lacking TOB were prone to spontaneous formation of tumors and the mRNA was decreased to 4.7-87.3% of the normal level in 13 of 18 human lung cancers. The mutation analysis revealed no point mutations or gross aberrations in the TOB gene (18). TOB also functions as a tumor suppressor in breast cancer

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Table I. TOB1 primers used for MassArray quantitative methylation analysis.

Primer	Sequences (5'-3')	Length (bp)
meth2s	aggaagagagAAGTTAAAAGTTTTAGGTTTGGTATG	429
meth2a	cagtaatacactcactatagggagaaggctTCAACTAAAAATATTACTCACAAATAACA	
meth4s	aggaagagagGGGTAGGTTGTGAAAAAGGTATTTAT	451
meth4a	agtaatacactcactatagggagaaggctTATTAATCACCCAAAACCTAAACC	
meth11s	aggaagagagTTTAGGTTTTTGATTTGGAAAGTGT	459
meth11a	cagtaatacactcactatagggagaaggctCCAACCTTCTCTAAACCTTTTTATTTC	
meth14s	aggaagagagGGAATAAGATTATTTAAGGTGAAGGA	474
meth14a	cagtaatacactcactatagggagaaggctCACTAATCCCTTTTCACCAATTTAATA	
meth17s	aggaagagagTTAAATTGGTGAAAAGGGATTAGTG	409
meth17a	cagtaatacactcactatagggagaaggctCTAACTACCCAAAACCAACCCATAC	

through the modulation and regulation of multiple signaling pathways and its expression is inversely correlated with breast cancer progression (12). However, whether TOB is a tumor suppressor in hepatocellular carcinoma (HCC) has yet to be elucidated. If it were a tumor suppressor, it is unclear what else would account for its downregulation, since no mutation was found in lung cancer.

In order to evaluate whether TOB is a tumor suppressor, the methylation profiles of 47 CpG sites were examined in the TOB promoter and nearby coding region and we performed MassArray methylation analysis in HCC and breast cancer tissues. No common CpG sites of significant difference in methylation level between tumor tissues and adjacent normal tissues were found. In addition, the mRNA expression of TOB was determined by real-time PCR and restoration experiments were performed with 5-aza-2'-deoxycytidine (5-aza-dC). The real-time PCR assay documented that the expression of TOB displayed no significant difference between tumor tissues and adjacent normal tissues and the restoration experiments demonstrated that methylation of TOB inhibited its expression in the HepG2 cell line. Generally speaking, these data illustrated that TOB may be a candidate non-tumor suppressor in HCC. In addition, survival analysis and analysis of clinical characteristics using the Chi-square test also suggested that the TOB gene was not a tumor suppressor in HCC.

Materials and methods

Materials. Informed, written consent regarding the use of the tissue samples was obtained from each patient prior to the study. Seventeen breast cancer samples [named as breast (tumor)] and adjacent matched normal tissues [named as breast (non-tumor)] from breast cancer patients were collected at Henan Province People's Hospital, China. Ethical approval was obtained from the research ethics committee of the hospital. The cell lines used in this study were obtained from the Shanghai cell bank of the Chinese Academy of Sciences (Shanghai, China) (A-375) or were kindly provided by Qing Sang (Fudan University, Shanghai, China) (MDA-MB-231 and HepG2).

Patients and follow-up. HCC samples [named as liver (tumor)] and their adjacent non-tumorous samples [named as liver (non-tumor)] were obtained from 43 consecutive patients who underwent curative liver resection for primary tumors between February 2004 and October 2005 at the Liver Cancer Institute (Zhongshan Hospital, Fudan University, Shanghai, China). HCC diagnosis was based on the criteria of the World Health Organization. Liver function was assessed using the Child-Pugh scoring system. Of the 43 patients, 32 had hepatitis B history. Tumor stage was determined according to the 2010 International Union Against Cancer tumor-node-metastasis (TNM) classification and the Barcelona Clinic Liver Cancer (BCLC) staging classification. Tumor differentiation was graded by the Edmondson grading system. Following surgery, the patients were monitored until April 2010, with a median follow-up of 53 months (range, 0.8-71.2 months). The detailed clinicopathological characteristics are displayed in Table I (19). Ethical approval was obtained from the research ethics committee of Zhongshan Hospital, and written informed consent was obtained from each patient. The follow-up procedures were carried out as described in the previous study (20).

DNA/RNA extraction. Genomic DNA was isolated using AxyPrep gDNA Isolation Mini kit (HD Biosciences Co., Ltd., Shanghai, China). RNA was extracted using Aqua-SPIN RNA Isolation Mini kit (Watson Biotechnologies, Inc., Shanghai, China). The concentration and quality of the isolated DNA and RNA were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Bisulfite conversion and MassArray quantitative methylation analysis. In total, 33 of the 43 HCC samples and 17 breast cancer samples were utilized for MassArray quantitative methylation analysis. Bisulfite treatment of genomic DNA was performed using the Ez DNA Bisulfite Treatment kit (Zymo Research, Irvine, CA, USA) as recommended by the manufacturer. Quantitative methylation was measured using the MassArray compact system, following the MassCLEAVE training protocol (Sequenom, San Diego, CA, USA) at CapitalBio Corporation (Beijing, China). The target CpG island in the promoter region and nearby coding region are

Table II. Primers used for real-time PCR.

Gene/ primer	Sequence (5'-3')	Length (bp)
TOB1		
tob1s	GAAAATGGATGTGAGTTGGATAAGG	198
tob1a	GGCAGCAAAAGTGGCAGTG	
GAPDH		
gapdhs	CAAGAAGGTGGTGAAGCAGG	116
gapdha	CGTCAAAGGTGGAGGAGTGG	

shown in Fig. 1 and the primer pairs in Table I. On the basis of bisulfite-converted genomic DNA, this system worked by combining MassCLEAVE base-specific cleavage with MALDI-TOF mass spectrometry, and the resultant methylation calls were analyzed with EpiTyper software (Sequenom) to generate quantitative CpG methylation results.

Real-time PCR. The 43 HCC samples were used for real-time PCR. The cDNA was synthesized by PrimerScriptRT Reagent kit (Takara Bio Inc., Otsu, Japan). The TOB1 gene was co-amplified with a fragment of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, which served as an internal standard. Specification of each pair of primers (Table II) was confirmed by agarose gel electrophoresis and melting curve analysis. Q-PCR was conducted by amplifying 1.0 μ l of diluted cDNA with the SYBR Premix Ex Taq kit (Takara Bio Inc.) on the ABI 7900HT Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). The cycling conditions of forty cycles of PCR were 95°C/5 sec, 55°C/30 sec and 72°C/30 sec. The amount of specific mRNA was quantified by determining the point at which the fluorescence accumulation entered the exponential phase (Ct), and the Ct ratio of the target gene to GAPDH was calculated for each sample. Each sample was run in four repeats and all the PCR data were analyzed with the ABI 7900HT system software version 2.3 (21).

5-aza-dC treatment. Human cell lines (MDA-MB-231, HepG2 and A-375) were incubated for 72 h with 50 μ M/1 5-aza-dC (Sigma-Aldrich, Steinheim, Germany) with a medium change every 24 h. RNA was isolated from treated cells as described above.

Statistical analysis. The methylation rates and real-time PCR results in two independent sample groups were compared using an independent samples t-test. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The cases with hierarchical cluster analysis clustered the 32 CpG sites in the TOB1 promoter based on Euclidean distances and the average linkage clustering algorithm. This clustering was implemented using Cluster 3.0 and viewed on Java Treeview. The correlation between the TOB1 expression ratio (tumor/non-tumor) and other clinicopathological characteristics was evaluated using Pearson's Chi-square test. Overall survival (OS) was defined as the interval between

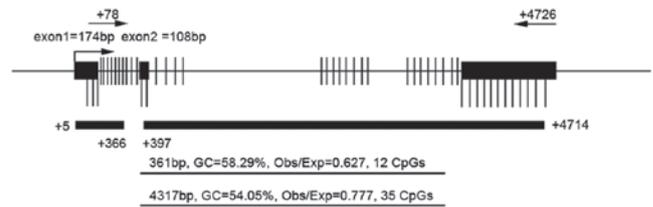


Figure 1. 5' end of the TOB1 gene, indicating the position of CpG islands and CpG sites used for DNA methylation analyses. The methylation analysis region is shown by inward facing arrows. The predicted transcriptional start site from the UC Santa Cruz Genome Browser is indicated by bent right arrows, and exon is the black filled bar. Vertical stripes indicate CpG sites. The bottom black filled bar shows the 5' CpG island; CpG island characteristics as determined using online EpiDesigner BETA software (<http://www.epidesigner.com>) are shown beneath the black bar.

HCC resection and mortality; patients alive at the end of follow-up were censored. The time to recurrence was calculated from the HCC resection to the first radiological evidence of recurrence. Patients who succumbed but did not experience recurrence were censored in determining recurrence (22). The cumulative recurrence and survival rates were carried out by the Kaplan-Meier method and analyzed by the log-rank test. All P-values were two-sided, and P<0.05 was considered to represent a statistically significant result.

Results

DNA methylation status of TOB gene promoter and nearby coding region in HCC and breast cancer. According to MassArray quantitative methylation analysis in 33 HCC samples and 17 breast cancer samples, the mean methylation level of each CpG site was used to compare between non-tumor and tumor tissues of the liver and breast (Fig. 2). In HCC tissue, the significant differences (P<0.05) were revealed at the following CpG sites: CpG_1.2.3, CpG_36, CpG_37, CpG_38, CpG_41, CpG_42, CpG_43, CpG_44, CpG_45, CpG_46 and CpG_47. In breast tissue, they were identified at the following CpG sites: CpG_31, CpG_34, CpG_35, CpG_41, CpG_42, CpG_43, CpG_44, CpG_45, CpG_46 and CpG_47. The common CpG sites of significant difference were CpG_41, CpG_42, CpG_43, CpG_44, CpG_45, CpG_46 and CpG_47. However, the common CpG sites shared the characteristic that the mean methylation degree in tumor tissues was lower than that of adjacent normal tissues and these common CpG sites were located in exon 3. These characteristics of CpG sites suggested that TOB may not be a tumor suppressor gene.

We then analyzed the general methylation feature profile of all examined CpG sites. The mean methylation range of different CpG sites was from 2.19% (at CpG_7) to 46.39% (at CpG_40) in liver tumor tissues and from 1.72% (at CpG_28.29) to 51.94% (at CpG_41) in liver non-tumor tissues. The mean methylation range of different CpG sites was from 2.94% (at CpG_1.2.3 and CpG_7) to 56.12% (at CpG_41) in breast tumor tissues and from 2.38% (at CpG_28.29) to 70.81% (at CpG_41) in breast non-tumor tissues. After unsupervised clustering, we observed that different CpG sites in the TOB promoter and nearby coding region shared a similar methylation pattern, namely, different CpG sites simultaneously had high or low

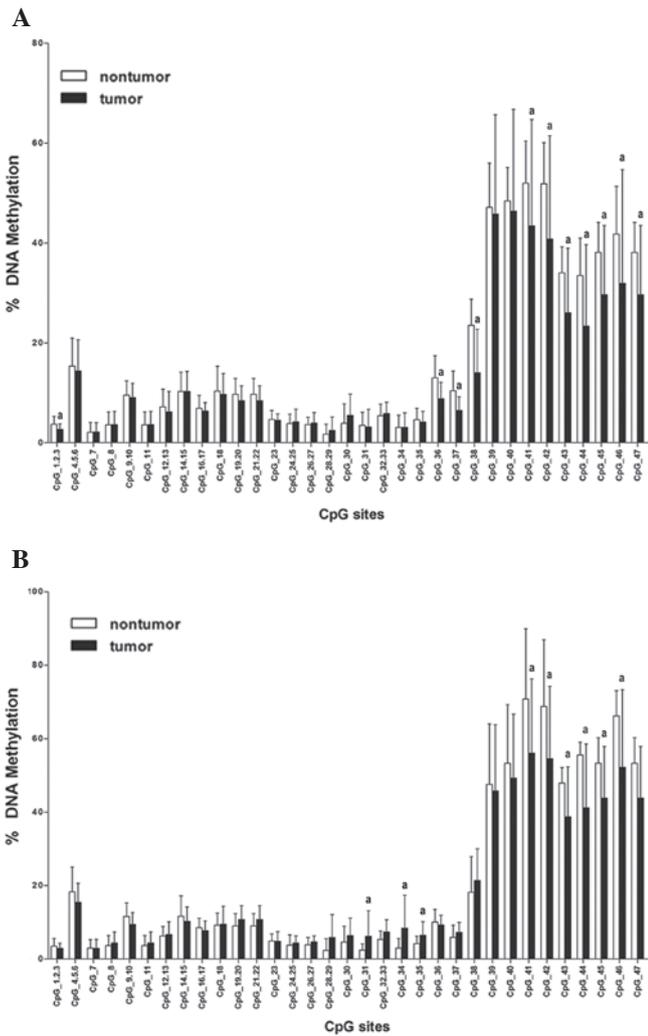


Figure 2. Comparison of mean methylation for each CpG site between non-tumor and tumor tissue in (A) liver cancer and (B) breast cancer. The X-axis represents 47 informative CpG sites within 5 MassArray amplicons for the TOB promoter and nearby region. The Y-axis shows the average methylation value of each CpG site (or clusters of CpG sites). Error bars, SD. *Indicates a significant difference ($P < 0.05$).

methylation levels in liver or breast tissues (Fig. 3A). However, with clustering ratios of methylation level (tumor: non-tumor) in both liver and breast tissues, we observed that many CpG sites possessed different methylation change patterns in liver and breast tissues (Fig. 3B; red represents upregulation and green represents downregulation). The mean methylation level of liver tumors was 14.18% and that of liver non-tumors was 16.90%. The mean methylation level of breast tumors was 18.58% and that of breast non-tumors was 20.70%. The mean methylation level in tumor tissues was compared to that of adjacent normal tissues and no significant difference was observed between them. Together with the above analysis of individual CpG sites, we observed that methylation variation may not be a major factor in the regulation of TOB expression.

Expression change of TOBI in tumor tissues versus non-tumor tissues and in cell lines following restoration experiments with 5-aza-dC. With real-time RT-PCR, TOB mRNA expression was quantified in 43 HCC samples. As shown in Fig. 4A, no

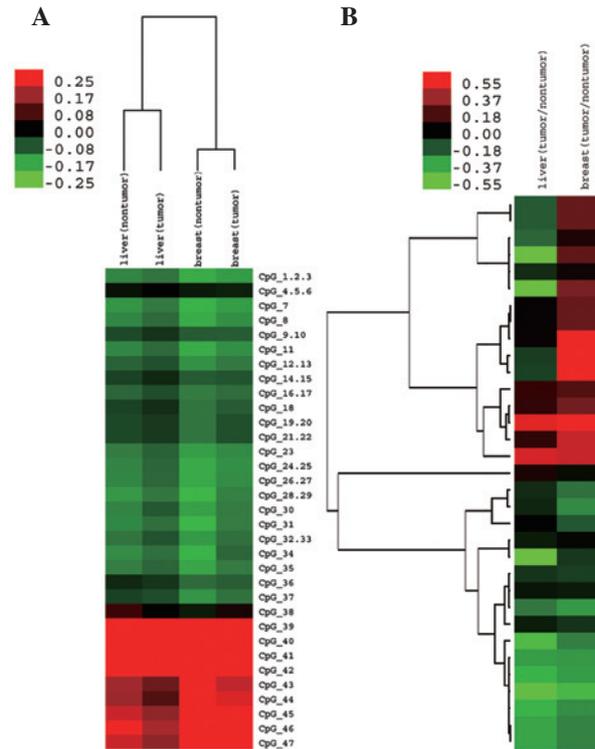


Figure 3. MassArray quantitative methylation of the TOBI promoter. (A) The hierarchical cluster analysis of methylation patterns of 47 CpG sites measured on 50 samples. The samples on the vertical axis form two distinct clusters separating the 33 liver samples (non-tumor and tumor) from the 17 breast samples (non-tumor and tumor). The methylation level (subtracting the general mean value) of each CpG site within each sample is shown in the plot with color ranging from green (indicating low methylation) to red (indicating high methylation). (B) The hierarchical cluster analysis of methylation patterns of 47 CpG sites measured in samples as above. The methylation level (\log_2 ratio tumor/non-tumor) of each CpG site within each sample is shown in the plot with color ranging from green (indicating methylation level of tumor lower than that of non-tumor) to red (indicating methylation level of tumor higher than that of non-tumor).

significance difference in mRNA levels was found in the tumor group compared to the non-tumor group (mean ratio of tumor group, 0.502; mean ratio of non-tumor group, 0.6680; $P > 0.05$).

To verify the functional association between the promoter and nearby coding region methylation increase and loss of TOB gene expression, mRNA expression levels were compared before and after treatment with 5-aza-dC in cell lines A-375, HepG2 and MDA-MB-231. In A-375 and MDA-MB-231 cells, the expression increased approximately ten and five-fold, respectively, following treatment. However, the expression in HepG2 decreased approximately 20% following treatment (mean ratio before treatment, 0.062; mean ratio after treatment, 0.052; $P < 0.01$; Fig. 4B). The expression variation in HepG2 was not consistent with that in A-375 and MDA-MB-231 cell lines. Many tumor suppressor genes were inhibited in expression by the hypermethylation in their promoter or nearby coding region. Therefore, our data suggested that TOB may be a candidate non-tumor suppressor gene in HCC.

Relative expression or expression ratio (tumor/non-tumor) of TOBI are not correlated with poorer prognosis in HCC patients. To explore whether TOB was a significant factor in

Table III. Correlation between TOB expression and clinicopathological characteristics in 42 HCC patients.

Variables	TOB expression (%)		P-value
	Low (n=21)	High (n=21)	
Age (years)			0.538
≤50	14	9	
>50	7	12	
Gender			0.107
Male	0	4	
Female	21	17	
HBsAg			1.000
Negative	1	1	
Positive	20	20	
Liver cirrhosis			1.000
No	16	16	
Yes	5	5	
Serum AFP (ng/ml)			0.758
≤20	10	11	
>20	11	10	
ALT (U/l)			1.000
≤75	19	19	
>75	2	2	
GGT (U/l)			0.116
≤54	6	2	
>54	15	19	
Tumor diameter (cm)			0.352
≤5	10	13	
>5	11	8	
Tumor number			0.634
Single	18	19	
Multiple	3	2	
Tumor encapsulation			0.204
Complete	11	15	
Incomplete	10	6	
Tumor differentiation			0.477
I/II	16	17 ^a	
III/IV	5	3	
Microvascular invasion			0.317
No	13	16	
Yes	8	5	
TNM stage			0.334
I	12	15	
II/III	9	6	
BCLC stage			0.495
0/A	5	7	
B/C	16	14	

^aDifferentiation status of one sample is missing.

Table IV. Correlation between TOB expression ratio and clinicopathological characteristics in 42 HCC patients.

Variables	TOB expression ratio (%)		P-value
	Low (n=21)	High (n=21)	
Age (years)			0.217
≤50	13	8	
>50	8	13	
Gender			0.107
Male	0	4	
Female	21	17	
HBsAg			1.000
Negative	1	1	
Positive	20	20	
Liver cirrhosis			1.000
No	16	16	
Yes	5	5	
Serum AFP (ng/ml)			0.355
≤20	9	12	
>20	12	9	
ALT (U/l)			1.000
≤75	19	19	
>75	2	2	
GGT (U/l)			0.045
≤54	7	1	
>54	14	20	
Tumor diameter (cm)			0.121
≤5	9	14	
>5	12	7	
Tumor number			0.634
Single	18	19	
Multiple	3	2	
Tumor encapsulation			0.525
Complete	12	14	
Incomplete	9	7	
Tumor differentiation			0.477
I/II	16	17 ^a	
III/IV	5	3	
Microvascular invasion			0.317
No	13	16	
Yes	8	5	
TNM stage			0.334
I	12	15	
II/III	9	6	
BCLC stage			0.172
0/A	4	8	
B/C	17	13	

^aDifferentiation status of one sample is missing.

determining clinical outcomes of HCC patients, we assessed its expression in 43 HCC patients. The expression of TOB was classified as either high or low group [median value of

relative expression (mvalue) was used as the cut-off value: TOB_{high}, value > mvalue; TOB_{low}, value < mvalue] or [median value of expression ratio (tumor/non-tumor) (mRatio)

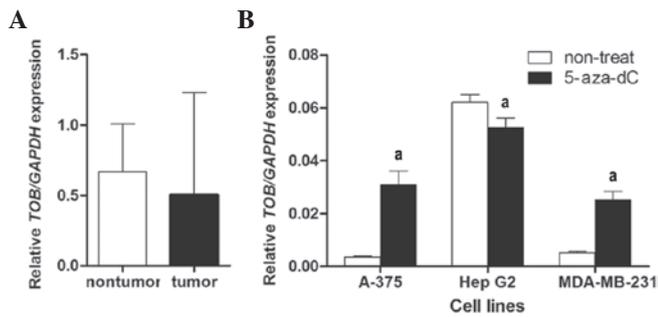


Figure 4. TOB1 expression analysis in tissues or cells before and after treatment with epigenetic-modifying agents. (A) Real-time PCR analysis of TOB1 expression in HCC tissues. The bar graphs show gene expression levels by the ratio of TOB1/GAPDH. Error bars, SD. (B) Quantitative analysis of TOB1 mRNA in cells before and after treatment with 5-aza-dC. The results are expressed as the ratio of copies of target gene relative to GAPDH. Error bars, SD. ^aIndicates a significant difference ($P < 0.01$).

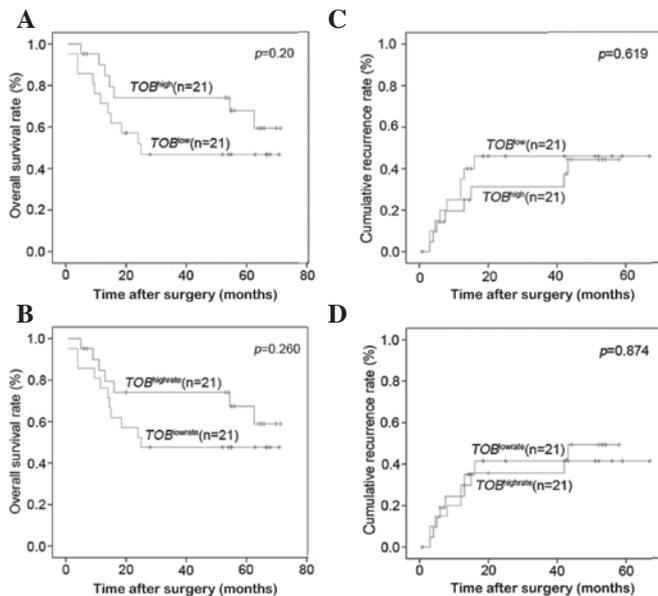


Figure 5. Prognostic significance assessed by Kaplan-Meier analysis and log-rank tests. (A and C) Overall survival curves and (B and D) cumulative recurrence curves in patients with TOB1 expression stratified by median TOB1 expression or TOB1 expression ratio (TOB1^{high} >0.278, TOB1^{low} ≤0.278; TOB1^{highrate} >0.464, TOB1^{lowrate} ≤0.464).

was used as the cut-off value: TOB1^{highrate}, ratio > mRatio; TOB1^{lowrate}, ratio < mRatio]. TOB1^{high} or TOB1^{highrate} accounted for 50% (21 of 42, expression data was not obtained in one sample) of all the patients. The Pearson's Chi-square test indicated that TOB1 was not associated with clinicopathological characteristics (Table III) and the expression ratio was not associated with clinicopathological characteristics (Table IV) with the exception of GGT. As of the last follow-up in April 2010, the OS and cumulative recurrence rates in the whole cohort were 60.4 and 28.6%, respectively. Furthermore, the OS rate showed no significant difference between the TOB1^{high} group and the TOB1^{low} group, or between the TOB1^{highrate} group and the TOB1^{lowrate} group (54.12 vs. 39.76%, $P = 0.20$; 53.74 vs. 40.41%, $P = 0.26$). The cumulative recurrence rates also had no significant difference

between them (40.39 vs. 40.13%, $P = 0.619$; 37.99 vs. 42.91%, $P = 0.874$) (Fig. 5).

Discussion

TOB1 has been reported to be a tumor suppressor gene and TOB expression is lost in human lung and thyroid cancers (12). However, whether it is a tumor suppressor gene in HCC remains unclear. In addition, whether its expression is decreased, and what is responsible for the downregulation in gene expression in HCC still need to be explored. In light of this, we performed MassArray quantitative methylation analysis in the promoter CpG island of TOB1 in breast cancer and HCC. Our results indicated that tumor and non-tumor tissues tended to share a common methylation pattern at different CpG sites (Fig. 3A). However, different CpG sites had a different change pattern following tumorigenesis in different tissues. This suggested that different CpG sites had different functions or only partial CpG sites were crucial for regulation in gene expression. With respect to DNA methylation, the decrease in CpG sites in tumors relative to their normal tissue counterparts was one of the first epigenetic alterations to be found in human cancer, particularly in the tumor suppressor gene promoter and nearby region (21,23). In our study, following comparison between tumor tissues and adjacent normal tissues at different CpG sites, we did not observe statistical significance at common CpG sites which shared the above phenomena in the methylation level. Based on these data, we speculated that TOB1 may be a candidate non-tumor suppressor gene in HCC.

To further evaluate whether TOB1 is a tumor suppressor gene in HCC, we investigated the correlation between TOB1 methylation variation and mRNA expression, and real-time PCR was conducted on a cohort of 43 patients with HCC. The results did not reveal a significant difference in tumor tissues compared to adjacent normal tissues. Our findings were not in agreement with a previous observation in breast cancer patients (12). Therefore, that TOB1 is not a tumor suppressor gene in HCC appears more likely. This concept was further supported by the fact that the TOB1 expression was inhibited in HepG2; however, in other two cell lines (A-375 and MDA-MB-231) TOB1 expression was elevated dramatically following 5-aza-dC treatment. Consequently, other mechanisms may account for the regulation of TOB1 gene expression, but no significant difference in gene expression between tumor tissues and adjacent normal tissues together with 5-aza-dC treatment assay provided a significant indication that TOB1 was not a tumor suppressor gene. Of course, the same gene having different functions in different tissues is normal for the TOB1 gene. For example, Ruan *et al* reported that TOB1 functioned in the deadenylation of mRNA decay and could interact with Caf1 and PABPC1 (24); Tzachanis *et al* reported that TOB1 functioned in enhancing Smad DNA-binding through associating with Smad2 and Smad4 in T cells (4). Therefore, it was logical to assume that TOB1 was not a tumor suppressor in HCC.

TOB1 has emerged as an important molecule correlated with tumorigenicity and metastasis in cell lines and expression inhibition in breast cancer tissues. This study did not reveal a significant difference in TOB1 expression between tumor tissues and adjacent normal tissues, and the TOB1 expression or expression ratio was not associated with the OS or

cumulative recurrence rates. Taken together, this may prove that TOB1 is not a tumor suppressor in HCC. The study also showed that TOB1 was associated with GGT. However, GGT is only an auxiliary marker in the diagnosis of HCC diagnosis, and further investigations are warranted to explore the role of TOB1 in HCC, such as a study in different populations. Therefore, it should be stated that our findings that TOB1 was not a tumor suppressor in HCC are still preliminary.

To conclude, our study found no methylation aberrance in global methylation level or single CpG site in the TOB1 promoter and nearby region between tumor tissues and adjacent normal tissues. In addition, the expression of the TOB1 gene displayed no significant difference between tumor tissues and adjacent normal tissues. We also observed that TOB1 expression had no correlation with the OS or cumulative recurrence rates. Based on these data, we hypothesize that TOB1 is not a tumor suppressor gene in HCC.

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References

- Maekawa M, Nishida E and Tanoue T: Identification of the anti-proliferative protein Tob as a MAPK substrate. *J Biol Chem* 277: 37783-37787, 2002.
- Yoshida Y, Hosoda E, Nakamura T and Yamamoto T: Association of ANA, a member of the antiproliferative Tob family proteins, with a Caf1 component of the CCR4 transcriptional regulatory complex. *Jpn J Cancer Res* 92: 592-596, 2001.
- Fletcher BS, Lim RW, Varnum BC, Kujubu DA, Koski RA and Herschman HR: Structure and expression of TIS21, a primary response gene induced by growth factors and tumor promoters. *J Biol Chem* 266: 14511-14518, 1991.
- Tzachanis D, Freeman GJ, Hirano N, *et al*: Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nat Immunol* 2: 1174-1182, 2001.
- Rouault JP, Rimokh R, Tessa C, *et al*: BTG1, a member of a new family of antiproliferative genes. *EMBO J* 11: 1663-1670, 1992.
- Rouault JP, Falette N, Guehenneux F, *et al*: Identification of BTG2, an antiproliferative p53-dependent component of the DNA damage cellular response pathway. *Nat Genet* 14: 482-486, 1996.
- Bradbury A, Possenti R, Shooter EM and Tirone F: Molecular cloning of PC3, a putatively secreted protein whose mRNA is induced by nerve growth factor and depolarization. *Proc Natl Acad Sci USA* 88: 3353-3357, 1991.
- Maekawa M, Yamamoto T and Nishida E: Regulation of subcellular localization of the antiproliferative protein Tob by its nuclear export signal and bipartite nuclear localization signal sequences. *Exp Cell Res* 295: 59-65, 2004.
- Suzuki T, K-Tsuzuku J, Ajima R, Nakamura T, Yoshida Y and Yamamoto T: Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. *Genes Dev* 16: 1356-1370, 2002.
- Kawamura-Tsuzuku J, Suzuki T, Yoshida Y and Yamamoto T: Nuclear localization of Tob is important for regulation of its antiproliferative activity. *Oncogene* 23: 6630-6638, 2004.
- Matsuda S, Kawamura-Tsuzuku J, Ohsugi M, *et al*: Tob, a novel protein that interacts with p185erbB2, is associated with anti-proliferative activity. *Oncogene* 12: 705-713, 1996.
- O'Malley S, Su H, Zhang T, Ng C, Ge H and Tang CK: TOB suppresses breast cancer tumorigenesis. *Int J Cancer* 125: 1805-1813, 2009.
- Iwanaga K, Sueoka N, Sato A, *et al*: Alteration of expression or phosphorylation status of tob, a novel tumor suppressor gene product, is an early event in lung cancer. *Cancer Lett* 202: 71-79, 2003.
- Yoshida Y, Tanaka S, Umemori H, *et al*: Negative regulation of BMP/Smad signaling by Tob in osteoblasts. *Cell* 103: 1085-1097, 2000.
- Yoshida Y, von Bubnoff A, Ikematsu N, *et al*: Tob proteins enhance inhibitory Smad-receptor interactions to repress BMP signaling. *Mech Dev* 120: 629-637, 2003.
- Xiong B, Rui Y, Zhang M, *et al*: Tob1 controls dorsal development of zebrafish embryos by antagonizing maternal beta-catenin transcriptional activity. *Dev Cell* 11: 225-238, 2006.
- Ezzeddine N, Chang TC, Zhu W, *et al*: Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. *Mol Cell Biol* 27: 7791-7801, 2007.
- Yoshida Y, Nakamura T, Komoda M, *et al*: Mice lacking a transcriptional corepressor Tob are predisposed to cancer. *Genes Dev* 17: 1201-1206, 2003.
- Liu H, Kong Q, Li B, He Y, Li P and Jia B: Expression of PEBP4 protein correlates with the invasion and metastasis of colorectal cancer. *Tumour Biol* 33: 267-273, 2012.
- Gao Q, Qiu SJ, Fan J, *et al*: Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 25: 2586-2593, 2007.
- Zhao Y, Kong X, Li X, *et al*: Metadherin mediates lipopolysaccharide-induced migration and invasion of breast cancer cells. *PLoS One* 6: e29363, 2011.
- Llovet JM, Di Bisceglie AM, Bruix J, *et al*: Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 100: 698-711, 2008.
- Tost J: DNA methylation: an introduction to the biology and the disease-associated changes of a promising biomarker. *Mol Biotechnol* 44: 71-81, 2010.
- Ruan L, Osawa M, Hosoda N, *et al*: Quantitative characterization of Tob interactions provides the thermodynamic basis for translation termination-coupled deadenylase regulation. *J Biol Chem* 285: 27624-27631, 2010.