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The transmembrane protein *TMEM7* is an IFN-α responsive gene that suppresses cell proliferation and is down-regulated in human hepatocellular carcinoma

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Abstract

The short arm of chromosome 3 contains multiple regions that are frequently affected by loss of heterozygosity in human cancers. The *TMEM7* gene, which encodes a transmembrane protein, is among the candidate tumor suppressor genes located at one of these regions, 3p21.3. This gene is expressed specifically in the liver, and the encoded protein shares substantial sequence homology with human and mouse 28-kDa interferon-alpha (IFN-α) responsive protein. On the basis of these observations, we have now investigated the possible role of *TMEM7* in the development of hepatocellular carcinoma (HCC). We examined *TMEM7* expression in 20 primary HCC and 18 HCC cell lines and found recurrent functional alterations. While *TMEM7* mRNA was expressed in normal hepatic cells, down-regulation or inactivation of the gene was detected in 85% and 33% of primary HCC and HCC cell lines, respectively. To identify the mechanisms responsible for down-regulation or silencing of *TMEM7*, we examined genomic deletion and mutation as well as the effect of inhibitors of DNA methyltransferase and histone deacetylase on cells with low levels or lacking endogenous *TMEM7* expression. Homozygous deletion of *TMEM7* was not detected in 17 pairs of human HCC and the corresponding non-cancerous liver tissues or in any of 18 HCC cell lines studied. Likewise, mutation of the gene was not detected in 18 HCC cell lines with low or normal *TMEM7* expression. Treatment of two out of six cell lines exhibiting down-regulation or loss of *TMEM7* with 5-aza-2'-deoxycytidine and trichostatin A resulted in a additive increase in *TMEM7* expression, implicating aberrant DNA methylation and histone deacetylation in the transcriptional silencing of this gene. Ectopic expression of *TMEM7* in two *TMEM7* deficient HCC lines suppressed cell proliferation, colony formation, and cell migration *in vitro* and reduced tumor formation in nude mice. Treatment of two highly invasive HCC cell lines with IFN-α for 7 days significantly increased *TMEM7* expression and in inhibited cell migration. These observations implicate loss of *TMEM7* expression in hepatocarcinogenesis and suggest that modification of TMEM7 expression by IFN-α may have potential therapeutic relevance in a subset of HCC.

Keywords

TMEM7; cell growth inhibition; cell migration inhibition; interferon alpha; reduction of tumorigenicity

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers worldwide and accounts for 90% of all liver carcinomas. It is estimated that more than 560,000 new cases of HCC are diagnosed annually, most of them having an unfavorable prognosis. The mortality rate of HCC is high because many tumors are asymptomatic until late stages of development. Various factors, including infection with hepatitis B or C viruses, dietary aflatoxin, alcohol consumption, and exposure to chemical carcinogens, have been implicated in the etiology of HCC [1]. In Western countries, the incidence of HCC has increased markedly in the last several years as a result of an increased incidence of hepatitis virus infection [2,3]. Both chemical carcinogens and oncogenic viruses induce DNA damage in liver cells that manifests at the chromosome level as deletions, duplications, or translocations. The progression to HCC is a slow process that includes several distinct stages that are associated with the sequential accumulation of genomic alterations [1]. In addition, epigenetic changes are thought to play an important role in hepatocarcinogenesis [4].

Chromosomal regions that are frequently deleted in cancer are thought to be the loci of tumor suppressor genes, whose loss or inactivation contribute to unrestricted cell proliferation. Tumor suppressor genes that are silenced by promoter methylation are thus often located at chromosomal regions that are deleted in cancer cells [5,6]. Deletions of the short arm of chromosome 3 are common in various types of cancer. Although region 3p21.3 is most frequently affected by homozygous and heterozygous deletions in many cancers, the identification of tumor suppressor genes at this site has been difficult [7]. Among bona fide and candidate tumor suppressor genes localized to this region of 3p, the gene for *TMEM7* (transmembrane protein 7) was identified by combined deletion mapping and by the "elimination test" i.e. SCID passage of chromosome 3 monochromosomal hybrids and mapping of the non-randomly lost regions [8,9].

We found that *TMEM7* shares 38% sequence identity and 45% sequence similarity with human and mouse 28-kDa interferon-α (IFN-α) responsive protein [10,11]. The antiviral, antiproliferative, apoptosis-inducing, anti-angiogenic, and immunologic effects of IFNs are mediated by transcriptional induction of various genes [12]. These observations, together with the *TMEM7* expression adult and fetal liver and its chromosomal location, prompted us to investigate whether this gene may contribute to the pathogenesis of HCC. We have now examined various HCC cell lines and primary tumors for alterations of *TMEM7*. Our results show that expression of *TMEM7* was down-regulated or silenced in both primary tumors and HCC cell lines, and that either ectopic expression of *TMEM7* or IFN-α induced up-regulation of endogenous *TMEM7* suppressed tumor cell proliferation or invasion.

Materials and Methods

Primary HCC specimens and PCR analysis of *TMEM7*

Primary tumor specimens were obtained by surgical resection of HCC from 17 patients in Qidong, China. Each tumor sample was matched with its surrounding noncancerous liver tissue. The tumors were positive for hepatitis B virus (HBV) surface antigen and /or PCR detection of the HBVx gene. DNA was extracted from tumor tissue samples, and portions of *TMEM7* and of the β-globin gene (internal control) were amplified by a multiplex polymerase chain reaction (PCR) with the primers 5'-GATCCTGAAAAACCTGGTGT-3' and 5'-TCCAAGGCTCATATAGCAGT-3' for *TMEM7* (sense and antisense, respectively, yielding a product of 369 bp) and 5'-GAAGAGCCAAGGACAGGTAC-3' and 5'-CAACTTCATCCACGT TCACC-3' for the β-globin gene (sense and antisense, respectively, yielding a product of 268 bp). PCR was performed with a Peltier Thermal Cycler (MJ Research, Watertown, MA) according to standard protocols.

HCC cell culture, mutation screening, and RT-PCR analysis of *TMEM7* expression

Eighteen HCC cell lines used in the present study were previously described and analyzed by comparative genomic hybridization (CGH) [13]. Cells were cultured under a humidified atmosphere of 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM)–F12 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). They were passaged by exposure to trypsin. Total RNA was isolated from the cells with the use of an RNeasy kit (Qiagen, Valencia, CA) and was subjected to reverse transcription (RT) and multiplex PCR analysis with the *TMEM7*-specifc primers described above and with the primers 5'-CTCGCGAACAAGGGATTATG-3' and 5'-ACACAGCCCAGCCACATTA-3' (sense and antisense, respectively, yielding a product of 507 bp) specific for *DLC1* (internal control). *TMEM7* PCR products amplified from the HCC cell lines were also purified and sequenced for detection of mutations. Total RNA from HCC cell lines and from 20 primary HCC samples were analyzed by quantitative real time PCR for expression of *TMEM7*. DNA from these primary HCC samples was not available. The *TMEM7* primer sets, probes, and an endogenous control GAPDH were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA). The ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA) was used with a universal cycling condition. The 2–{Delta}{Delta}Ct method was used to calculate the relative fold difference of *TMEM7* mRNA expression in all samples. Two-fold increased or decreased expression was considered significant.

Tissue distribution of *TMEM7* mRNA

The abundance of *TMEM7* mRNA in human organs and tissues was determined with the use of Rapid-Scan Gene Expression Panels (OriGene Technologies, Rockville, MD). *TMEM7* primers used as above and 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3' (sense and antisense, respectively, yielding a product of 540 bp) for the actin gene (internal control). PCR was performed with a Peltier Thermal Cycler, and 10 μl of each reaction mixture were then subjected to electrophoresis.

Plasmid construction and cell transfection

A full-length *TMEM7* cDNA was subcloned into pcDNA-DEST40 and pcDNA-DEST47-GFP (Invitrogen, Carlsbad, CA), vectors designed for high-level stable or transient expression in mammalian cells. The sequence and orientation of the constructs were confirmed by DNA sequencing. Cells were transfected with the vectors using Lipofectamine 2000 (Invitrogen) and were subjected to selection 48 h after transfection by incubation in complete medium containing G418 (Invitrogen) at 200 or 250 μg/ml. Expression of *TMEM7* cDNA in the transfected cell lines was assessed by RT-PCR analysis. The efficiency of transient transfection was evaluated under the same experimental conditions with pcDNA-DEST47-GFP vectors. Selected stable transfectants were expanded for analysis of tumorigenicity.

Assay of cell growth, colony formation, and cell migration

Exponentially growing cells were transfected with either pcDNA-DEST40 or pcDNA-DEST40-*TMEM7*, harvested 2 days later, and transferred to six-well plates (3×10^4 cells per well) in G418 selection medium. The cells were harvested with trypsin at 48-h intervals during incubation for up to 10 days and the number of viable cells was determined by staining with trypan blue. Colony formation was assayed with a cell transformation detection kit (Chemicon, Temecula, CA). The transfected cells were thus suspended in 0.4% agar, layered onto a 0.8% agar base, and incubated at 37°C until colonies formed. The colonies were counted after staining with the solution included with the kit. For assay of cell migration in vitro, transiently transfected cells (2×10^5) were suspended in serum-free medium and seeded into the upper compartment of BIO-COAT invasion chambers containing a membrane (pore size, 8 μm) that

had been coated with Matrigel (Becton Dickinson, Bedford, MA). The lower compartment of the chambers was filled with complete medium. After incubation for 24 h at 37°C, cells that had migrated to the bottom surface of the membrane were fixed, stained with Diff-Quick (Dade Behring, Newark, DE), and counted.

Detection of IFN-α responses

Two highly invasive HCC cell lines, HLF and MHCC97, that express moderate levels of *TMEM7* were cultured in 100-mm dishes and incubated with native IFN-α (Schering, Kenilworth, NJ) at 100 or 1000 IU/ml or with vehicle for 7 days, with a change of medium every 2 days. The cells were subjected to RT and real-time PCR analysis for measurement of *TMEM7* mRNA or were assayed for cell migration as described above. For quantitative RT-PCR analysis, total RNA was isolated from the cells and converted to cDNA, 200 ng of which were then subjected to real-time PCR with TaqMan Universal PCR Master Mix and an ABI Prism Sequence Detection Instrument 7900 (Applied Biosystems, Foster City, CA). Primers and TaqMan probes for *TMEM7* (Assay ID: Hs00230189-m1) and for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (internal control) were obtained from Applied Biosystems.

Tumorigenicity assay

HCC cells (PLC/PRF/5 and SNU 398) stably transfected with pcDNA-DEST40-*TMEM7* or with the empty vector were harvested. The cell viability was assessed by staining with trypan. Viable cells (2×10^6) were injected subcutaneously at the proximal dorsal midline of 6-week-old male athymic nu/nu mice (Harlan). The size of the resulting tumors was measured in two dimensions twice a week for up to 6 weeks.

Effects of DNA methylation and histone deacetylation inhibitors on TMEM7 expression

HCC cell lines with undetectable or low levels of *TMEM7* expression were cultured in six-well plates to 30 to 40% confluence and were then incubated with 1 μM 5-aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis, MO), a DNA methyltransferase inhibitor, for 72 h; with 500 nM trichostatin A (Sigma-Aldrich), a histone deacetylase inhibitor, for 12 h; or with the combination of 1 μM 5-aza-2'-deoxycytidine (for 72 h) and 500 nM trichostatin A (added only during the last 12 h). Total RNA was then extracted from the cells and subjected to RT and real-time PCR analysis for quantitation of *TMEM7* mRNA as described above.

Statistical analysis

All data are representative of at least three independent experiments. Quantitative data are presented as means ± SD, and the significance of differences between means was determined by Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

Structure of *TMEM7*

Human *TMEM7* encompasses ~ 3 kbp of genomic DNA, comprises two exons consisting of a total of 791 bp, and encodes a protein of 232 amino acids with a predicted molecular size of 27 kDa (Figure 1A). The SMART program predicts that the TMEM7 protein contains a single transmembrane domain that comprises amino acids 211 to 228 (LSIFCCCVILIVIVVIVV). Sequence alignment with the use of GCG Gap-Global revealed that human TMEM7 shares 38% sequence identity and 45% sequence similarity with human or mouse 28-kDa IFN-α responsive protein (Figure 1B). No conserved domains were detected in *TMEM7* by the NCBI Conserved Domain Search program.

Expression and mutation screening of *TMEM7* in HCC cell lines

The expression of *TMEM7* in 18 HCC cell lines was examined by RT-PCR analysis. The loss or near loss of *TMEM7* expression in the absence of homozygous deletion of the gene was apparent in six (33%) of the eighteen cell lines (Figure 2A). Sequencing of *TMEM7* exon 2 in these HCC cell lines did not detect any mutations (data not shown).

Expression of *TMEM7* in human tissues and primary HCC

Expression of *TMEM7* in 24 human organs or tissues was examined with an RT-PCR–based system. *TMEM7* mRNA was most abundant in adult liver but was also present in smaller amounts in kidney, testis, and fetal liver (Figure 2B). It was not detected or was present at only low levels in the other organs and tissues examined. Quantitative real time PCR analysis of 20 primary HCC tumors showed down regulation of *TMEM7* expression in 17 tumors (85%) as compared normal liver tissue (Figure 2C). Homozygous deletion of *TMEM7* was not detected by Southern blotting in 17 primary HCC specimens and their matched normal tissue samples (data not shown).

Effects of *TMEM7* on cell growth, colony formation, and cell migration

We examined the effects of *TMEM7* on various cellular activities by transient transfection of two HCC cell lines, SNU 398 and PLC/PRF/5, which contain only a small amount of endogenous *TMEM7* mRNA with either pcDNA-DEST40-*TMEM7* and pcDNA-DEST47-GFP-*TMEM7* or the corresponding empty vectors. Using fluorescent microscopy, we detected approximately 65 to 75% transfection efficiency in SNU 398 and PLC/PRF/5 cells transfected with pcDNA-DEST47-GFP-*TMEM7*. Transfection of each of the two HCC cell lines with the *TMEM7* vector resulted in suppression of cell growth that was first apparent 4 days after transfection compared with the rate of increase in cell number observed in cells transfected with the empty vector (Figure 3A). Transfection with the *TMEM7* vector also resulted in a marked reduction in both the number and size of colonies formed by each cell line compared with that apparent with cells transfected with the empty vector (Figure 3B). We also examined the effect of *TMEM7* expression on the invasiveness of SNU 398 and PLC/PRF/5 cells in vitro. Although the proportions of PLC/PRF/5 and SNU 398 cells transfected with the empty vector that migrated through a Matrigel-coated membrane differed by a factor of >2, ectopic expression of *TMEM7* inhibited cell migration by >50% in both HCC cell lines (Figure 3C).

Effects of IFN-α on *TMEM7* expression in and migration of HCC cells

To determine whether *TMEM7* is an IFN-α responsive gene, we examined the effect of IFN-α on the abundance of *TMEM7* mRNA in HLF and MHCC97 cells, both of which are highly invasive and express *TMEM7* at a moderate level. Quantitative RT-PCR analysis revealed that incubation of cells with IFN-α for 7 days resulted in a concentration-dependent increase in the amount of *TMEM7* mRNA in each cell line (Figure 4A). We also examined the effect of IFN-α on cell migration in vitro. Although incubation of cells with IFN-α at 100 IU/ml for 7 days had no effect on cell migration (data not shown), incubation with IFN-α at 1000 IU/ml resulted in >50% inhibition of cell invasiveness (Figure 4B).

Effect of *TMEM7* on tumorigenicity

To examine the effect of *TMEM7* on tumorigenicity in vivo, we injected HCC cells stably transfected with pcDNA-DEST40-*TMEM7*, which showed high levels of TMEM7 expression as quantified by real time PCR, or with the empty vector into athymic nude mice. Restoration of *TMEM7* expression reduced the ability of SNU 398 and PLC/PRF/5 cells to form tumors in nude mice. Compared with cells transfected with the empty vector, those transfected with the *TMEM7* vector showed an increased latency to tumor formation and formed markedly smaller and fewer tumors (Table 1).

Inactivation of *TMEM7* by DNA methylation and histone deacetylation in HCC cells

Given that homozygous deletion and mutation were not responsible for down regulation or loss of *TMEM7* expression in the HCC cell lines studied, we examined the possible role of DNA methylation or histone deacetylation in *TMEM7* silencing by treating four of the affected cell lines with 5-aza-2'-deoxycytidine or trichostatin A, respectively. Whereas these agents alone or together had no effect on *TMEM7* expression in PLC/PRF/5 or HLE cells, 5-aza-2'-deoxycytidine induced 6.4- and 4.5-fold increases in the amount of *TMEM7* mRNA in SNU 398 and SNU449 cells, respectively (Figure 5). Although trichostatin A alone had no effect on *TMEM7* expression in the latter two cell lines, it potentiated the effect of 5-aza-2'-deoxycytidine about 27 to 34% more.

Discussion

The identification of cancer-related genes, which constitute only a small fraction of the human genome, is fundamental to the development of new therapeutics. We have now shown that expression of *TMEM7* is down-regulated in 85% of the primary HCC and in 33% of the HCC cell lines studied. Restoration of the expression of this gene in such cell lines by vector transfection results in inhibition of tumorigenic properties in vitro and in vivo. Ectopic expression of *TMEM7* thus suppressed cell growth and migration *in vitro* as well as tumor formation in vivo, showing that *TMEM7* acts as a tumor suppressor gene in HCC cells.

About 30% of HCC patients exhibit loss of heterozygosity on chromosome 3p, and loss of DNA copy number at 3p has been detected by CGH in the HCC cell lines used in the present study [13]. Current analysis showed that homozygous deletion and mutation of *TMEM7* were not responsible for down-regulation of the expression of this gene in the affected cell lines and were also not detected in the primary HCC tumor specimens. These results are not surprising given the fact homozygous deletions are rare in primary HCC tumors and HCC cell lines [14,15] and that none of the other 3p21.3 genes appear to be mutational targets in cancers. However, as in other types of cancer, aberrant DNA methylation and histone deacetylation also contribute to the inactivation of tumor suppressor genes in HCC [4,16]. Down-regulation or complete silencing of the expression of *RASSF1A*, *BLU*, *SEMA3B*, *FHIT*, *RIZ*, and *VHL*, all of which are candidate or bona fide tumor suppressor genes on chromosome 3p, has been detected in HCC and shown to be the result of genetic or epigenetic mechanisms [17,18–21]. In the present study, treatment of *TMEM7*-deficient HCC cell lines with trichostatin A alone had no effect on *TMEM7* expression. However, this histone deacetylase inhibitor potentiated the stimulatory effect of 5-aza-2-deoxycytidine on *TMEM7* expression in two HCC cell lines. A similar effect of these two agents was recently observed in prostate cancer cell lines and is consistent with the notion that optimal reexpression of genes silenced by promoter methylation and histone deacetylation can be achieved by treatment with inhibitors of both processes [22]. Most recently the molecular mechanisms responsible for such optimal response by sequential treatment with methyltransferase inhibitors and histone deacetylase inhibitors have been reported [23]. Drugs that bring about DNA demethylation or histone acetylation have been shown to have therapeutic potential in cancer and the responses to their combination justify the testing in randomized clinical trials [6,23,24]. Other mechanisms responsible for *TMEM7* deregulation, such as phosphorylation and ubiquitination remain to be examined .

Ectopic expression of *TMEM7* in *TMEM7*-deficient HCC cells resulted in inhibition of cell proliferation, colony formation, and cell migration *in vitro* as well as of tumor formation in nude mice. Although restoration of *TMEM7* expression in HCC cells did not prevent tumor formation in most injected mice, it reduced both the number of mice that developed tumors and tumor size as well as increased the latency to tumor formation. We previously showed that ectopic expression of *DLC-1*, which functions as tumor suppressor gene, resulted in a similar

reduction in the frequency and size of tumors formed by HCC cells in this model system [25]. In breast and lung cancer, *DLC-1* abolished the in vivo tumorigenicity [26,27].

Based on the 38% sequence identity and 45% sequence similarity of the 28-kDa IFN-α-responsive protein with *TMEM7* we addressed the question whether there are functional similarities and/or cooperation between them. IFN-α manifests antiviral as well various antitumor activities that include immunomodulatory, antiproliferative, and anti-angiogenic effects [28,29]. Chemoadjuvant therapy with IFN-α has been shown to be highly effective, inhibiting dissemination to secondary sites, in individuals with advanced and metastatic HCC. We have shown that exposure of highly invasive HCC cell lines to IFN-α resulted in a marked increase in the abundance of *TMEM7* mRNA as well as inhibition of cell migration in vitro. A similar effect of IFN-α on cell invasiveness has been observed in HLF cells [29]. IFN-α also exerts antiproliferative and antimetastatic effects on HCC through inhibition of matrix metalloproteinase expression. The migration of and invasion by HCC cells require matrix metalloproteinase activity [29,30]. Lactoferrin, which is encoded by another candidate tumor suppressor gene located in the region of deletion at 3p21.3 in cancer cells, exerts an immunomodulatory effect through up-regulation of IFN-α expression in healthy individuals [31,32].

In summary, we have shown that *TMEM7* appears to be an IFN-α responsive gene and manifests tumor suppressor activity that may be important in the pathogenesis of HCC. Our observations suggest that *TMEM7* has potential therapeutic applications not only for cancer but also for inflammatory or infectious diseas

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References

1. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular carcinoma. Nat Genet 2002;31:339–46. [PubMed: 12149612]
2. El-Serag HB, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. N Engl J Med 1999;340:745–50. [PubMed: 10072408]
3. Tanaka Y, Hanada K, Mizokami M, Yeo AE, Shih JW, Gojobori T, Alter HJ. Inaugural Article: A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. Proc Natl Acad Sci U S A 2002;99:15584–9. [PubMed: 12438687]
4. Katoh H, Shibata T, Kokubu A, Ojima H, Fukayama M, Kanai Y, Hirohasi S. Epigenetic instability and chromosomal instability in hepatocellular carcinoma. Am J Pathol 2006;168:1375–84. [PubMed: 16565510]
5. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28. [PubMed: 12042769]
6. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003;349:2042–54. [PubMed: 14627790]
7. Huebner K. Tumor suppressors on 3p: a neoclassic quartet. Proc Natl Acad Sci U S A 2001;98:14763–5. [PubMed: 11752421]
8. Kiss H, Yang Y, Kiss C, Andersson K, Klein G, Imreh S, Dumansky JP. The transcriptional map of the common eliminated region 1 (C3CER1) in 3p21.3. Eur J Hum Genet 2002;10:52–61. [PubMed: 11896456]
9. Imreh S, Klein G, Zabarovsky ER. Search for unknown tumor-antagonizing genes. Genes Chromosomes Cancer 2003;38:307–21. [PubMed: 14566849]
10. Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, Wagner L, Shenmem CM, Schuler DG, Altschul SF, Zeeberg B, Buetow KH, et al. Generation and initial analysis of more

than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A 2002;99:16899–903. [PubMed: 12477932]

11. Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, Kiyosawa H, et al. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 2002;420:563–73. [PubMed: 12466851]
12. Leaman DW, Chawla-Sarkar M, Jacobs B, Vyas K, Sun Y, Ozdemir A, Taolin Yi, Williams BR, Borden EC. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. J Interferon Cytokine Res 2003;23:745–56. [PubMed: 14769151]
13. Zimonjic DB, Keck CL, Thorgeirsson SS, Popescu NC. Novel recurrent genetic imbalances in human hepatocellular carcinoma cell lines identified by comparative genomic hybridization. Hepatology 1999;29:1208–14. [PubMed: 10094966]
14. Pineau P, Marchio A, Nagamori S, Seki S, Tiollais P, Dejean A. Homozygous deletion scanning in hepatobiliary tumor cell lines reveals alternative pathways for liver carcinogenesis. Hepatology 2003;37:852–61. [PubMed: 12668978]
15. Thorgeirsson SS. Hunting for tumor suppressor genes in liver cancer. Hepatology 2003;37:739–41. [PubMed: 12668963]
16. Yang B, Guo M, Herman JG, Clark DP. Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma. Am J Pathol 2003;163:1101–7. [PubMed: 12937151]
17. Du Y, Carling T, Fang W, Piao Z, Sheu JC, Huang S. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. Cancer Res 2001;61:8094–9. [PubMed: 11719434]
18. Schagdarsurengin U, Wilkens L, Steinemann D, Flemming P, Kreipe HH, Pfeifer GP, Schlegelberger B, Damman R. Frequent epigenetic inactivation of the RASSF1A gene in hepatocellular carcinoma. Oncogene 2003;22:1866–71. [PubMed: 12660822]
19. Tischoff I, Markwarth A, Witzigmann H, Uhlmann D, Hauss J, Mirmohammadsadegh A, Wittekind C, Hengge UR, Tannapfel A. Allele loss and epigenetic inactivation of 3p21.3 in malignant liver tumors. Int J Cancer 2005;115:684–9. [PubMed: 15704097]
20. Yu J, Ni M, Xu J, Zhang H, Gao B, Gu J, Chen J, Zhang L, Wu M, Zhen S, Zhu J. Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis. BMC Cancer 2002;2:29. [PubMed: 12433278]
21. Yuan BZ, Keck-Waggoner C, Zimonjic DB, Thorgeirsson SS, Popescu NC. Alterations of FHIT gene in human hepatocellular carcinoma. Cancer Res 2000;60:1049–53. [PubMed: 10706123]
22. Guan M, Zhou X, Soulitzis N, Spandidos DA, Popescu NC. Aberrant methylation and deacetylation of deleted in liver cancer-1 gene in prostate cancer: potential clinical applications. Clin Cancer Res 2006;12:1412–9. [PubMed: 16533763]
23. Gore SD, Baylin S, Sugar E, Carraway H, Miller CB, Carducci M, Grever M, Galm O, Dauses T, Karp JE, Rudek MA, Zhao M, et al. Combined DNA methyltransferase and histone deacetylase inhibition in the treatment of myeloid neoplasms. Cancer Res 2006;66:6361–9. [PubMed: 16778214]
24. Marks P, Rifkind RA, Richon VM, Breslow R, Miller T, Kelly WK. Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 2001;1:194–202. [PubMed: 11902574]
25. Zhou X, Thorgeirsson SS, Popescu NC. Restoration of DLC-1 gene expression induces apoptosis and inhibits both cell growth and tumorigenicity in human hepatocellular carcinoma cells. Oncogene 2004;23:1308–13. [PubMed: 14647417]
26. Yuan BZ, Zhou X, Durkin ME, Zimonjic DB, Gumundsdottir K, Eyfjord JE, Thorgeirsson SS, Popescu NC. DLC-1 gene inhibits human breast cancer cell growth and in vivo tumorigenicity. Oncogene 2003;22:445–50. [PubMed: 12545165]
27. Yuan BZ, Jefferson AM, Baldwin KT, Thorgeirsson SS, Popescu NC, Reynolds SH. DLC-1 operates as a tumor suppressor gene in human non-small cell lung carcinomas. Oncogene 2004;23:1405–11. [PubMed: 14661059]
28. Bogdan C. The function of type I interferons in antimicrobial immunity. Curr Opin Immunol 2000;12:419–24. [PubMed: 10899033]
29. Kaneko F, Saito H, Saito Y, Wakabayashi K, Nakamoto N, Tada S, Suzuki S, Tsunematsu S, Kumagai N, Ishii H. Down-regulation of matrix-invasive potential of human liver cancer cells by type I

interferon and a histone deacetylase inhibitor sodium butyrate. Int J Oncol 2004;24:837–45. [PubMed: 15010820]

30. Giannelli G, Bergamini C, Fransvea E, Marinosci F, Quaranta V, Antonaci S. Human hepatocellular carcinoma (HCC) cells require both alpha3beta1 integrin and matrix metalloproteinases activity for migration and invasion. Lab Invest 2001;81:613–27. [PubMed: 11304581]
31. Yang Y, Li J, Szeles A, Imreh MP, Kost-Alimova M, Kiss H, Kholodnyuk I, Fedorova L, Darai E, Kein G, Imreh S. Consistent downregulation of human lactoferrin gene, in the common eliminated region 1 on 3p21.3, following tumor growth in severe combined immunodeficient (SCID) mice. Cancer Lett 2003;191:155–64. [PubMed: 12618328]
32. Ishikado A, Imanaka H, Kotani M, Fujita A, Mitsuishi Y, Kanemitsu T, Tamura Y, Makino T. Liposomal lactoferrin induced significant increase of the interferon-alpha (IFN-alpha) producibility in healthy volunteers. Biofactors 2004;21:69–72. [PubMed: 15630172]

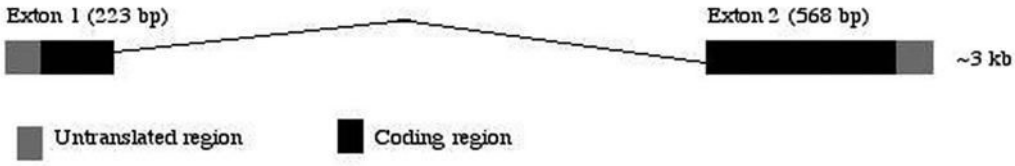
Nonstandard abbreviations

TMEM7
: transmembrane protein 7

HCC
: hepatocellular carcinoma

IFN-α
: interferon alpha

A.



B.

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HSA TMEM7 M--AGDTEVWKQMFQELMREVKPWHRWTLRPDKGLLPNVLKPGWMQYQQW 48
HSA INF28 MVV--DFWTWEQTFQELIQEAKPRATWTLKLDGNLQLDCLAQGWKQYQQR 48
Mu. INF28 MLFPDDFSTWEQTFQELMQEEKPGAKWSLHLDKNIVPDGAALGWRQHQQT 50

HSA TMEM7 TFARFQCSSCSRNWASAQVLVLFHMNWSEEKSRGQVKMRVFTQRCKKCPQ 98
HSA INF28 AFGWFRCSSCQRSWASAQVQILCHTYWEHWTSQGQVRMRLFGQRCQKCSW 98
Mu. INF28 VLGRFQCSRCCRSWTSAQVMILCHMYPDTLKSQGQARMRIFGQKCQKCFG 100

HSA TMEM7 PLFEDPEFTQENISRILKNLVFRILKKCYRGR-FQLIEEVPMIKDISLEG 147
HSA INF28 SQYEMPEFSSDSTMRILSNLVQHILKKYYGN-GMRKSPEMPVILEVSLEG 147
Mu. INF28 CQFETPKFSTEIIKRILNNLVNYILQRYYGHRKIALTSNASLGEKVTLDG 150

HSA TMEM7 PHNSDNCEACLQGFCAGPIQVTSLPPSQTPRVH----------SIYKVE 186
HSA INF28 SHDTANCEACTLGICGQGLKSYMTKPSKSLLPHLKTGNSSPGIGAVYLAN 197
Mu. INF28 PHDTRNCEACSLNSHGRCALAHKVKPRSPSPLPKSSSPSKSCPP---PP 197

HSA TMEM7 EVVKPWASGENVYSYACQN-HICRN---LSIFCCCVILIVIVVIVVK--- 230
HSA INF28 QAKNQSDEAKEAKGSGYEKLGPSRDPDPLNI--CVFILLLV--FIVVKCF 243
Mu. INF28 QTRNTDFGNKTFQDFGNRTFQGCREPPQREIEPPLFLFLSIAAFALFSLF 247

HSA TMEM7 TAI 232
HSA INF28 TSE 246
Mu. INF28 TR 249
```

Figure 1.

Structure of human *TMEM7* (**A**) and sequence alignment of human *TMEM7* with human (HSA) or mouse (Mu) 28-kDa IFN-α responsive proteins (**B**). Identical residues in the sequence alignment are shaded, and dashes represent gaps introduced to optimize the alignment.

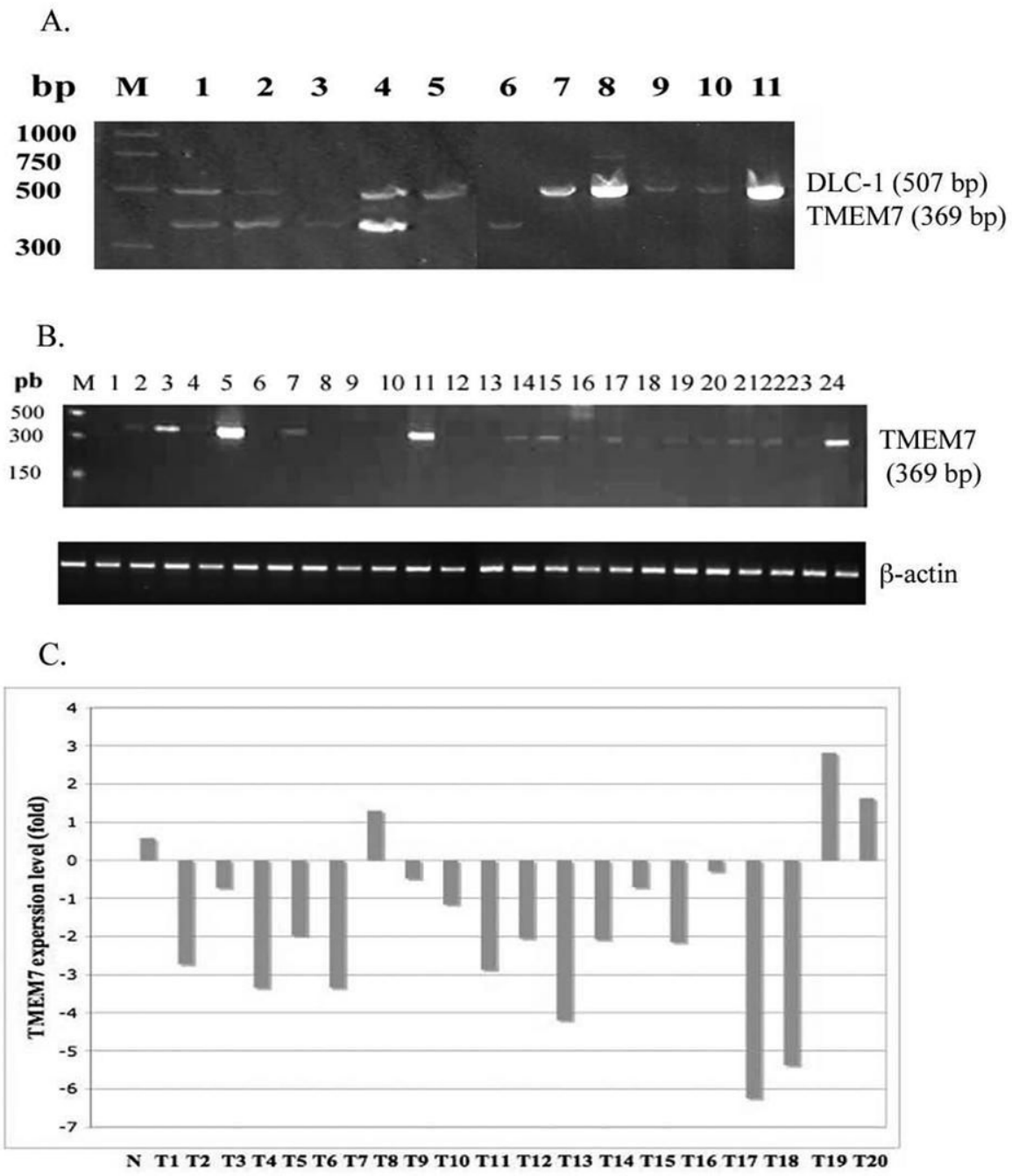

Figure 2.

RT-PCR analysis of *TMEM7* expression in human organs or tissues, primary HCC samples, and in HCC cell lines. (**A**) Expression of *TMEM7* in HCC cell lines. Total RNA was isolated from the cell lines and subjected to RT-PCR analysis with primers specific for *TMEM7* and for *DLC1* (internal control). The cell lines Huh 6, HuH7, Chang, SK-Hep-1, and Focus (lanes 1 to 4 and lane 6, respectively) manifested a moderate level of *TMEM7* expression, whereas *TMEM7* mRNA was undetectable or present at low levels in SNU449, PLC/PRF/5, HLE, SNU 387, SNU 398, and SNU 475 (lane 5 and lanes 7 to 11, respectively). A moderate level of *TMEM7* expression was also detected in the HCC cell lines HLF, MHCC97, 7703K, HepG2, Hep3B, Hep40, and SNU 182 (data not shown). (**B**) Expression of *TMEM7* in human organs

and tissues measured with a panel of cDNAs prepared from brain, heart, kidney, spleen, liver, colon, lung, small intestine, skeletal muscle, stomach, testis, placenta, salivary gland, thyroid, adrenal gland, pancreas, ovary, uterus, prostate, skin, plasma leukocytes, bone marrow, fetal brain, and fetal liver (lanes 1 to 24, respectively). PCR was performed with primers specific for *TMEM7* and for the β-actin gene (internal control). Lane M, molecular size standards. (C). Expression of *TMEM7* in primary HCC samples. N; normal liver sample and T1–T20, primary HCC tumor samples. Among 20 tumors examined, 17 displayed down regulation of *TMEM7* expression.

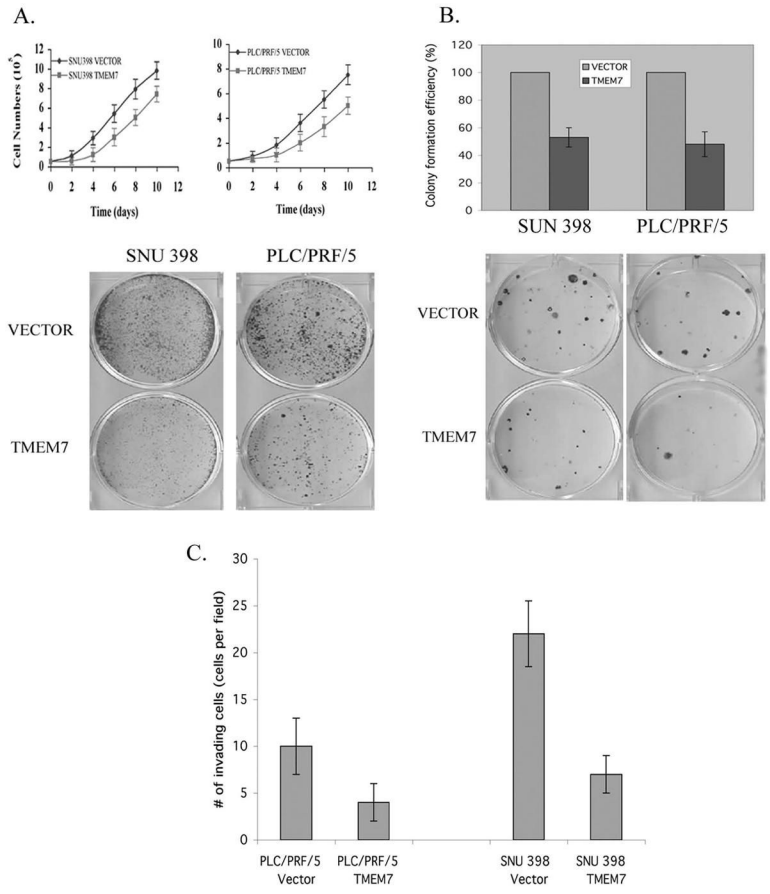

Figure 3.

Inhibition of HCC cell growth, colony formation, and cell migration by ectopic expression of *TMEM7*. (**A**) Cell growth. PLC/PRF/5 or SNU 398 cells were transiently transfected with pcDNA-DEST40-*TMEM7* or pcDNA-DEST40, harvested after 48 h, and transferred to six-well plates (3×10^4 cells per well). The number of viable cells was determined at various times thereafter (upper panel) and the wells were photographed after 10 days (lower panel). (**B**) Colony formation. Cells transfected as in (**A**) were harvested and assayed for colony formation. Colonies formed after 12 days were stained, counted (upper panel), and photographed (lower panel). Data are expressed as colony formation efficiency relative to that of each cell line transfected with the empty vector. (**C**) Cell migration. Cells transfected as in (**A**) were harvested and assayed for cell invasiveness with a Matrigel-coated membrane. Cells that had migrated through the membrane after incubation for 24 h were stained and counted . Data are expressed as the average number of invading cells per microscopic field (400×) determined from 10 fields per membrane. Quantitative data in (**A**) through (**C**) are means ± SD of values from three independent experiments.

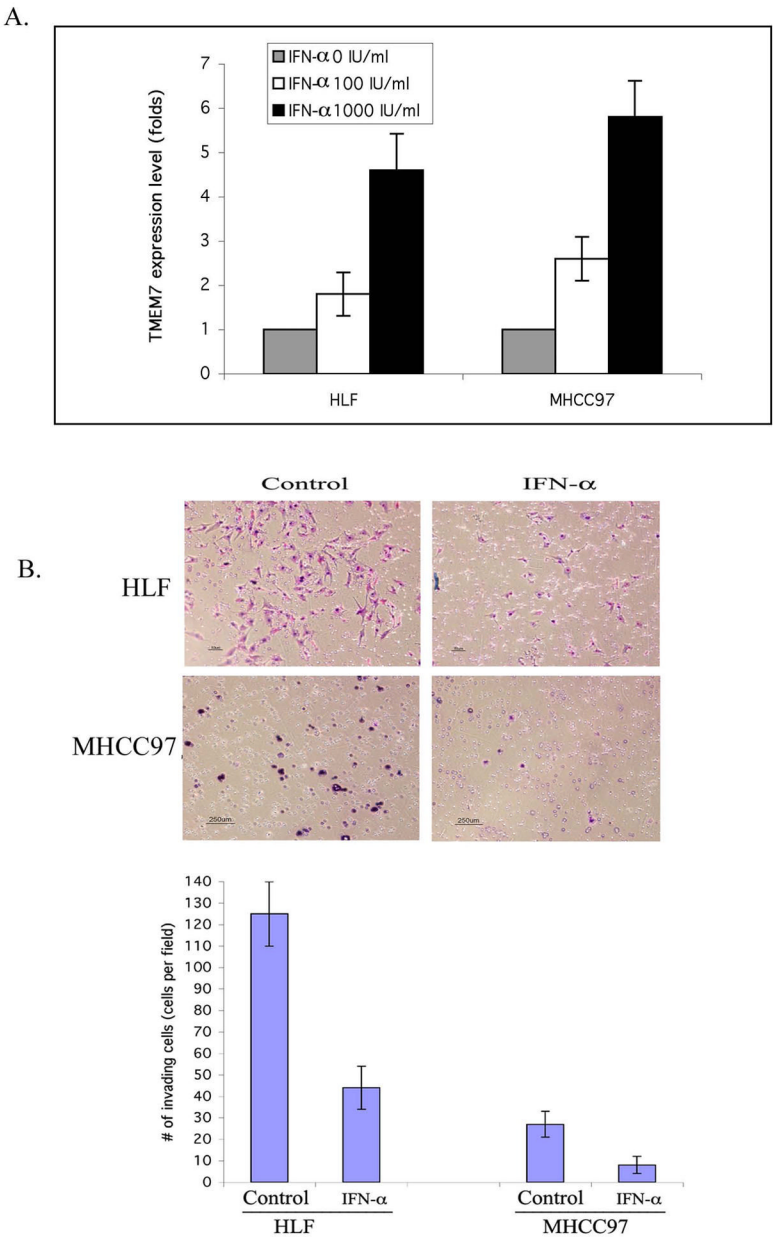

Figure 4.

Induction of *TMEM7* expression in and inhibition of migration of HCC cells by IFN-α. (**A**) *TMEM7* expression. HLF and MHCC97 cells were incubated in the absence or presence of IFN-α (100 or 1000 IU/ml) for 7 days, after which the abundance of *TMEM7* mRNA was determined by quantitative RT-PCR analysis. (**B**) Cell migration. HLF and MHCC97 cells were exposed to IFN-α (1000 IU/ml) for 7 days and were assayed for cell invasiveness with a Matrigel-coated membrane during the final 24 h of exposure. Cells that had migrated through the membrane were stained, photographed (upper panel), and counted (lower panel). Data are expressed as the average number of invading cells per microscopic field (400×) determined from 10 fields per membrane. Quantitative data in (**A**) and (**B**) are means ± SD of values from three independent experiments.

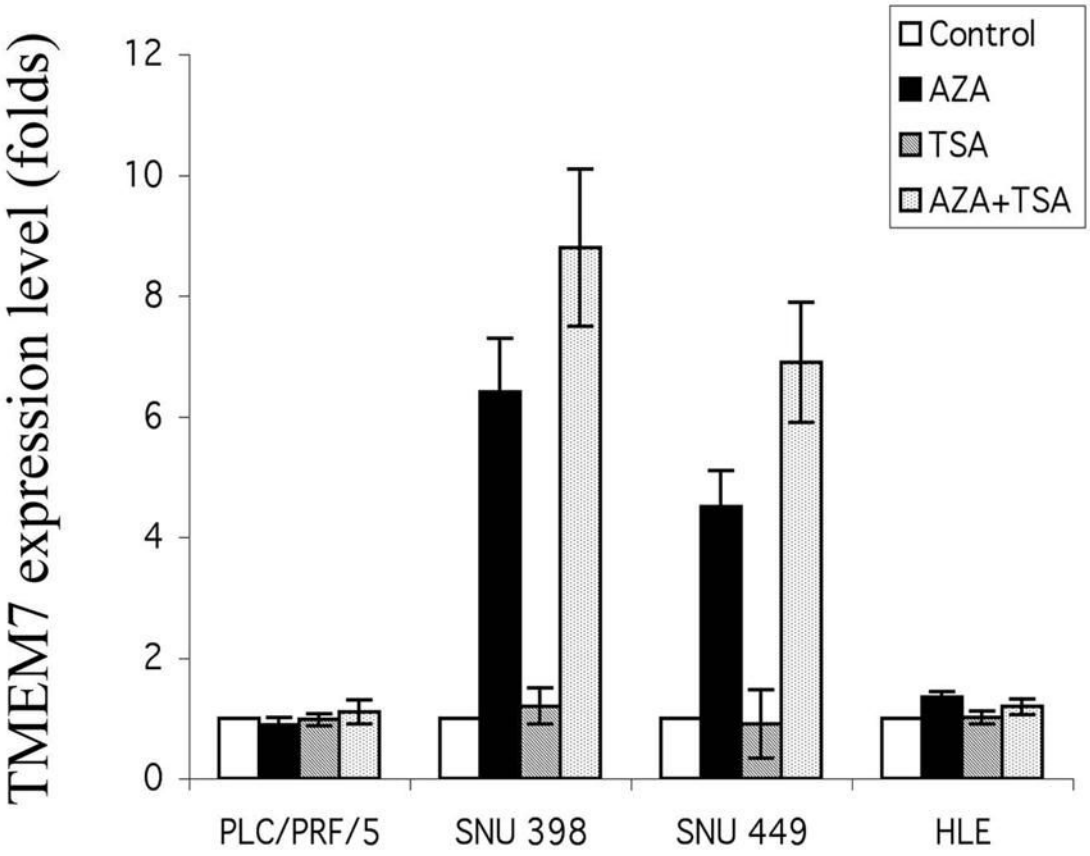

Figure 5.

Effects of DNA methyltransferase and histone deacetylase inhibitors on *TMEM7* expression in HCC cells. The indicated cell lines were incubated with 5-aza-2'-deoxycytidine (AZA), with trichostatin A (TSA), or with both agents as described in Materials and Methods, after which the abundance of *TMEM7* mRNA was determined by quantitative RT-PCR analysis.

Table 1

Inhibition of in vivo tumorigenicity of HCC cells by restoration of TMEM7 expression.

Cell Line	number of cells inoculated	Latency (days)	Tumor size (*W*×*L* mm)	# of tumors/# of inoculations
SNU 398 Vector	2×10^6	30	12×14	20/20
SNU 398 TMEM7	2×10^6	45	6×8	10/20
PCL/PRF/5 Vector	2×10^6	45	13×16	19/20
PCL/PRF/5 TMEM7	2×10^6	60	8×10	13/20