TIP30 Mutant Derived from Hepatocellular Carcinoma Specimens Promotes Growth of HepG2 Cells through Up-Regulation of N-cadherin

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Abstract

TIP30 is a tumor suppressor whose expression is altered in human liver, prostate, lung, colon, and breast cancers. Mice lacking TIP30 spontaneously developed hepatocellular carcinomas (HCC) and other tumors at a higher incidence than wild-type mice. Somatic missense mutations in the TIP30 gene were identified in human HCC tissue specimens, which resulted in instability or abnormal cellular distribution of TIP30 protein in cells. Here, we show that TIP30 mutants are able to promote cell growth and invasion and inhibit cisplatin-induced apoptosis in the HCC cell line HepG2 negative for endogenous TIP30. Moreover, one of the TIP30 mutants can dramatically accelerate tumor formation in immunodeficient mice. Analysis of gene expression in HepG2 cells, ectopically expressing either wild-type TIP30 or mutant TIP30, by Affymetrix GeneChip array, real-time quantitative PCR, and Western blotting assays reveals that TIP30 mutants can alter expression of genes involved in the regulation of tumorigenesis. This includes up-regulation of expression of N-cadherin and c-MYC and down-regulation of expression of p53 and E-cadherin. N-cadherin knockdown with small interfering RNA in HepG2 cells expressing mutant TIP30 resulted in a profound reduction in cell viability. Taken together, our data indicate that somatic mutations in the TIP30 gene may abolish its native tumor-suppressor activity and gain oncogenic activities partially through up-regulation of N-cadherin, thereby potentiating the pathogenesis of HCC in patients.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary hepatic tumor and a leading cause of cancer death worldwide. The highest incidence of HCC is seen in Asia and Africa, but the HCC incidence is increasing in the United States and Western Europe (1, 2). It was estimated that ~600,000 people in the People’s Republic of China die from HCC annually (3). HCC is possibly caused by both genetic and nongenetic factors. Risk factors, including hepatitis B and C viruses, cirrhosis, tobacco and alcohol consumption, aflatoxins, and sex hormones are linked to the development of HCC (4, 5). Genetic aberrations associated with HCC include somatic mutations in the p53 tumor-suppressor gene (TP53), resulting in the disruption of its function; activation of ras, Myc Met, and c-fos oncogenes; increased expression of growth factors and their receptors; altered expression of genes for cell cycle control; and activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase signaling pathway (2). However, the mechanisms by which these risk factors and genetic variations contribute to the carcinogenesis and pathogenesis of HCC still remain largely unknown. A better understanding of genetic alterations in HCC may lead to the discovery of new etiologic, prognostic or therapeutic targets that will improve the survival rate of HCC patients.

TIP30, also called CC3 or HTATIP2, is a tumor suppressor that can promote apoptosis and inhibit angiogenesis (6–9). Abnormal expression of TIP30 is implicated in the pathogenesis of human prostate, lung, colon, and breast cancers (6, 10–12). Mutations were frequently found in TIP30 exon 2 in various cancer cells but not in normal cells when the TIP30 sequences in the National Center for Biotechnology Information databases were analyzed (13). Our laboratory previously showed that mice lacking TIP30 spontaneously developed HCCs and other tumors at a higher incidence than wild-type mice (13). Somatic missense mutations in the TIP30 gene were identified in human HCC tissue specimens, which resulted in either instability or the abnormal cellular distribution of TIP30 protein in cells (13), suggesting that these TIP30 mutations may contribute to the pathogenesis of HCC development through inactivation of TIP30 function. However, it has not been known whether these mutations in cells affect the function of TIP30 and influence pathogenesis of HCC.

Previous studies have revealed that TIP30 acts as a transcription cofactor to enhance expression of genes involved in apoptosis in small-cell lung carcinoma cells (6) and to repress c-MYC expression in breast cancer cells (14). Other studies suggest that TIP30 may act as an inhibitor of protein transportation to regulate apoptosis in NIH3T3 and HeLa cells (15). Therefore, it is reasonable to hypothesize that mutations in the TIP30 gene identified in HCC patients may disturb TIP30-regulated apoptosis, thereby affecting pathogenesis of HCC. To test this hypothesis, we investigated the effects of two mutations on the function of TIP30 that were separately identified in one of eight HCC specimens. Previous studies showed that G134V mutation significantly shortened the half-life of TIP30 protein in HepG2 cells, whereas R106H mutation altered the subcellular distribution of TIP30, suggesting that both mutations might be loss-of-function mutations (13). Using both cultured cells and an in vivo murine xenograft model, we show that TIP30 mutants not only loss the function of tumor suppression but also gain functions such as acceleration of tumorigenesis in nude mice, promotion of proliferation, and inhibition of apoptosis in HepG2 cells. Furthermore, we show that TIP30R106H mutant can alter expression of the N-cadherin gene.
These observations suggest that the TIP30 mutants play an important role in the pathogenesis of HCCs by regulating expression of genes important in the regulation of tumorigenesis. TIP30 mutants may represent new targets for developing a therapy for human HCCs.

Materials and Methods

Plasmids and cell lines. The bacterial expression plasmids pRSET-his-TIP30G134V and pRSET-his-TIP30R106H were generated by changing DNA sequences of the TIP30 gene on pRSET-his-TIP30 with the site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. pFlag-TIP30, pFlag-TIP30G134V, and pFlag-TIP30R106H were constructed by insertion of DNA fragments encoding TIP30, TIP30G134V, and TIP30R106H isolated from pRSET-his-TIP30 and pRSET-his-TIP30 mutant vectors into pFlag (8) at NdeI and BamHI sites. The retroviral vector pBabe Puro-TIP30, pBabe Puro-TIP30G134V, and pBabe Puro-TIP30R106H were created by cloning of BglII-BamHI DNA fragments from pFlag-TIP30 and pFlag-TIP30 mutants into pBabe Puro vector at BamHI sites.

Retroviral infection. HepG2 cells were purchased from the American Tissue Culture Collection. HepG2 cells were grown as described previously (13). Infectious retroviruses (pBabe-Puro-TIP30, TIP30G134V, and empty retroviral vector) were produced by transfection of retroplasmids into a packaging cell line, 293T. Supernatants from transfected packaging cells were collected 48 h after transfection, filtered through 0.45-μm filter, and used for infection. HepG2 cells were infected with viral supernatants supplemented with 10 μg/mL polybrene (Sigma). Two days later, infected cells were given puromycin (1 μg/mL) and selected for 3 days. Mixed populations of puromycin-resistant stable cells resulting from infection and puromycin selection were subjected to the assays described in the figures. Effective infection was confirmed by Western blot analysis.

Cell proliferation assays. For cell counting assay, cells were plated on a 24-well plate at 1.2 × 105 per well in 10% fetal bovine serum (FBS) DMEM medium. Viable cells identified by trypan blue exclusion were counted after each 24-h period. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were done as described (16), and cells were plated on a 96-well plate at 2,000 per well. Twenty microliters of MTT (5 mg/mL in PBS) stock solution were added to each well, and the plates were incubated for 4 h at 37°C. Then, 50 μL of triplex solution (10% SDS-5% isoprobutanol-0.012% N HCl) was added and the plates were incubated for 12 to 20 h at 37°C. Absorbance readings at 570 nm were determined using FLUOstar OPTIMA and POLARstar OPTIMA (BMG Labtech, Germany). Differences were analyzed by Student’s t test as described in the figure legends.

Anchorage-independent growth and cell invasion assays. For anchorage-independent growth, 5 × 104 cells were plated in 0.3% low melting point agarose/growth medium onto 60-mm dishes with a 0.65% agarose underlay. After 2 weeks, colonies that were ≥2 mm in diameter were counted. For cell invasion assay, cells in the density of 1.25 × 104 in 1 mL of 0.1% FBS DMEM were seeded on each upper chamber of the six-well Matrigel invasion chamber plate (Becton Dickinson). In the lower chamber, 1.5 mL of 16% FBS DMEM was added. After incubation at 37°C in 5% CO2 incubator for 24 h, the cells that remained on the upper chamber, 1.5 mL of 0.1% FBS DMEM was added. Twenty microliters of MTT stock solution were added to each well, and the plates were incubated for 4 h at 37°C. Then, 50 μL of triplex solution (10% SDS-5% isoprobutanol-0.012% N HCl) was added and the plates were incubated for 12 to 20 h at 37°C. Absorbance readings at 570 nm were determined using FLUOstar OPTIMA and POLARstar OPTIMA (BMG Labtech, Germany). Differences were analyzed by Student’s t test as described in the figure legends.

Detection and quantitation of apoptosis. Trypan blue exclusion was used to determine cell viability. Cells were plated on six-well plates at 2.5 × 105 per well treated with 6 μg/mL cisplatin in DMEM supplemented with 0.1% FBS. Viable cells identified by trypan blue exclusion were counted after 24 h. Apoptosis was examined by assessing nuclear changes indicative of apoptosis using the DNA-binding dye Hoechst 33342 (Molecular Probes). Cells were plated in 60-mm culture dishes at appropriate densities, exposed to 6 μg/mL cisplatin (Sigma) for 24 h in DMEM supplemented with 0.1% FBS, and then stained by Hoechst 33342 and examined by fluorescence microscopy (Nikon Eclipse E600). Annexin V–Cy3 apoptosis detection kit (Sigma) was used to further examine these cells. This kit detects early apoptotic cells stained with yellow [Annexin V positive, 6-carboxyfluorescein diacetate (CFDA) positive], dead cells stained with red (Annexin V positive, 6-CFDA negative), and viable cells stained with green (Annexin V negative, 6-CFDA positive).

Immunoblot analysis for mitochondrial cytochrome c release. Cells were grown in DMEM containing either 10% FBS or 0.1% FBS plus cisplatin for 24 h. Cytosolic fractions used for immunoblot analysis of cytochrome c were prepared using the method described by Yang et al. (17) and immunoblotted with mouse monoclonal anti-cytochrome c antibody (BD PharMingen) and mouse anti-β-actin antibody (Sigma).

Tumorigenicity in nude mice. Single-cell suspensions of each of the infected cell lines and control cell lines were trypsinized and collected. The cell viability was >95% as determined by trypan blue staining. Cells (1 × 107) in 0.1 mL of PBS were s.c. injected into the right and left back of 6-week-old female athymic NCr-nu/nu (National Cancer Institute Frederick). Five mice were injected for each cell line. Once palpable tumors were established, tumor volume measurements were taken every 3 days using calipers. Tumor volume was calculated as follows: V = (4/3)π × R1 × R2, where R1 and R2 are radius and R1 < R2. All of the animal experimentation was done according to the NIH guidelines in the University of Nebraska Medical Center.

Evaluation of proliferation and apoptosis in tumors. Two unainted tumor sections for each cell type were used for the analyses of proliferation and apoptosis in tumors. Ki67 expression was measured with Ki67 antigen kit (Novocastra, United Kingdom). Terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assay for apoptotic cells was done using the Apoptag Peroxidase In Situ Apoptosis Detection kit (Chemicon). Positive cells were counted from three arbitrary fields (500 total cells counted) under high power (×400). Differences were analyzed by Student’s t test and P values ≤ 0.05 were considered as significant.

RNA isolation and microarray analysis. Affymetrix U133 GeneChip (Affymetrix, Santa Clara, CA) arrays were used to study the differential gene expression between HepG2-TIP30G134V and HepG2-TIP30. Five micrograms of total RNA from the pool of three dishes of cultured cells, isolated with the Trizol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions, were used to prepare double-stranded cDNA. The cRNA was transcribed with biotin-labeled nucleotides (Enzo Diagnostics, New York, NY) and hybridized to microarray U133A (Affymetrix). Data were analyzed with Microarray Suite 5.0 (MASS) software (Affymetrix).

Quantitative real-time reverse transcription-PCR. Differential expression of selected genes between HepG2-TIP30 mutants and HepG2-TIP30 wild-type were confirmed by quantitative real-time reverse transcription-PCR (RT-PCR). Two micrograms of the DNA-free total RNA were reverse transcribed into cDNA using oligo(dT)12-18 and SuperScript II RT (Invitrogen) following manufacturer’s instruction. The same amounts of resulting cDNA were used for PCR amplification. The primers used for PCR and their sequences are as follows:

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); sense 5′-GAAGGT-GAAGGTCGGAGTCT-3′; antisense 5′-GAAGATGTTGATGGGATTCT-3′ (225 bp)

Myc: sense 5′-CGGTTGTGCAACAGAATCCTAGCAGAGG-3′; antisense 5′-CTCAGGCGTCCAGCCCTGTCATTAAAAG-3′ (307 bp)

TP53 sense 5′-GTCCAGAGTCTGAGTGGAGG-3′; antisense 5′-TCTGAGT-CAGGCCCTTCTGTG-3′ (159 bp)

E-cadherin sense 5′-TGCCCCAAGATAAAGG-3′; antisense: 5′-GTTGATGGCAGTCTGTTG-3′ (200 bp)

N-cadherin sense 5′-CCGGAACAGTCTCACAACC-3′; antisense: 5′-CCCAAAAGACGACAGTC-3′ (110 bp)

All real-time PCR reactions were done in a 25 μL mixture containing 1× reaction buffer (0.01 nmol of each primer, 0.25× SYBR green I, 200 μM/μL deoxynucleotide triphosphate, 2 μM/mL MgCl2, and 1.5 units of TaqDNA polymerase) using a smart cycle IIR PCR system (Cepheid). The following PCR program was used: 95°C for 150 s, then 40 cycles of denaturation at 95°C.
95°C for 15 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s, followed by a temperature gradient between 54°C and 95°C. The last step was for dissociation analysis of the PCR product to monitor the amplicon identity. Serial 10-fold dilutions of each vector (pTA-GAPDH, pTA-myc, pTA-N-cadherin, pTA-TIP3, and pTA-E-cadherin) were used as reference molecules for the standard curve calculations. Quantitative results were normalized for the level of GAPDH.

**Western blot analysis.** Extract was prepared as described (13). Cells were sonicated in buffer containing 50 mmol/L Tris-HCl (pH 7.4), 120 mmol/L NaCl, 1 mmol/L EDTA, and 1% NP40. One hundred micrograms of lysate were subjected to SDS-PAGE followed by Western blot analysis using antibodies for TIP30, TP33 (Sigma), N-cadherin, and E-cadherin (gifts from James Wahl) or β-actin (Sigma), respectively. Immunoblots were developed using ECL-enhanced chemiluminescence reagents (Perkin-Elmer Life Sciences).

**Small interfering RNA transfection.** N-cadherin small interfering RNA and nonspecific small interfering RNA (siRNA) were obtained from Dharmacon Research, Inc. (Dallas, TX). siRNAs were transfected with DharmaFECT 1 into HepG2-TIP30R106H cells at 10 nmol/L following manufacturer’s protocol. Forty-eight or 96 h after the transfection, the cells were harvested and subjected to Western blotting, cell proliferation, or apoptosis analysis as described above.

**Results**

**Mutant TIP30 promotes cell proliferation.** TIP30 G134V mutation (Fig. 1A) was identified from HLC472 specimen, but it was not detected in the adjacent tissues from the same HCC specimen, as described previously (13). TIP30 R106H was identified in HLC576 by using the same method. We have further confirmed that R106H mutation is present in the same HCC specimen by performing a mutant PCR amplification assay (see Supplementary Fig. S1). It was previously shown that G134V mutation shortens the half-life of TIP30 protein in cells, whereas R106H alters the subcellular distribution of TIP30, suggesting a possible disruption of TIP30 function by these mutations (13). To examine the effect of these TIP30 mutants on their function in cells, we infected HepG2 cells, lacking endogenous TIP30 expression, with retroviral vectors expressing wild-type TIP30, TIP30 mutants, and empty viral vector control. Cell viability and proliferation of HepG2 cells ectopically expressing TIP30 mutants (Fig. 1B) were analyzed by trypan blue exclusion assay and MTT. As shown in Fig. 1C, HepG2-TIP30 cells grew slower than HepG2-vector cells, showing a 43.5% decrease in total numbers after 7 days of growth (P < 0.01). Both of the HepG2-TIP30 mutant cells grew much faster than HepG2-vector cells, showing 60% to 86.5% increases in total numbers (P < 0.01). Consistent with the result from the trypan blue exclusion assay, MTT analysis of these cells also revealed that HepG2-TIP30R106H and HepG2-TIP30G134V cells had an increase growth (P < 0.001 and P < 0.05), whereas HepG2-TIP30 cells had a decrease in growth (P < 0.05) compared with HepG2-vector cells (Fig. 1D).

![Figure 1](https://example.com/figure1.png) **Figure 1.** TIP30 mutants promote the growth of HepG2 cells. A, schematic representation of the TIP30 gene. B, the levels of TIP30 and TIP30 mutants in virally infected HepG2 cells were examined by Western blot analysis with anti-TIP30 antibodies. β-Actin was used as a loading control. C, graph of growth curves of HepG2 cells expressing the indicated genes. Average numbers of viable HepG2 cells infected with a pBabe viral vector or retroviruses expressing the indicated genes were determined and counted with trypan blue staining. Points, mean of three independent experiments each done in triplicate. *, P ≤ 0.01 (TIP30R106H, TIP30G134V, or TIP30 versus pBabe). D, analysis of proliferation with MTT assay. Absorbance (O.D.) of 570 nm for each type of cultured HepG2 cells infected with the indicated genes was measured in triplicate. One of three representative experiments. *, P ≤ 0.05; **, P ≤ 0.001.
TIP30 mutants promote the growth of HepG2 cells, suggesting that TIP30R106H and TIP30G134V have gained oncogenic activities that are not possessed by wild-type TIP30.

Expression of mutant TIP30 in HepG2 cells promotes anchorage-independent growth and cell invasion. To assess whether TIP30 mutants affect anchorage-independent growth of HepG2 cells, retrovirus-infected cells were subjected to soft agar assays. As expected, whereas expression of wild-type TIP30 did not promote colony formation in soft agar compared with HepG2-vector cells, ectopic expression of TIP30 mutants resulted in a moderate increase (2- to 5-fold increase) in the number (Fig. 2A) and size of colonies (data not shown) compared with HepG2-vector cells. We next analyzed whether TIP30 mutants affected cell ability to invade through the basement membrane, an important component in the process of tumor invasion and metastasis. As shown in Fig. 2B, HepG2-TIP30 cells exhibited significantly less (55.7% decrease) invasion through Matrigel-coated filters than HepG2-vector cells. HepG2-TIP30R106H cells exhibited more invasion (41.4% increase) than vector control. However, TIP30G134V did not promote cell invasion.

Mutant TIP30 confers resistance to cisplatin-induced cell death on HCC cells in mitochondria-dependent pathway. Because HCC cells often develop resistance to chemotherapeutic drugs, we next investigated whether TIP30 mutants in HepG2 cells conferred resistance to cisplatin-induced apoptosis (Fig. 3A). Consistent with the role of TIP30 in lung cancer cells described previously (8), expression of TIP30 in HepG2 cells resulted in a 62% reduction of viable cells in comparison with control vector. In contrast, expression of TIP30 mutants protect cells from cisplatin-induced cell death as the numbers of viable HepG2-TIP30R106H and HepG2-TIP30G134V cells are increased by 2.24- and 2.78-fold relative to the number of HepG2-vector cells, respectively. We also observed that the numbers of viable cells were similar in all three groups of HepG2 cells without cisplatin treatment (data not shown). Inhibition of cisplatin-induced apoptosis by TIP30 mutants was confirmed by staining cells with Hoechst 33342 (Fig. 3B) or Annexin V–Cy3 combined with 6-CFDA (Fig. 3C). The numbers of dead cells (red) and early apoptotic cells (stain with both red and green) were significantly increased in HepG2-TIP30 cells treated with cisplatin in low serum medium compared with control HepG2-vector cells treated with cisplatin, whereas the numbers of dead cells and early apoptotic cells were slightly decreased in HepG2-TIP30R106H and HepG2-TIP30G134V cells treated with cisplatin in low serum medium (Fig. 3C). Taken together, these results indicate that expression of TIP30 mutants in HepG2 cells confers resistance to cisplatin-induced cell death, whereas expression of wild-type TIP30 sensitizes cells to cisplatin.

To explore if the action of TIP30 and its mutants are involved in a mitochondria-facilitated apoptotic pathway, we examined the presence of cytochrome c, an important apoptogenic factor in the mitochondria-dependent apoptosis pathway, in the cytosolic fraction of the cells. As shown in Fig. 3D, when HepG2 cells expressing TIP30R106H or wild-type TIP30 were grown in normal medium, cytochrome c release from mitochondria was not detected. After these treatments with cisplatin for 24 h, cytochrome c release from the mitochondria to the cytosol was significantly increased in HepG2-TIP30 cells relative to HepG2-vector control. In contrast, expression of TIP30R106H inhibited cytochrome c release, suggesting that wild-type TIP30 enhances mitochondria-dependent apoptosis, whereas TIP30 mutants can inhibit cisplatin-induced cytochrome c release.

TIP30R106H can dramatically accelerate tumorigenesis in immunodeficient mice. Having observed that TIP30 mutants can promote cell growth and reduce cell death, we then sought to assess the oncogenic potential of TIP30 mutant cells. HepG2-pBabe, HepG2-TIP30, HepG2-TIP30G134V, and HepG2-TIP30R106H cells were injected s.c. into the back of nude mice and monitored them for tumor growth. Mice injected with HepG2 cells containing pBabe vector control exhibited exponential tumor growth after 15 days with an average volume of 590 mm$^3$ and average size of 265 mg by day 30. In contrast, all mice injected with HepG2-TIP30R106H cells exhibited significantly tumor growth after only 7 days, with an average volume of 1,167 mm$^3$ and average size of 375 mg by day 15, indicating that TIP30R106H dramatically accelerated tumor formation. As expected, wild-type TIP30 exhibited a substantial decrease in tumorigenicity with an average volume of 280 mm$^3$ and average size of 150 mg by day 41. Expression of TIP30G134V in HepG2 cells did not promote in vivo tumorigenicity compared with HepG2 vector controls. However, the average volume and size of tumors generated by TIP30G134V cells were still substantially greater than tumors formed by HepG2-TIP30 cells. Thus, these results suggest that TIP30G134V has lost the function of tumor suppression possibly because its half-life is shortened in cells (13) and TIP30R106H has gained a tumor-promoting function. To determine the effects of TIP30 and TIP30R106H on proliferation and apoptosis in vivo, we
analyzed proliferative and apoptotic cells in tumor sections by immunostaining for the proliferation marker Ki67 and for apoptosis using the TUNEL assay. We found that 33.73 ± 7.43% of HepG2-TIP30R106H cells expressed Ki67, whereas only 0.57 ± 0.27% of HepG2-TIP30 cells and 10.87 ± 7.71% of HepG2-pBabe cells expressed Ki67 (Fig. 4C). The mean value of the proliferation index in HepG2-TIP30R106H tumors was much higher than that in HepG2-TIP30 tumors (P = 0.0001) and HepG2-pBabe tumors (P = 0.0004). In contrast, TUNEL assay revealed no significant difference in apoptosis between the tumors of HepG2-pBabe and HepG2-TIP30R106H or HepG2-TIP30 cells (Fig. 4D). These results further suggest that TIP30R106H is able to promote proliferation of HepG2 cells in vivo.

**Mutant TIP30 can up-regulate expression of N-cadherin.** To further investigate the molecular basis of the action of TIP30R106H, we initially used Affymetrix GeneChip to examine differential gene expression between HepG2-vector, HepG2-TIP30R106H, and HepG2-wild-type TIP30. We observed that the expression of 51 genes was increased 1.7-fold or greater in HepG2-TIP30R106H compared with HepG2-TIP30 (described in Supplementary Data). Eleven are metastasis-related genes or angiogenic inducers, including S100A4, Cyr61, MMP3, N-cadherin, EVA1, ITGA6, and oncogenes/proto-oncogenes, including PIM1, FYN, AXL, PTTG1, as well as tumor markers such as TMSB10. There were also 25 genes whose expression was decreased 1.7-fold or greater in HepG2-TIP30R106H compared with HepG2-TIP30. These included tumor suppressor genes TP53 and E-cadherin, as well as putative tumor suppressors Septin1, A1M, and Pcgf2. Because DNA microarray analysis may underestimate changes in gene expression, and because the promoter of c-MYC gene was previously shown as a direct target of TIP30 in breast cancer cells (14), we selectively analyzed the expression of TP53, c-MYC, E-cadherin, and N-cadherin in HepG2-TIP30R106H mutant and HepG2-wild-type TIP30 cells by real-time RT-PCR and Western blot analysis. As shown in Fig. 5A, expression of either TIP30R106H in HepG2 cells resulted in decreases in mRNA levels of TP53 and E-cadherin and increases in mRNA levels of

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**Figure 3.** TIP30 mutants inhibit HepG2 cells sensitivity to cisplatin. A, trypan blue exclusion assay of cells treated with cisplatin. Average numbers of viable HepG2 cells infected with a pBabe viral vector or retroviruses expressing the indicated genes were determined and counted with trypan blue staining. Columns, mean of three independent experiments each done in triplicate. *, P < 0.05 (TIP30R106H or TIP30G134V versus pBabe). B, apoptosis in the same series of cells was determined with Hoechst staining. Photographs are representative microscopic fields from each population of HepG2 cell expressing the indicated genes after Hoechst staining. C, apoptosis in the same series of cells was determined using Annexin V–Cy3 apoptosis detection kit. Photographs are representative microscopic fields from each type of HepG2 cells expressing the indicated genes after Annexin V–Cy3 staining. Cells stained green were viable; cells stained red were dead; cells stained both green and red were early apoptotic. D, analysis of cytochrome c levels in the cytosol. The cytosolic fractions from HepG2-pBabe (lane 1), HepG2-TIP30 (lane 2), or HepG2-TIP30R106H (lane 3) cells treated with (right) or without (left) cisplatin as indicated were immunoblotted with anti–cytochrome c (Cyto C) antibodies. h-Actin was used as a loading control.
c-MYC and N-cadherin compared with expression of wild-type TIP30. Consistent with this, HepG2 expressing wild-type TIP30 had higher mRNA levels of TP53 and E-cadherin than the parent cells containing the control vector.

In agreement with Affymetrix GeneChip and quantitative RT-PCR studies, Western blot analysis also showed that TIP30R106H increase expression of N-cadherin and decrease expression of E-cadherin and TP53 compared with wild-type TIP30 (Fig. 5B). Interestingly, we noticed that the mRNA level of E-cadherin in TIP30 mutant cells was higher than that in the control cells without TIP30 in both Affymetrix array and quantitative PCR assays. However, E-cadherin protein was undetected in TIP30R106H cells, suggesting that inhibition of E-cadherin induced by TIP30R106H is likely at the level of posttranscription (Fig. 5B). Together, our data suggest that TIP30R106H increase expression of c-MYC and N-cadherin, and decrease expression of TP53 and E-cadherin.

Down-regulation of N-cadherin in cells expressing TIP30R106H led to a profound inhibition in cell viability associated with apoptosis. Because N-cadherin expression was significantly increased in HepG2-TIP30R106H cells and because its role in tumorigenesis is known, we selected specific siRNA to down-regulate N-cadherin expression in HepG2-TIP30R106H cells to further investigate the mechanism in which TIP30R106H promotes the tumorigenesis in HepG2 cells. As shown in Fig. 6A, N-cadherin protein was dramatically knocked down after siRNA treatment, compared with the untreated or nonspecific siRNA treatment. Cell viability was measured with trypan blue exclusion assay as described. As shown in Fig. 6B, cells with N-cadherin siRNA treatment had significantly decreased viability compared with cells with nonspecific siRNA treatment. As expected, there was no difference in viability between cells without siRNA treatment and cells with nonspecific siRNA treatment. This result suggests that the effect of TIP30R106H on cell growth is mediated, at least, partially through up-regulation of N-cadherin.

To determine whether increased N-cadherin expression in HepG2-TIP30R106H cells also conferred resistance of cells to cisplatin, we treated these siRNA treatment cells with 6 μg/mL cisplatin for 24 h and stained with Hoechst 33342. As shown in Fig. 6C, cells with down-regulation of N-cadherin expression were more sensitive to cisplatin-induced apoptosis compared with control or no-siRNA treatment cells; it showed a 50% increase of apoptotic cells. Thus, blocking of N-cadherin by siRNA in HepG2-TIP30R106H cells can sensitize the cells to cisplatin-induced apoptosis, thereby conferring HepG2 cells resistance to cisplatin-induced apoptosis.

Discussion

Human cancer is a complex disease that often progresses by accumulation of genetic abnormalities in somatic cells, allowing...
them to gain growth advantages and escape from control mechanisms involved in cell cycle or apoptosis. It is well known that HCC cells often contain the alterations in many cellular genes involved in the control of carcinogenic pathways. These include activation of oncogenes, mutations in tumor-suppressor genes, and changes in expression of hepatocyte growth factors and protease and matrix metalloproteinases. However, the mechanisms by which these factors contribute to the development and progression of HCCs remain exclusive. Three lines of evidence support the hypothesis that TIP30 mutants play important roles in hepatocellular carcinogenesis. First, in our previous study, aberrant expression of TIP30 and mutations in the TIP30 gene were frequently detected in human HCC specimens. Second, we showed previously that loss of TIP30 increases the incidence of HCCs in mice. In the present study, we show that these identified mutations in the TIP30 gene can abolish the tumor-suppressive activities of wild-type TIP30. Importantly, we show here that TIP30 mutants have gain of functions that include the enhancement of cell proliferation, the conferment of cisplatin resistance to cancer cells, and the promotion of tumor formation in vivo by ectopic expression of TIP30 mutants in a HCC cell line that lacks expression of endogenous TIP30. Finally, we show that N-cadherin is at least partly responsible for increasing the proliferation of HCC cells expressing a tumor-promoting TIP30 mutant. Together, our data provide the first demonstration that somatic missense mutations in the TIP30 gene, which occurred in HCC cells, can convert TIP30, a tumor suppressor, to an oncogenic protein by conferring oncogenic activities on TIP30 and subsequently promote carcinogenesis. A similar scenario has been previously observed for TP53, which normally acts as a tumor suppressor to inhibit tumorigenesis; it is converted to an oncoprotein by mutations in the development or progression of tumors (19). Therefore, our discovery further supports the hypothesis that mutations in tumor-suppressor genes play important roles in the development and progression of human cancer.

TIP30, also called CC3, was initially characterized as a putative metastasis suppressor in lung cancer cells by sensitizing lung carcinoma cells to apoptosis (6). Available evidence have established that TIP30 acts as a transcriptional cofactor in lung and breast cancer cells to regulate expression of genes involved in apoptosis and cell growth (8, 14). In the present study, we used Affymetrix arrays and quantitative RT-PCR to identify genes that were differentially expressed in the isogenic HCC cell lines HepG2-vector, HepG2-TIP30, and HepG2-TIP30R106H. Subsequently,
alteration of a subset of oncogenes and tumor-suppressor genes that have been previously shown to promote cell proliferation and carcinogenesis were identified in HepG2-TIP30R106H cells. Based on the functions of these genes in the literature, we hypothesized that some of them may have facilitated TIP30 mutant–induced tumor growth. Among these genes, we selected N-cadherin for further testing because it is significantly elevated in both mRNA and protein levels in TIP30 mutant cells. In addition, recent evidence indicates that N-cadherin is dominant over E-cadherin in metastatic progression and is overexpressed in a subset of cancer types in addition to the loss of E-cadherin (20, 21). It was proposed that switching expression from E-cadherin to N-cadherin in tumor cells can enhance invasive and survival capabilities of tumor cells and also can promote cooperation between tumor cells and the surrounding microenvironment, a critical event in metastatic progression. Indeed, up-regulation of N-cadherin could confer breast tumor cells more resistant to apoptotic stimuli and more motile, invasive, and metastatic in nude mice (22, 23). Consistent with previous studies on the role of N-cadherin in prostate and melanoma cells (24, 25), our results show that reduction of expression of N-cadherin in HepG2-TIP30 mutant cells attenuates TIP30 mutant–induced cell proliferation and sensitizes the apoptotic response of cells to cisplatin. These findings thus support a hypothesis that TIP30 mutants contribute to the pathogenesis of HCC partially through up-regulation of N-cadherin.

In summary, the data presented here suggest that TIP30 mutants have gain of functions to promote cell growth, resistance to cisplatin-induced cell death, and tumorigenesis, most likely due to the up-regulation of N-cadherin expression. Thus, TIP30 and TIP30 mutants play important roles in the development and progression of HCC. TIP30 mutants may provide specific and effective targets for therapeutic intervention in HCC patients that express mutant TIP30.

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**Figure 6.** Down-regulation of N-cadherin in HepG2-TIP30R106H cells leads to decreased cell survival and increased apoptosis. HepG2-TIP30R106H cells were plated in a six-well plate (A) and a 12-well plate (B and C) and transfected with N-cadherin–specific siRNA (siN-cadherin) or nonspecific siRNA controls (siCTRL) 48 h after the transfection. A, immunoblots showing protein levels for N-cadherin and actin in HepG2-TIP30R106H cells following siRNA. B, effect of down-regulation of N-cadherin expression on HepG2-TIP30R106H cells. The number of viable cells was counted every 2 d after transfection. Points, mean of two experiments done in triplicate; bars, SD. *, P < 0.05 (siN-cadherin versus siCTRL). C, down-regulation of N-cadherin sensitizes HepG2-TIP30R106H cells to cisplatin-induced apoptosis. Cells transfected with or without RNA interference were also treated with 6 μg/mL for 24 h and stained with Hoechst 33342. About 500 cells from five random fields were counted. Results are presented as the percentage of apoptotic cells over viable cells. Columns, mean of two experiments done in triplicate; bars, SD. *, P < 0.05 (siN-cadherin versus siCTRL).
References

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