TIP30 Deficiency Increases Susceptibility to Tumorigenesis

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ABSTRACT

TIP30, also called CC3 or Htatip2, is a putative metastasis suppressor that promotes apoptosis and inhibits angiogenesis. Although TIP30 has several characteristic features of a tumor suppressor in vitro analyses, tumor development as a result of TIP30 inactivation has not been demonstrated in vivo, and abnormal expression of TIP30 in human cancer has not been reported. Using genetically engineered mice and cells deficient in TIP30, we show that TIP30-deficient mice have a high incidence of hepatocellular carcinoma and other tumors, and loss of TIP30 enhances susceptibility of fibroblasts to transformation by the SV40 large T antigen. Furthermore, immunohistochemical analysis indicates that reduced TIP30 expression is associated with 33% of human hepatocellular carcinomas. Some of these carcinomas harbor missense mutations in the TIP30 gene, which cause abnormal expression of TIP30. Together, these results demonstrate that the Tip30 gene is a tumor susceptibility gene playing an important role in the suppression of hepatocarcinogenesis.

INTRODUCTION

HIV-1 Tat-interacting protein TIP30, identical to the putative metastasis suppressor CC3, has been implicated in the regulation of tumor cell growth and metastasis (1–4). Recent experiments demonstrated that a reduced level of TIP30 mRNA has been found in a number of tumor cell lines that include variant-small cell lung carcinoma (V-SCLC), classic-SCLC, neuroblastoma, colon cancer, and melanoma cell lines (2–6). Ectopic expression of the TIP30/CC3 gene in a variant SCLC line lacking TIP30/CC3 expression led to a significant suppression of the metastatic potential of these cells in SCID-hu mice. In addition, metastases in melanoma-bearing mice are significantly reduced after i.v. treatment with the TIP30/CC3 gene-cationic liposome-DNA complex (6). Subsequent work has led to the proposal that the suppression of cell growth and metastasis by ectopic expression of TIP30 may result, in part, from a propensity of cells to undergo apoptosis (2–4).

TIP30 was believed to be a transcription cofactor because it was demonstrated to potentiate Tat-mediated transcription in cooperation with components of elongation factor P-TEFb in transient transfection assays and is essential for Tat-mediated transcription in cell-free transcription assays (1, 3). TIP30 displays a serine-threonine kinase activity that can phosphorylate the carboxyl terminal domain of RNA polymerase II in a Tat-dependent manner in vitro (3). The intrinsic kinase activity is essential for TIP30 to enhance Tat-activated transcription and to sensitize NIH3T3 and v-SCLC cells to apoptosis (3). Consistent with the role of TIP30 in the suppression of tumor growth and metastasis via transcription mechanisms, studies revealed that the ectopic expression of TIP30 in v-SCLC cells and other tumor cell lines up-regulates the expression of proapoptotic factors (3) and angiogenic inhibitors, and down-regulates expression of angiogenic stimulators (4). Therefore, TIP30 may function as a tumor suppressor or tumor modifier that controls expression of genes involved in tumor growth and metastasis.

Although TIP30 has several characteristic features of a tumor suppressor, tumor development as a result of TIP30 inactivation has not been established in vivo, and abnormal expression of TIP30 in human cancer has not been reported. In this study, the generation of mice lacking TIP30 has allowed us to demonstrate a role of TIP30 in the suppression of tumor development in vivo. In addition, we have identified missense mutations in the TIP30 gene in the clinical human hepatocellular carcinomas (HCCs), which resulted in aberrant expression of TIP30.

MATERIALS AND METHODS

Constructions of the Tip30 Targeting Vector and TIP30-Expressing Plasmids. To isolate the mouse Tip30 gene, a mouse 129Sv1 genomic library (Stratagene, La Jolla, CA) was screened with a mouse Tip30 cDNA probe. Fourteen overlapping clones contained a 49-kb genomic region that included five coding exons of the Tip30 gene locus. The HindIII-SacI 2.7-kb genomic fragment was replaced by LacZ in-frame and a phosphoglycerate kinase neo-cassette (Fig. 1A). This replacement ablated the two exons that encode the NH2-terminal portion of Tip30, except for 10 amino acids after the translation start site. The targeting vector (7) included a 5.2-kb upstream homologous liposome-DNA complex (7). Subsequent work has led to the proposal that the suppression of cell growth and metastasis by ectopic expression of TIP30 may result, in part, from a propensity of cells to undergo apoptosis (2–4).

TIP30 was believed to be a transcription cofactor because it was demonstrated to potentiate Tat-mediated transcription in cooperation with components of elongation factor P-TEFb in transient transfection assays and is essential for Tat-mediated transcription in cell-free transcription assays (1, 3). TIP30 displays a serine-threonine kinase activity that can phosphorylate the carboxyl terminal domain of RNA polymerase II in a Tat-dependent manner in vitro (3). The intrinsic kinase activity is essential for TIP30 to enhance Tat-activated tran-
heterozygous crossings of mice that had been backcrossed 10 times with C57BL6/J mice.

**Northern Blot Analysis.** Northern blot analysis was performed as described previously (7). The full-length Tip30 cDNA was used as a probe, and labeled with [α-32P]dCTP and a random primer DNA labeling system (Invitrogen, Carlsbad, CA).

**Growth and Soft Agar Assays of Immortalized MEFs.** Primary Tip30+/−, Tip30−/−, and Tip30−/− MEFs in passage 2 were used to introduce the SV40 T-antigen expression vector by LipofectAMINE reagent (Invitrogen) and be immortalized after 20 passages. For growth curves, immortalized cells (10^5 per well of 12-well plates) were seeded, and cells were stained with trypan blue and counted daily. For soft agar assays, immortalized MEFs (5 × 10^5) were grown in DMEM supplemented with 10% fetal bovine serum and 0.53% agarose on top of a layer containing 0.4% agarose for 21 days. The clones were stained with crystal violet, and the clones with diameters >1 mm were counted.

**Histology and Immunohistochemistry.** Tissues were fixed with 10% buffered-formalin and embedded in paraffin blocks. Tissue sections were deparaffinized, rehydrated, and stained with H&E. For immunohistochemistry, rehydrated sections were incubated overnight at 4°C, rehydrated sections were incubated overnight at 72°C, and 72°C for 30 s; 60°C were carried out for 25 cycles of 94°C, 30 s; 60°C, 30 s; and 72°C, 30 s. The PCR products were incubated with Taq polymerase for 10 min at 72°C for adding A to the ends, and purified with Qiagen PCR purification columns (Qiagen, Valencia, CA), then cloned to the TA vector pCR2.1 (Invitrogen). Ten clones for each sample were sequenced for both DNA strands by the Eppeley core facility at the University of Nebraska Medical Center using M13 reversal and universal primers. At least three clones containing identical mutations were sequenced for each patient. The HepG2 cell line was purchased from the American Type Culture Collection (Manassas, VA). Experiments in this study have been approved by the Institutional Review Board for the protection of human subjects at the University of Nebraska Medical Center.

**RESULTS AND DISCUSSION**

To investigate the roles of Tip30 in both cultured cells and living animals, we generated mice carrying inactivated Tip30 gene by homologous recombination. ES cell clones with a disrupted Tip30 locus were used to obtain germ-line chimeras that, in turn, were used to generate heterozygous F1 mutant 129SvJ/C57BL6Jd hybrid mice (Fig. 1A). Crosses between mice heterozygous for the Tip30 gene led to the expected Mendelian ratios of live-born progeny, thus indicating that the Tip30 gene is not essential for development. The loss of Tip30 expression was confirmed by Southern blot analyses of tail genomic DNAs, and by Northern blot analyses of liver and stomach total RNAs (Fig. 1, B and C). Histological examinations of the Tip30−/− mice before the age of 1 year did not reveal obvious gross alterations or developmental abnormalities in organs other than the mammary glands. Both Tip30−/− males and females were fertile and generated normal litter sizes.

To determine whether Tip30 is a tumor suppressor, we have kept a cohort of Tip30-deficient and wild-type animals (50% of C57BL6J and 50% 129SvJ of genetic background) for long-term observation of the development of spontaneous malignancy. At the age of 18–20 months, 31 females and 16 males of the F2 littermates were sacrificed. Autopsy and histological analyses revealed tumor development in 9 of 18 (50%) Tip30-deficient female mice and 2 of 9 Tip30-deficient male mice. In contrast, none of the 13 female and 7 male wild-type mice displayed tumors (Table 1A; wild-type mice versus mutant mice; P < 0.005; X^2 = 11.3). The spectrum of tumor types in Tip30-mutant mice (Table 1B) is distinct from that seen in older wild-type mice described by others (10–12), as many of the tumors found in Tip30-deficient mice were carcinomas. Among those tumors, HCC showed the highest incidence, constituting 30% of the total tumors. The relatively high incidence of HCC indicates that Tip30 has a prevalent role in tumor suppression in hepatocytes. Interestingly, 1 Tip30−/− mouse had two tumors, a lipoid cell tumor at the right ovary and a neuroblastoma that arose at the right adrenal gland, with multiple metastases to tissues that included spleen, thymus, and salivary glands. The metastases were also found in the neck, mesentery, and mediastinum. In addition, 1 mouse developed a transitional cell carcinoma in the urinary bladder, and exhibited metastases along the epithelium that involved the ureters and kidneys, whereas 2 male mice

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5 Jill Pecha and Hua Xiao, unpublished observations.
developed sarcomas that metastasized to the liver, spleen, and pancreas. These data demonstrate that TIP30-deficient mice are prone to tumor development.

To reveal the basis for the susceptibility of Tip30-mutant mice to tumor development, we analyzed Tip30 wild-type and mutant MEFs. In an examination of the growth rates of MEFs, no significant difference was detected (data not shown). We then asked if TIP30-deficient MEFs may be more susceptible to transformation by SV40 large T antigen, which is able to inactivate p53 and retinoblastoma protein (reviewed in Ref. 13). These cells were immortalized by ectopic expression of the T antigen, and growth rates were measured. Immortalized Tip30+/− and Tip30+/+ MEFs grew much faster than immortalized Tip30+/+ MEFs (Fig. 2A). Tip30−/− and Tip30+/− MEFs also grew in a more disorganized fashion than Tip30+/+ MEFs (data not shown). Therefore, we assessed the ability of each type of MEF to grow in soft agar. Tip30+−/− and Tip30+/−/− MEFs showed a marked increase in the number and size of colonies as compared with Tip30+/−/− MEFs (Fig. 2, B and C). Western blot analysis revealed similar levels of T-antigen expression in Tip30+/−/− and Tip30+/−/− MEFs (Fig. 2D), and slightly higher expression of T antigen in Tip30+/−/− MEFs. These results demonstrate that a lack of TIP30 in cells enhances transformation by the SV40 T antigen.

To determine whether our studies in mice are relevant to human disease, we analyzed 24 surgical specimens of human HCC and compared the expression of TIP30 in cancerous cells with expression in adjacent benign hepatocytes on formalin-fixed, paraffin-embedded tissues with antihuman TIP30 antibody (1). Undetectable or significantly decreased TIP30 expression was found in cancerous cells in 8 specimens (33%) in comparison with the adjacent tissues. An example of the immunohistochemical analyses is shown (Fig. 3A). These data indicate that abnormal expression of TIP30 is implicated in human HCC. We next sought to examine whether these HCC cells harbor mutations in the TIP30 gene. Because the SNP database has listed four single nucleotide polymorphisms (SNPs) in the TIP30 coding regions, we first compared the TIP30 cDNA sequences in National Center for Biotechnology Information databases (14) to identify bp changes other than these SNPs in the human TIP30 coding region. Initially, we examined the sequences of TIP30 cDNA clones to identify insertion, deletion, or bp changes that will result in amino acid substitution. Besides those reported SNPs in the TIP30 gene that were found in both normal and cancer cells, we only identify missense mutations in exon 3. We found that none of the normal cells in the database (0 of 75) harbored a single base-pair substitution in exon 3, and 24% of various types of cancer cells (21 of 52) harbored at least a single base-pair substitution in the same region. Interestingly, the amino acid sequences encoded by Tip30 exon 3 are conserved among human, mouse, and Caenorhabditis elegans TIP30 proteins, and this region is important for the nuclear localization of TIP30. Therefore, we focused our mutational study on Tip30 exon 3 for those liver cancer patients who have abnormal TIP30 expression in their cancer cells. Three of 8 cancer samples contained at least a single bp change in the Tip30 gene (Table 1). These bp changes were not found in normal cells according to the National Center for Biotechnology Information database. In addition, whereas analyzing the sequences of cloned exon 3 DNA, we found that some of clones (20–80%) from the same cancer sample did not have any bp change, and individual clones from 1 HCC even harbored different bp changes. Importantly, we noted that 2 patients even had the same mutations in their carcinoma cells. Therefore, it is very likely that these base changes are somatic mutations that occurred during cancer development.

To explore the functional consequences of these amino acid substitutions in TIP30, we first investigated whether these mutations affected expression of TIP30 in cells. We transiently transfected a HCC cell line, HepG2, with mammalian expression plasmids encoding wild-type TIP30 and a mutant form of TIP30 with substitutions in G134V (TIP30G134V) identified from the HCC showing in Fig. 3A. On Western blot analysis showed in Fig. 3B,

![Fig. 2. Deletion of the TIP30 gene enhances fibroblast transformation by the SV40 large T antigen. A, Tip30+/− and Tip30+/+ mouse embryonic fibroblasts (MEFs) proliferate faster than wild-type MEFs after immortalization by SV40 T antigen. Immortalized Tip30+/−, Tip30+/−, and Tip30−/− MEFs were counted each day. Each value represents the mean of a representative experiment performed in triplicate. B, T antigen-immortalized Tip30+/− and Tip30+/+ MEFs develop more colony foci than Tip30−/− immortalized MEFs in soft agars. Each value represents the mean of a representative experiment performed in triplicate; bars, ± SD. C, an example of a colony from each MEF genotype is shown. D, Western blot analysis of T-antigen expression in Tip30+/−, Tip30+/−, and Tip30−/− MEFs. Equivalent amounts of whole cell extracts from each of the indicated MEFs were analyzed by immunoblotting with an anti-T-antigen monoclonal antibody and antitubulin monoclonal antibody.

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Table 1 TIP30

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tumor rate</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tip30+/−/−</td>
<td>0/20 (0 %)</td>
<td>0/13 (0 %)</td>
<td>0/7 (0 %)</td>
<td></td>
</tr>
<tr>
<td>Tip30+/− or −/−</td>
<td>11/27 (41%)</td>
<td>9/18 (50 %)</td>
<td>2/22 (22 %)</td>
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</tr>
<tr>
<td>Tip30+/−</td>
<td>7/13</td>
<td>6/8</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>Tip30−/−</td>
<td>4/13</td>
<td>3/10</td>
<td>1/3</td>
<td></td>
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</table>

B. Spectrum of tumors in Tip30-deficient mice

<table>
<thead>
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<th>Sex</th>
<th>Genotype</th>
<th>Histological type</th>
<th>Anatomic site</th>
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</thead>
<tbody>
<tr>
<td>F</td>
<td>+/−</td>
<td>Hepatocellular carcinoma</td>
<td>Liver</td>
</tr>
<tr>
<td>F</td>
<td>+/−</td>
<td>Hepatocellular carcinoma</td>
<td>Liver</td>
</tr>
<tr>
<td>F</td>
<td>−/−</td>
<td>Hepatocellular carcinoma</td>
<td>Liver</td>
</tr>
<tr>
<td>F</td>
<td>+/−</td>
<td>Thymoma</td>
<td>Thymus</td>
</tr>
<tr>
<td>F</td>
<td>+/−</td>
<td>Transitional cell carcinoma, grade III</td>
<td>Ureter, bladder, renal pelvis</td>
</tr>
<tr>
<td>F</td>
<td>+/−</td>
<td>Neuroblastoma lipid cell tumor</td>
<td>Adrenal gland ovari</td>
</tr>
<tr>
<td>F</td>
<td>−/−</td>
<td>Adenocarcinoma</td>
<td>Duodenum</td>
</tr>
<tr>
<td>F</td>
<td>−/−</td>
<td>Leiomyoma</td>
<td>Uterus</td>
</tr>
<tr>
<td>M</td>
<td>+/−</td>
<td>Hemangiosarcoma</td>
<td>Retropertioneum, liver, spleen, subcutis</td>
</tr>
<tr>
<td>M</td>
<td>−/−</td>
<td>Undifferentiated sarcoma</td>
<td>Retropertioneum, liver, spleen, pancreas</td>
</tr>
</tbody>
</table>

C. Amino acid substitutions in TIP30

<table>
<thead>
<tr>
<th>Name</th>
<th>Codon/nucleotide</th>
<th>Base change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLC 472</td>
<td>134/341</td>
<td>G → T</td>
<td>Gly → Val</td>
</tr>
<tr>
<td>HLC 485</td>
<td>109/325</td>
<td>C → T</td>
<td>Arg → stop</td>
</tr>
<tr>
<td>106/316</td>
<td>115/344</td>
<td>C → A</td>
<td>Arg → Ser</td>
</tr>
<tr>
<td>106/316</td>
<td>108/324</td>
<td>C → A</td>
<td>Arg → Ser</td>
</tr>
<tr>
<td>116/346</td>
<td>144/430</td>
<td>C → A</td>
<td>Leu → Ile</td>
</tr>
</tbody>
</table>

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* C. Jiang and H. Xiao, unpublished observations.
TIP30G134V was less expressed compared with the level of the wild-type TIP30. We then investigated whether this mutation affects the stability of the TIP30 protein in HepG2 cells. The relative level of TIP30 was monitored after addition of cycloheximide, a protein synthesis inhibitor. As shown in Fig. 3, C and D, G134V substitution markedly reduces the half-life of TIP30. This result suggests that G134V substitution reduces the stability of TIP30, thereby resulting in a lower level of TIP30 in cells. To investigate whether mutations affect the cellular localization of TIP30, we transfected HepG2 cells with mammalian expression vectors encoding GFP-TIP30 and mutant fusion proteins (GFP-TIP30R106H and GFP-TIP30G134V). The GFP tag had no influence on the cellular distribution of TIP30, because GFP-TIP30 had a subcellular distribution similar to endogenous TIP30 in HeLa and MCF7 cells (data not shown). In cells expressing wild-type GFP-TIP30 (Fig. 3E, panel 1), ~90% of transfected cells contained GFP-TIP30 in the cytoplasm, and 10% of transfected cells expressed GFP-TIP30 in the nucleus. When cells were treated with LMB, a CRM1-dependent nuclear export inhibitor (15), nuclear localization of GFP-TIP30 (Fig. 3E, panel 2) was increased to ~30% of transfected cells, suggesting that TIP30 is exported to the cytoplasm from the nucleus. However, cells expressing GFP-TIP30R106H showed an exclusively cytoplasmic staining (Fig. 3E, panel 5), and LMB treatment did not increase the nuclear localization of GFP-TIP30R106H (Fig. 3E, panel 6). This indicates that the R106H change may abrogate nuclear localization of TIP30 through inhibition of nuclear import. In contrast, GFP-TIP30G134V (Fig. 3E, panel 3) was expressed in both the cytoplasm and nucleus similar to the expression of GFP alone, suggesting that this mutation may affect its nuclear export. However, LMB treatment did not increase accumulation of GFP-TIP30G134V in the nucleus (Fig. 3E, panel 4), suggesting that the mutation may affect the nuclear import of TIP30. Therefore, it is possible that the mutation alters the protein structure, and results in abnormal nuclear import and export of TIP30. Given that TIP30 acts as a transcription coactivator in the nucleus and predisposes cells to apoptosis (1–5), it is conceivable that these mutations may impair the ability of TIP30 to regulate gene expression and apoptosis. Therefore, it is possible that TIP30 deficiency as a result of mutations in the TIP30 gene may inhibit its function in the liver cells and contribute to the pathogenesis of human HCC.

Cellular gene products can function as tumor suppressors or tumor modifiers to regulate tumorigenesis (16, 17). In this study, we showed that TIP30-deficient mice spontaneously develop tumors in their second year of life. Some tumors were carcinomas such as HCC that rarely occurs in 129Sv/J/C57BL6/J mice (18). In addition, 1 Tip30 mutant mouse even had two different types of tumor. These data clearly represent a significant enhancement of susceptibility to tumorigenesis in TIP30-deficient mice. The same incidences of tumors in Tip30−/−/ and Tip30−/− mice is similar to the observations described in previous studies with DMP1, an Arf transcription activator, and p27kip1, a potent tumor suppressor, that are haploinsufficient for tumor suppression (10, 19). We found that TIP30 mRNA and protein were expressed in both primary and metastatic sites of the neuroblastoma and hemangiosarcoma arisen in Tip30−/− mice by reverse transcription-PCR and immunohistochemical analyses (data not shown). Although we are not sure that the second allele in the tumors of Tip30−/− mice encodes a normal TIP30, based on the similar tumor incidence and latency in Tip30−/− and Tip30−/− mice, we suggest that TIP30 is haploinsufficient for tumor suppression.

The role of TIP30 in tumorigenesis is also suggested by the observation of reduced expression of TIP30 in human HCC and Tip30 mutations in those HCC. One mutation results in significantly decreased expression of TIP30, and another mutation
changes cellular localization of TIP30. Therefore, it is possible that Tip30 deficiency as a result of mutations in the Tip30 gene may contribute to the pathogenesis of human HCC. Together, these results strengthen a conclusion that TIP30 plays an important role in tumorigenesis.

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