# Wilms' Tumor 1 Gene Expression in Hepatocellular Carcinoma Promotes Cell Dedifferentiation and Resistance to Chemotherapy

**Maria J. Perugorria,[1] Josefa Castillo,[1] M. Ujue Latasa,[1] Saioa Goñi,[1] Victor Segura,[2] Bruno Sangro,[3] Jesús Prieto,[1,3] Matías A. Avila,[1] and Carmen Berasain[1]**

[1]Division of Hepatology and Gene Therapy, and [2]Genomics, Proteomics and Bioinformatics Unit. CIMA and [3]CIBERehd, University Clinic, University of Navarra, Pamplona, Spain

## Abstract

**The *Wilms' tumor 1* gene (*WT1*) encodes a transcription factor involved in cell growth and development. As we previously reported, WT1 expression is hardly detectable in normal hepatic tissue but is induced in liver cirrhosis. Although WT1 has been found to be overexpressed in a number of malignancies, the role of WT1 in hepatocarcinogenesis has not been clarified. We found that WT1 is expressed in several human hepatocellular carcinoma (HCC) cell lines, including PLC/PRF/5 and HepG2, and in HCC tumor tissue in 42% of patients. WT1 small interfering RNAs did not affect proliferation rate of HCC cells but abrogated their resistance to anoikis. Transcriptome analysis of PLC/PRF/5 cells after WT1 knockdown showed up-regulation of 251 genes and down-regulation of 321. Ninety percent of the former corresponded to metabolic genes, mostly those characterizing the mature hepatocyte phenotype. On the contrary, genes that decreased upon WT1 inhibition were mainly related to defense against apoptosis, cell cycle, and tumor progression. In agreement with these findings, WT1 expression increased the resistance of liver tumor cells to doxorubicin, a compound used to treat HCC. Interestingly, doxorubicin strongly enhanced WT1 expression in both HCC cells and normal human hepatocytes. Among different chemotherapeutics, induction of WT1 transcription was restricted to topoisomerase 2 inhibitors. When WT1 expression was prohibited, doxorubicin caused a marked increase in caspase-3 activation. In conclusion, WT1 is expressed in a substantial proportion of HCC contributing to tumor progression and resistance to chemotherapy, suggesting that WT1 may be an important target for HCC treatment.** [Cancer Res 2009;69(4):1358–67]

## Introduction

*Wilms' tumor 1* gene (*WT1*) is an important nuclear factor involved in organ development and cell growth (1). In addition to regulate nuclear transcription, WT1 is involved in RNA splicing and metabolism (2, 3). *WT1* encodes for 10 exons and generates various mRNA species. There are 2 main splicing events: one causing the splicing of exon 5 and the other leading to the splicing of 9 nucleotides at the 3′end of exon 9 (codifying for lysine, threonine, and serine: KTS). These splicing events, together with the use of alternative start codons and RNA editing may give raise to the generation of 24 different isoforms (3). The role of WT1 in cell biology is equally complex, and it has been shown that the repression or activation function of WT1 is dependent on the cell type and on its level of expression (3). Moreover, WT1 has been described as a tumor suppressor and as an oncogene (1, 3). During the last years, accumulating evidence shows that overexpression of WT1 exerts an oncogenic effect in different forms of tumors (1, 4, 5), and in fact, high levels of WT1 have been linked to poor prognosis in leukemia and breast cancer (6, 7).

In the adult, WT1 is detected in the kidney, mesothelium, ovary, and testis (8). In the liver, WT1 is expressed during the fetal stage of development but not after birth (9). Although WT1 is not found in the healthy adult liver, it is detected in the nuclei of hepatocytes in patients with chronic hepatitis and significant fibrosis and, at high levels, in the cirrhotic liver (9). We found that transforming growth factor-β is a key factor that promotes the expression of WT1 in hepatocytes. Also, we observed that WT1 was able to down-regulate HNF4 in parenchymal liver cells, which may contribute to the loss of specific hepatocellular functions and to the development of hepatic insuficiency in cirrhotic patients (9).

In this work, we show that WT1 is also expressed in tumor tissue in a high proportion of patients with hepatocellular carcinoma (HCC), a finding that is in agreement with a very recent report from Japan showing positive immunohistochemical staining in 95% of HCC specimens (10). Although in this report, the authors observed a correlation between WT1 expression and patient survival, there are no data showing the implication of WT1 in HCC biology. Here, we show that WT1 knockdown in HCC cells reduces antiapoptotic genes and genes favoring cell dedifferentiation, whereas this maneuver enhances the expression of genes that characterize the mature hepatocyte phenotype. Moreover, we show that the inhibition of WT1 expression increases the sensitivity of the cells to chemotherapeutic compounds such as doxorubicin. Interestingly, we also observed that among different chemotherapeutic compounds topoisomerase 2 (Topo2) inhibitors used in the treatment of HCC were able to promote WT1 gene expression in liver cancer cells. These data implicate WT1 in HCC progression and resistance to chemotherapy.

## Materials and Methods

**Patients.** Liver tissue was obtained from control individuals ($n$ = 19; all males; mean age, 58 y; range, 45–70 y) with normal or minimal changes in the liver. Tissue samples were collected at surgery of digestive tumors or from percutaneous liver biopsy performed because mild alteration of liver function tests. Cancerous liver tissues ($n$ = 19) were obtained during surgical resection. This study was approved by the University of Navarra Human Research Review Committee.

**Cell culture and treatments.** The human HCC cell lines HepG2, Hep3B, Huh7, and PLC/PRF/5 were grown in DMEM supplemented with 10% fetal

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

M.J. Perugorria and J. Castillo contributed equally to this work.

C. Berasain and M.A. Avila share senior authorship.

**Requests for reprints:** Carmen Berasain, Division of Hepatology and Gene Therapy, CIMA, University of Navarra, Avda. Pio XII, n55, 31008 Pamplona, Spain. Phone: 34-948-194700; Fax: 34-948-194717; E-mail: cberasain@unav.es.

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bovine serum, glutamine, and antibiotics. Human hepatocytes were from CellzDirect and were cultured in Williams' E medium supplemented as above. Cells were treated with doxorubicin (Sigma), etoposide (VP-16; Teva Pharmaceuticals), merbarone (Merb; Calbiochem), cisplatin (CDDP; Chiesi Farmaceutici), or topotecan (Topot; GlaxoSmithKline). Reduced glutathione mono-ethyl ester (E-GSH), catalase (CATA), N-acetyl-L-cysteine (NAC), and deferoxamine (DFO) were from Sigma. UV-C irradiation (254 nm) was performed using an UV cross-linker from UVItec Ltd.

**RNA interference.** Small interfering RNAs (siRNA) targeting different regions of the human WT1 gene (*siWT1*) were from Sigma (exons 4 and 10) or from Dharmacon Research (exon 7). Control siRNA (siGL) was from Dharmacon Research. Transfections with the different siRNA duplexes were carried out with the Dharmafect reagent as recommended by manufacturer (Dharmacon Research). Silencing of WT1 was confirmed by quantitative real-time PCR and Western blotting.

**Microarray analysis.** Microarray data were analyzed using RMA (Robust Multichip Average) algorithm (11). Linear Models for Microarray Data (12) was used to find probesets with differential expression between experimental conditions. We have followed the Minimum Information About a Microarray Gene Experiment guidelines, and deposited the microarray raw data in the National Center for Biotechnology Information Gene Expression Omnibus, accession number GSE12886.

**Cell growth and apoptosis assays.** Cell proliferation and viability was estimated using the WST-1 reagent (Roche) as reported (13). Apoptosis was estimated with the Cell Death Detection Assay (Roche) as described (14). Anchorage-independent growth was evaluated as described (13).

**RNA isolation and analysis of gene expression.** Total RNA was extracted using the TRI Reagent (Sigma). Reverse transcription and real-time PCR were carried out as reported (14). The amount of each transcript was expressed as the *n*-fold difference relative to the control gene β-actin ($2^{\Delta Ct}$; ref. 14). Primers for WT1 amplification were as described (9).

**Transient transfections.** Hep3B cells were transfected as described (9) with an equimolar mixture of pCV5 plasmids encoding the four major isoforms of WT1 kindly provided by Dr. Jochemsen (Leiden University Medical Center, Leiden, the Netherlands). Hep3B and Huh7 cells were transfected as described (9) with the pCAT-proximal-promoter plasmid encompassing 0.65 kb of the 5′ region of human *WT1* gene (15), kindly provided by Dr. Frazier (M. D. Anderson Cancer Center, Houston, TX). Reporter gene expression was assessed by measuring CAT mRNA levels by real-time PCR.

**Western blot analysis, immunohistochemistry, and immunocytochemistry.** Cell lysis and Western blotting were performed as reported (13). Antibodies used were as follows: 9664S for caspase-3 p17 subunit and 9718 for phosphorylated histone H2A ($Ser^{139}$; γH2AX) from Cell Signaling; AB17003 antibody from Chemicon for Bim. Equal loading was shown with an anti-histone H4 antibody (Santa Cruz), or anti-actin antibody (Calbiochem). Immunohistochemistry, immunocytochemistry and Western blot analysis of WT1 were performed as reported (9) with antibody C19 (Santa Cruz) and a preimmune rabbit IgG as control (Santa Cruz).

**Statistical analysis.** Normally distributed data were compared among groups using the Student's *t* test. Nonnormally distributed data were compared using the Mann-Whitney test. Data are means ± SE. Unless otherwise indicated experiments were performed at least twice in duplicates. A *P* value of $<0.05$ was considered significant.

## Results

**Expression of WT1 in human HCC tissues and HCC cell lines.** The expression of WT1 was assessed by real-time PCR in samples from control livers and HCC tissues. As shown in Fig. 1*A*, the average level of WT1 gene expression was significantly higher in HCC tissues than in control livers (614.2 ± 190.0 versus 80.0 ± 27.0 $P < 0.01$, mRNA levels expressed in arbitrary units). In agreement with our previous observations (9), WT1 expression was very low or undetectable in most control tissue samples. However, WT1 mRNA was detected in all but two HCC samples and showed high expression in ∼40% of tumor tissue samples. In all but one of these tumor samples with high WT1 expression, the levels of WT1 mRNA were higher than in the surrounding nontumoral tissue (data not shown). To further characterize the status of WT1 in human HCC, we cloned WT1 cDNAs from a representative number of tumors ($n = 9$). Upon sequencing, we did not find any mutations in these cDNAs, and we could detect the presence of isoforms including and excluding both exon 5 and the KTS epitope in all HCC samples. In agreement with the gene expression data, immunohistochemical analysis of control and HCC tissues showed intense WT1 immunoreactivity in neoplastic hepatocytes, whereas WT1 staining was undetectable in normal liver (Fig. 1*A, bottom*). As we previously observed in the cirrhotic liver tissue, WT1 immunoreactivity localized mainly to the nuclei, although some staining was also evident in the cytoplasm of tumor cells (Fig. 1*A*). We also tested whether high WT1 mRNA levels were accompanied by enhanced WT1 immunostaining in HCC tissues. To this end, we examined three HCC samples with high and three HCC samples with low WT1 mRNA levels. In agreement with a recent report (10) describing a close correlation between WT1 mRNA and protein expression in liver tumors, we found that those HCC samples with higher WT1 mRNA levels also showed enhanced WT1 immunostaining. These observations, together with our previous findings in chronic hepatitis and cirrhosis (9), indicate that WT1 expression in the liver is associated with chronic tissue injury and neoplastic transformation, situations in which liver parenchymal cells display enhanced proliferation. To evaluate whether WT1 up-regulation could be related to hepatocellular proliferation, we examined WT1 expression in rat liver samples taken at different time points after partial hepatectomy. We did not observe up-regulation of WT1 gene expression under this condition in which active proliferation of parenchymal cells occurs (data not shown). Next, we examined WT1 expression in human HCC cell lines. We could identify two groups of HCC cell lines, one with high WT1 expression, which included HepG2 and PLC/PRF/5 cells, and another with very low levels of WT1 mRNA including Huh7 and Hep3B cells (Fig. 1*B, inset*). Immunocytochemical analyses confirmed the gene expression data by showing intense WT1 staining in HepG2 and PLC/PRF/5 cell lines, which was mainly localized to the cell nuclei (Fig. 1*B, bottom*).

**Role of WT1 in HCC cell growth and survival.** To examine the potential role of WT1 in HCC cells, we knocked down WT1 expression using specific siRNAs. As shown in Fig. 2*A*, transfection of PLC/PRF/5 cells with siRNAs targeting WT1 exons 7 and 10 resulted in a significant reduction in WT1 mRNA levels. A similar down-regulation of WT1 expression was obtained when exons 4 and 10 were simultaneously targeted (data not shown). Down-regulation of WT1 mRNA was accompanied by a significant reduction in WT1 protein levels (Fig. 2*B*). When we examined the growth under standard conditions of PLC/PRF/5 cells transfected with siWT1 or control siGL siRNAs, we did not appreciate any significant differences (Fig. 2*C*). Similar observations were made in HepG2 cells (data not shown). We also tested the anchorage-independent growth of HCC cells in which WT1 expression was down-regulated. As shown in Fig. 2*D*, PLC/PRF/5 siWT1 transfectants presented significantly inhibited anchorage-independent growth in soft-agar compared with control cultures transfected with siGL (control siRNAs). This inhibitory effect was observed with different combinations of WT1-specific siRNAs: siWT1 A, targeting exons 4 and 10; siWT1 B, targeting exons 7 and 10; and siWT1 C, targeting exons 4, 7, and 10. These findings indicate that

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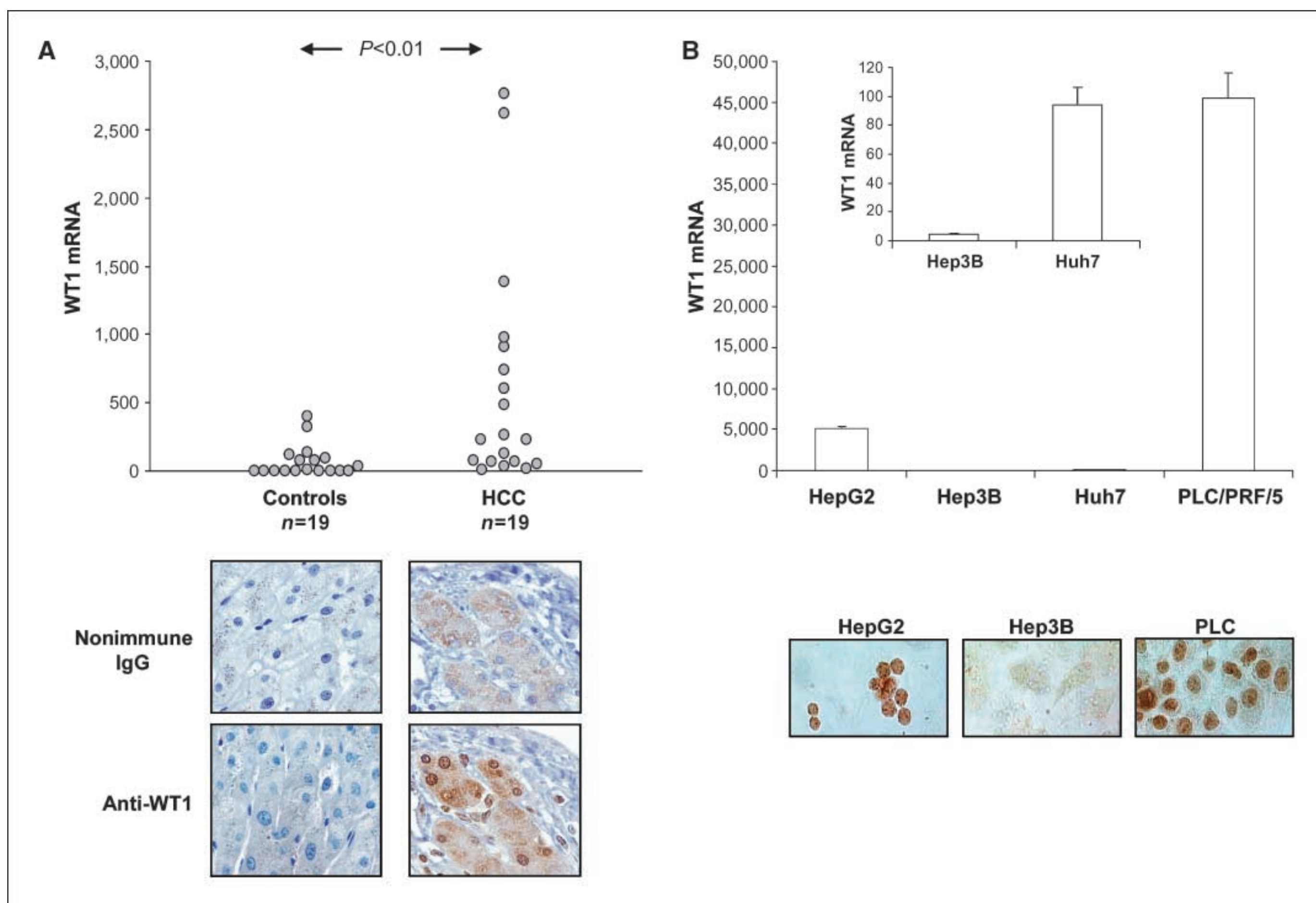


Figure 1. *A,* expression of WT1 gene in normal human liver and HCC as assayed by real-time PCR. *Bottom,* representative immunohistochemistry of control liver and a HCC specimen. Nonimmune IgG is shown as control. *B,* expression of WT1 gene in human HCC cell lines determined by real-time PCR. Low levels of WT1 mRNA were detected in Hep3B and Huh7 cells, which are shown in the inset graph at a bottom scale. *Bottom,* representative immunocytochemical analyses of WT1 protein in different human HCC cell lines. There was no staining when the anti-WT1 antibody was omitted (data not shown).

WT1 expression it is essential to preserve the viability of liver tumor cells grown under nonadherent conditions.

**Analysis of the influence of WT1 expression on the HCC cell transcriptome.** To gain further insight on the influence of WT1 gene expression on HCC cell biology, and to learn about the mechanisms behind its survival-promoting effects, we performed a microarray transcriptome analysis in PLC/PRF/5 cells transfected with siWT1 (siWT B) or control siGL siRNAs. Total RNA was isolated after 72 hours of transfections, and samples were hybridized on Affymetrix HG-U133-Plus-2 Chips. Microarray data analysis identified a total of 251 genes up-regulated and 321 genes inhibited upon WT1 silencing. By analysis with the Ingenuity Pathway Analysis Network, we found that the significantly up-regulated genes were mainly involved in metabolic pathways, including lipid, amino acid, and vitamin metabolism, as well as cell death and cancer (Supplementary Table S1). The inhibited genes were mostly associated with cellular development and cell cycle, and also with cell death and cancer (Supplementary Table S1). The differential expression of specific genes, selected by their potential physiopathologic significance and/or the magnitude of their change upon WT1 silencing, was validated by quantitative real-time PCR. This was done in the same RNA samples used for microarray analysis, and also in samples from independent transfections. The results were reproducible in both sets of samples and are shown in Fig. 3*A* and *B*. Same data were obtained in HepG2 cells (data not shown). Up-regulated genes upon WT1 silencing included markers of hepatocellular differentiation such as HNF4, albumin, and the epidermal growth factor receptor (EGFR). Conversely, WT1 knockdown reduced the expression of genes related to HCC disease progression and cell survival such as insulin-like growth factor binding protein1 (IGFBP1), H19, Tbx3, BclX, connective tissue growth factor (CTGF), Aurora kinase-2, and ID1, among others. Together, these observations further confirm the involvement of WT1 in promoting hepatocellular dedifferentiation, and contribute to explain apoptosis resistance in HCC cells.

**Down-regulation of WT1 expression sensitized HCC cells to doxorubicin-induced apoptosis.** The ability to evade apoptosis induced by chemotherapeutic agents is a common trait of HCC cells; however, the underlying mechanisms are not well-understood. We tested the influence of WT1 expression on apoptosis induced by the clinically relevant chemotherapeutic doxorubicin. Down-regulation of WT1 expression by itself had only a modest effect on cell survival; however, WT1 knockdown markedly sensitized both PLC/PRF/5 (Fig. 4*A*) and HepG2 cells (data not shown) to apoptosis induced by doxorubicin. The levels of the active caspase-3 p17 subunit correlated well with the extent of

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apoptosis induced by WT1 silencing and doxorubicin treatment (Fig. 4*B*). BH3-only proteins such as Bim prime HCC cells for apoptosis elicited by cytotoxic drugs, including doxorubicin (16, 17). We observed that enhancement of HCC cell apoptosis upon WT1 silencing in PLC/PRF/5 cells was accompanied by the up-regulation of Bim (Fig. 4*B*). Drug resistance in tumor cells, including HCC cells, is also associated with the expression of the *multidrug resistance 1* (*MDR1*) gene (18). Moreover, repetitive treatment with doxorubicin is known to enhance multidrug resistance of cancer cells (19). These notions prompted us to test whether WT1 could contribute to the expression of the MDR1 gene. To this end, we undertook two complementary approaches. First, we measured MDR1 gene expression upon doxorubicin treatment in PLC/PRF/5 cells transfected with control (siGL) or WT1-specific (siWT1) siRNAs. As shown in Fig. 4*C*, the up-regulation of MDR1 elicited by doxorubicin was significantly inhibited when WT1 was knocked down. Conversely, in Hep3B cells, transfection with an equimolar mixture of plasmids encoding the four major isoforms of WT1 elicited a clear induction of MDR1 gene expression (Fig. 4*D*).

**The expression of WT1 can be induced by Topo2 inhibitors in HCC cells and primary human hepatocytes.** Activation of survival mechanisms in normal and transformed cells is a common adaptive response to cell stress caused by a variety of agents, including chemotherapeutics such as doxorubicin (17, 19, 20). We have shown above that WT1 expression confers resistance to cell death in HCC cells. To further examine the involvement of WT1 in the response of liver tumor cells to cytotoxic compounds, we measured WT1 gene expression in four HCC cell lines upon treatment. We found that doxorubicin significantly up-regulated WT1 mRNA levels not only in PLC/PRF/5 and HepG2 cells but also in Huh7 and Hep3B cells, which displayed very low basal levels of WT1 mRNA (Fig. 5*A*). Remarkably, this response was not restricted to transformed cells because doxorubicin also elicited WT1 gene expression in normal cultured human hepatocytes (Fig. 5*A*). Induction of WT1 gene expression by doxorubicin was further characterized in Hep3B cells. We found that doxorubicin effects on WT1 transcription were dose- and time-dependent, with a potent induction of the gene at doses as low as 50 ng/mL (Fig. 5*B*). Although we cannot exclude potential effects of doxorubicin on WT1 mRNA stability, we showed that doxorubicin was able to transactivate the proximal WT1 gene promoter (15). We showed this in transient transfection experiments with a reporter CAT gene under the control of WT1 proximal promoter in Hep3B and Huh7 cells (Fig. 5*C*). In view of these findings, we wanted to know whether WT1 up-regulation upon doxorubicin treatment could participate in chemoresistance. To this end, Hep3B cells were first transfected with either control siGL or siWT1 siRNAs, and

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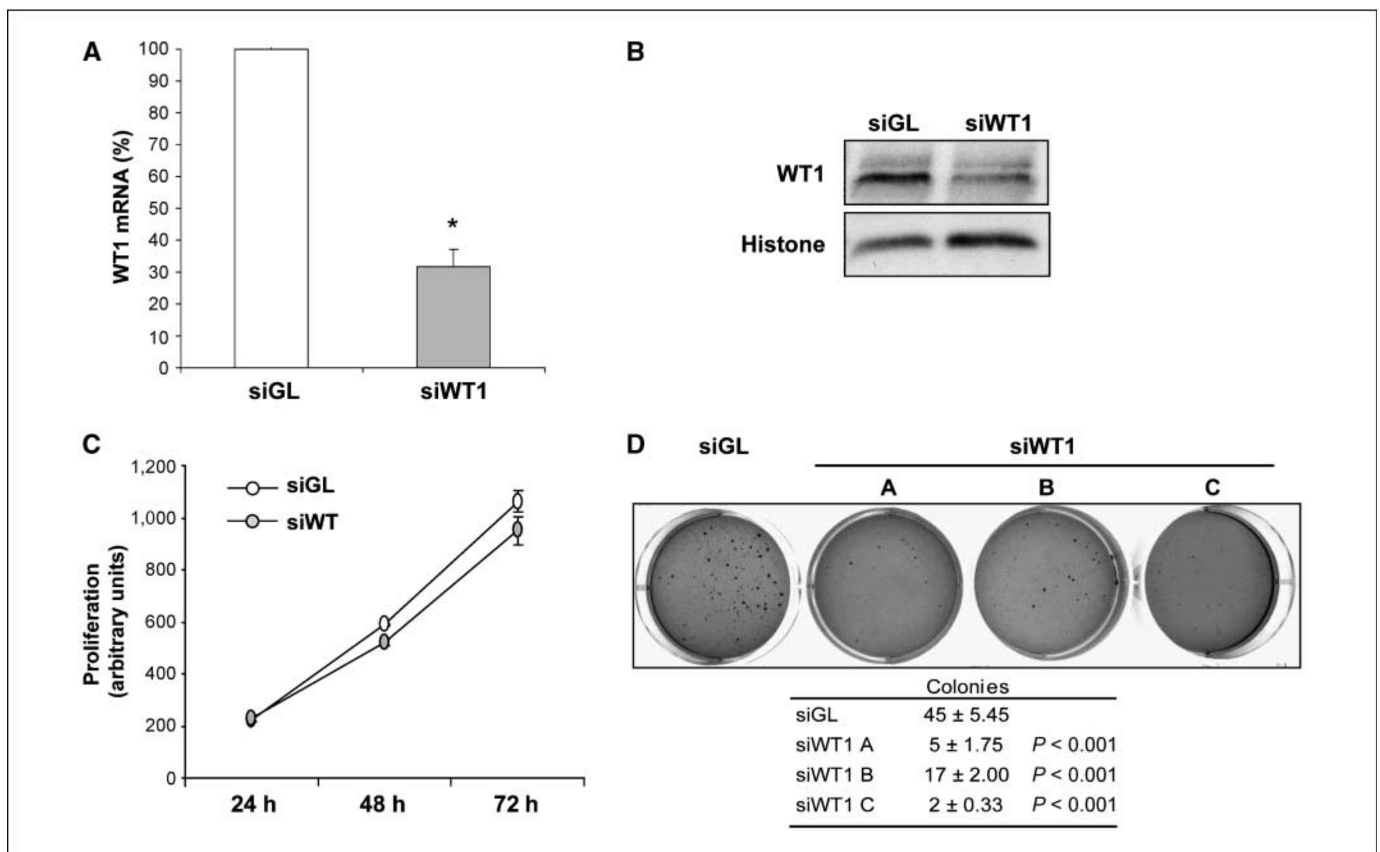


**Figure 2.** WT1 knockdown in human PLC/PRF/5 HCC cells. *A,* WT1 was knocked down by specific siRNA transfection (*siWT1*, 100 nmol/mL) in PLC/PRF/5 cells as described in Materials and Methods. Down-regulation of WT1 mRNA was confirmed 72 h after transfection by real-time PCR analysis. Values were normalized to WT1 expression in cells transfected with a control siRNA (*siGL*). *B,* WT1 protein levels as determined by Western blotting in PLC/PRF/5 cells 72 h after siWT1 or control siGL transfections. *C,* effect of siWT1 knockdown on PLC/PRF/5 cell proliferation. Cells were transfected with either siWT1 or control siGL siRNAs, and 24 h after, transfection cell proliferation was assessed as described in Materials and Methods. *D,* effect of WT1 knockdown on anchorage-independent growth of PLC/PRF/5 cells in soft agar. Cells were transfected with various combinations of siRNAs targeting different WT1 exons; *A*, exons 4 and 7; *B,* exons 7 and 10; and *C,* exons 4, 7, and 10, or with control siGL siRNAs. After 24 h, cells were harvested, counted, resuspended in 0.2% soft agar, and seeded onto 0.4% soft agar in DMEM supplemented with 10% FCS ($10^4$ per plate). After 4 wk, colonies were stained with crystal violet and counted.

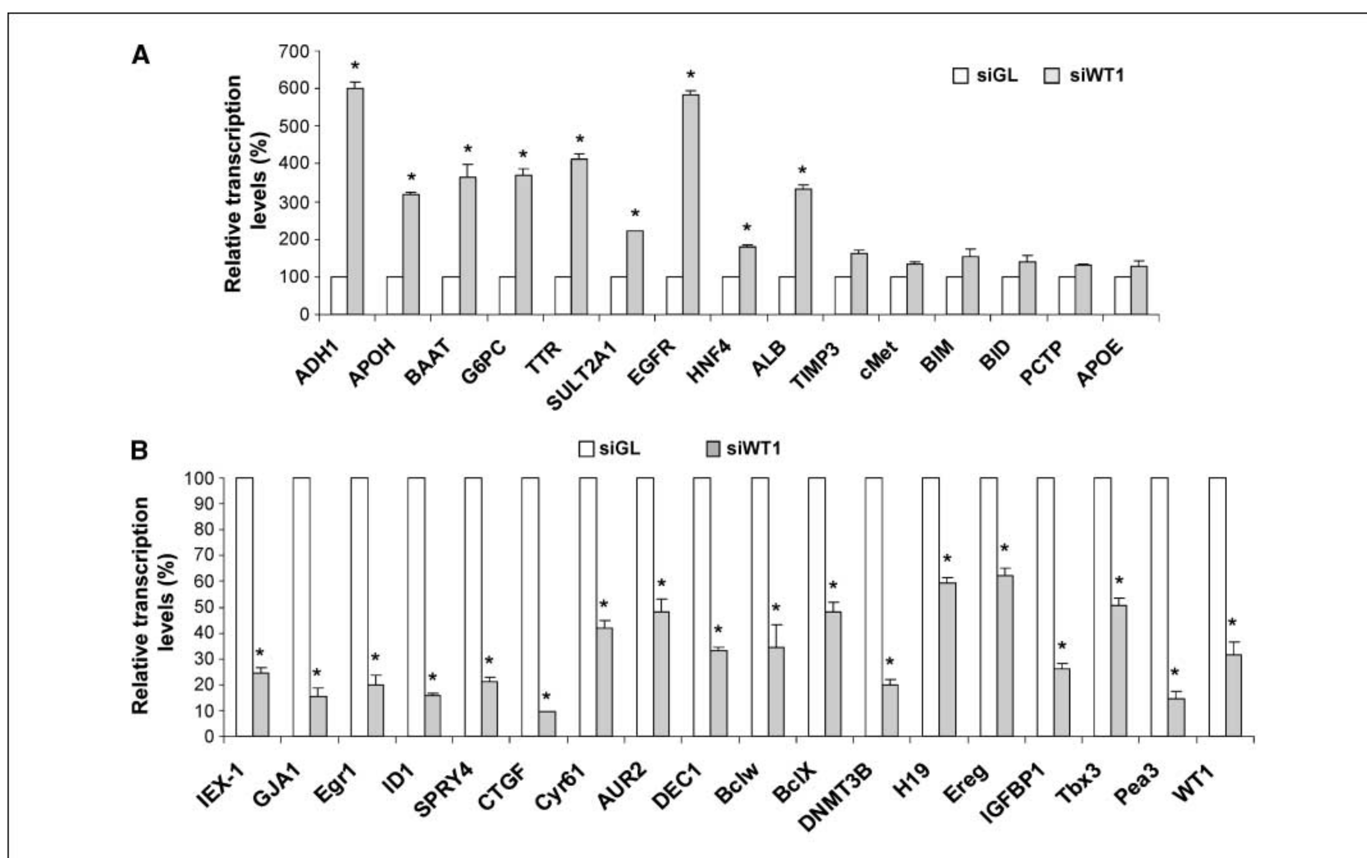


**Figure 3.** Validation of selected genes identified by microarray analysis as differentially expressed in PLC/PRF/5 cells upon WT1 knockdown. Quantitative real-time PCR analysis of gene expression in total RNA samples from PLC/PRF/5 cells 72 h after transfection with siWT1 or control siGL siRNAs. Values are expressed in relative transcript levels compared with control siGL siRNA. *A,* genes up-regulated or (*B*) down-regulated upon WT1 knockdown. *, $P < 0.05$ with respect to control transfections.

subsequently treated with doxorubicin. We observed that the apoptotic response to doxorubicin, as indicated by caspase-3 activation and elevated Bim protein levels, was more prominent when WT1 up-regulation was prevented (Fig. 5*D*).

We have also examined the potential mechanisms through which doxorubicin may stimulate WT1 gene expression. Doxorubicin has complex biological activities, including the generation of free radicals and the induction of DNA damage (21). As shown in Fig. 6*A*, doxorubicin-induced up-regulation of WT1 gene expression in Hep3B cells was unaffected in the presence of different free radicals scavengers. Doxorubicin treatment may also trigger DNA damage and genotoxic stress through the interference with Topo2 (21). We observed that WT1 expression elicited by relatively low concentrations of doxorubicin was indeed accompanied by the up-regulation of p21 and the phosphorylation of histone H2AX (γH2AX), both hallmarks of DNA damage (Fig. 6*B*; ref. 22). This led us to examine whether other genotoxic agents could modulate WT1 gene expression. As shown in Fig. 6*B*, treatment with the Top1 inhibitor Topot, the cytotoxic compound CDDP, or the exposure to UV irradiation had no effect on WT1 expression, whereas all three elicited a genotoxic response as shown by increased p21 and γH2AX levels (Fig. 6*B*). Collectively, these observations suggest that WT1 induction in HCC cells is not mediated through the DNA damage response pathway. Furthermore, WT1 induction in HCC cells seems to be a specific response to doxorubicin, and not to other chemotherapeutic agents such as Topot and CDDP (Fig. 6*B*), or 5-fluorouracil and gemcitabine (data not shown). Top2 is a major target of doxorubicin, to confirm whether WT1 induction could be dependent on interference with this enzyme, we tested other Top2 inhibitors. Figure 6*C* shows that WT1 up-regulation was induced in Hep3B cells treated with VP-16 or Merb, two different Top2 inhibitors. The effects of VP-16 and Merb on WT1 expression were reproduced in normal cultured human hepatocytes (data not shown). Contrary to doxorubicin and VP-16, Merb is a catalytic inhibitor of Top2, a kind of inhibitors that do not induce significant DNA damage (23). Consistent with this, we observed that Merb did not elicit p21 expression or γH2AX levels (Fig. 6*C*) but still triggered WT1 gene expression. This finding further confirms that WT1 up-regulation is independent from DNA damage but requires Top2 inhibition. Top2 inhibitors are known to promote the proteasome-mediated degradation of this enzyme (24). In agreement with this, we observed that treatment of Hep3B cells with VP-16 or Merb significantly reduced the protein levels of the two isozymes Top2α and Top2β (Fig. 6*D*). However, doxorubicin treatment did not reduce Top2 protein levels (Fig. 6*D*), suggesting that the effect of this drug on WT1 gene expression is dependent on the inhibition of Top2 enzymatic activity but not on the cellular Top2 protein contents.

## Discussion

Previously, we reported that WT1 is not expressed in normal liver but it is induced in patients with chronic liver injury and cirrhosis (9), conditions associated with HCC development (25). In

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the present work, we found that WT1 is overexpressed in 2 of 4 HCC cell lines analyzed and in tumor tissue in 42% of patients with HCC. Overall, these observations are in agreement with a very recent report that examined WT1 expression in HCC tissues (10). To evaluate the functional role of WT1 in liver cancer, we performed knockdown experiments in cells with high WT1 expression. In contrast to what has been reported in other types of tumors, such as leukemia and breast cancer cells (1, 5), neither in PLC/PRF/5 nor in HepG2 cells WT1 suppression modified cell proliferation. However, abrogation of WT1 expression markedly reduced the aggressiveness of HCC cells, as shown by the impaired anchorage-independent growth in soft agar. The ability to survive and grow under nonadhesive conditions by inhibiting anoikis-related apoptotic pathways is a characteristic of transformed cells that is absent in normal epithelial cells (26). To investigate the mechanisms through which WT1 could mediate its prosurvival effects, we carried out a genome-wide expression profile analysis in PLC/PRF/5 cells after WT1 silencing. Among the genes that expression was down-regulated upon WT1 silencing, we found several candidates with antiapoptotic activity, such as the Bcl-2 family members Bcl-w and Bcl-X (26), or DEC1 a potent inhibitor of apoptosis overexpressed in colon carcinoma cells (27). Other antiapoptotic genes that were dependent on WT1 expression included the transcriptional repressor Tbx3 (28) and IGFBP1 (29), both recently described to enhance the survival of HCC cells, and the immediate early response gene X-1, a stress-inducible gene that codes for an intracellular membrane-associated protein with prosurvival properties (30). Interestingly, the protective mechanisms associated with WT1 expression were not only dependent on the expression of intracellular prosurvival genes. Indeed, WT1 silencing compromised the expression of growth and survival factors such as epiregulin (Ereg), cysteine-rich61, and CTGF, secretable mediators previously associated with cytoprotection and HCC development, which can function in an autocrine or paracrine manner on transformed cells (14, 31, 32). Other WT1-dependent genes up-regulated in liver cancer, and that are relevant

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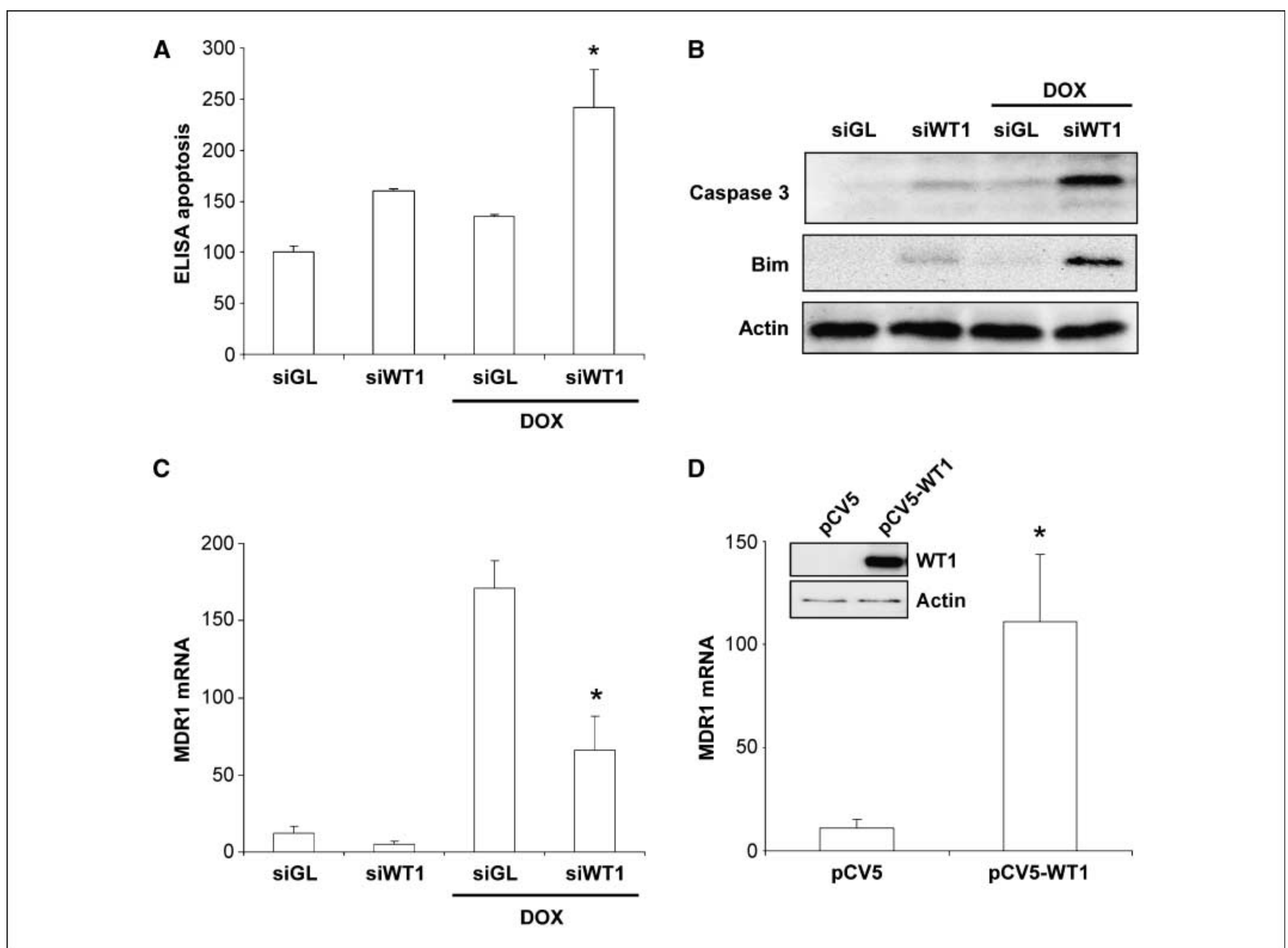


**Figure 4.** Effect of WT1 knockdown on doxorubicin-induced apoptosis and MDR1 expression in PLC/PRF/5. *A,* treatment with doxorubicin (*DOX*; 0.25 μg/mL) was started 48 h after transfection with either control siGL or siWT1 siRNAs. Apoptosis was measured 24 h later as described in Materials and Methods. *, $P < 0.05$ versus cells transfected with siGL and treated with doxorubicin. *B,* levels of the active caspase-3 p17 subunit and Bim protein as assessed by Western blotting in PLC/PRF/5 cells transfected with control siGL or siWT1 siRNAs. *C,* effect of WT1 knockdown on doxorubicin-induced MDR1 gene expression. PLC/PRF/5 cells were transfected with control siGL or siWT1 siRNAs and, after 48 h, were treated with doxorubicin (0.25 μg/mL) for another 24 h, then MDR1 gene expression was assessed by real-time PCR. *, $P < 0.05$ with respect to control transfection. *D,* effect of transfection with an equimolar mixture of plasmids encoding the 4 isoforms of WT1 (*pCV5-WT1*) or control plasmid (*pCV5*) on the expression of MDR1 in Huh7 cells. MDR1 gene expression was measured by real-time PCR 48 h after transfections. *, $P < 0.05$ versus transfections with control plasmid. WT1 expression upon transfection with pCV5 or pCV5-WT1 plasmids was confirmed by Western blotting (*inset*).

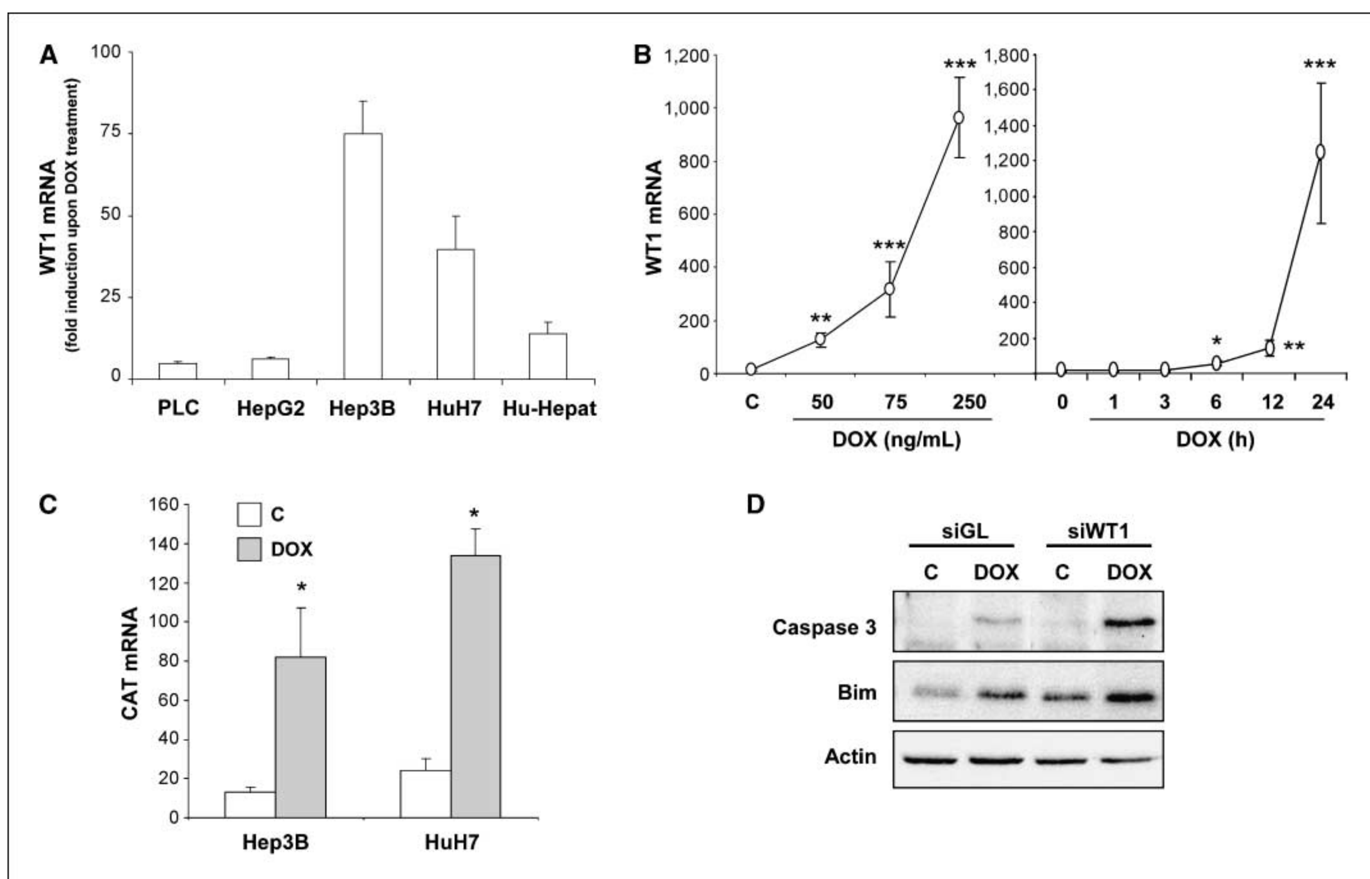


**Figure 5.** Doxorubicin up-regulates WT1 gene expression in human HCC cells and normal hepatocytes. *A,* PLC/PRF/5, HepG2, Hep3B, Huh7, and normal human hepatocytes (*Hu-Hepat*) were treated with doxorubicin at 0.25 μg/mL during 24 h, and mRNA levels of WT1 were measured by real-time PCR. Data are means of three independent experiments. *B,* dose- and time-dependent induction of WT1 gene expression in Hep3B cells treated with doxorubicin. Dose-dependent effects were measured after 24 h of treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus untreated cells or t = 0 h. *C,* doxorubicin treatment stimulates WT1 gene transcription in Hep3B and Huh7 cells. HCC cells were transfected with a reporter plasmid harboring CAT cDNA under the control of WT1 proximal promoter. Twenty-four hours after transfections cells were treated with doxorubicin (0.25 μg/mL) for another 24 h. Transactivation of WT1 promoter was assessed by measuring CAT mRNA by real-time PCR. *, $P < 0.05$ versus untreated cells. *D,* knockdown of doxorubicin-induced WT1 enhances caspase 3 activation and Bim expression in Hep3B cells. Hep3B cells were transfected with control (*siGL*) or siWT1 siRNAs, 24 h after transfection cells were treated with doxorubicin (0.1 μg/mL) for another 48 h. Representative Western blots are shown.

to the development and prognosis of this disease, included the serine/threonine kinase Aurora-A (33), the inhibitor of differentiation/DNA binding protein 1 (ID1; ref. 34), connexin 43 (GJA1; ref. 35), PEA3/ETV4 (36), and DNA-methyltransferase 3b (37). Interestingly, silencing of WT1 expression in HCC cells resulted in the induction of a number of genes characteristic of the adult and differentiated hepatocyte that are progressively down-regulated during carcinogenesis. These included albumin, transthyretin, alcohol dehydrogenase, glucose-6-phosphatase, DHEA sulfotransferase (SULT2A1), the EGFR, and HNF4 (38, 39). Although some of the differentially expressed genes, such as Ereg, H19, CTGF, Egr1, and the EGFR, are recognized transcriptional targets of WT1 (1, 8, 40, 41), most of the genes identified in our study have not been functionally linked to WT1, and therefore may be indirect targets of this transcription factor. Indeed, many of the modified genes upon WT1 silencing were transcription factors such as Egr1, ID1, PEA3, and DEC1, the alteration of which may effect on a wide range of target genes. In normal hepatocytes, we have previously shown that WT1 represses the expression of HNF4 (9), a crucial factor for the maintenance of metabolic functions typical of differentiated parenchymal liver cells (38). Thus, it seems possible that a repressor effect of WT1 on key liver-enriched transcription factors such as HNF4 may mediate and amplify some of the effects of WT1 that contribute to cellular dedifferentiation. This would be also in agreement with the developmentally regulated expression of WT1, which is detected in the fetal hepatocyte but is subsequently lost in the adult cell (9). Together these data suggest that in the HCC cell WT1 is sustaining a gene expression program leading to resistance to apoptosis, dedifferentiation and tumor progression.

Another relevant finding in this study was the protective effect exerted by WT1 on apoptosis induced by the chemotherapeutic agent doxorubicin. Doxorubicin is frequently used in the treatment of HCC, although the observed response rate is only around 10% (42). The resistance toward doxorubicin toxicity granted by WT1 may be attributed in part to the WT1-dependent expression of genes, such as *Tbx3*, recently reported to protect from apoptosis induced by this drug (27). However, although it was not detected in our microarray analysis, here, we show that WT1 was able to promote the expression of the MDR1 gene in HCC cells. MDR1 codes for the drug-transporting membrane-bound P-glycoprotein, a key gene in doxorubicin resistance (18, 19). The mechanisms involved in WT1 regulation of MDR1 expression in HCC cells are not fully understood at the moment. These may include a direct interaction of WT1 with MDR1 promoter as described in leukemia

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cells (43), or could involve indirect mechanisms such as the expression of H19, an inducer of MDR1 transcription in liver cancer cells (18) identified in our microarray experiments as a WT1-dependent gene.

Of interest was also the observation that doxorubicin can stimulate WT1 gene transcription in HCC cells. This was observed in all four cell lines tested but most prominently in those with low basal levels of WT1 mRNA. This may imply that although WT1 gene expression is detected in a subset of human HCC samples, perhaps all liver tumors may become WT1 positive upon doxorubicin treatment. These findings suggest that WT1 could play an important role in the acquisition of drug resistance in HCC.

We have addressed the potential mechanisms responsible for WT1 up-regulation by doxorubicin. The biological effects of doxorubicin have been essentially attributed to two mechanisms. Being an anthraquinone, it has redox cycling ability leading to the formation of free radical species (21). On the other hand, doxorubicin is a potent inhibitor of Top2 isozymes, Top2α and Top2β, and induces the formation of stable Top2-doxorubicin-DNA complexes capable of producing double DNA strand breaks, having thus a genotoxic effect (21, 22, 44). The involvement of reactive free radicals was ruled out when we observed that doxorubicin-mediated up-regulation of WT1 was unaffected in the presence of antioxidant compounds. We could also dissociate the production of DNA damage from the activation of WT1 gene expression. We observed that other DNA-damaging agents, including other chemotherapeutic compounds and UV radiation, had no effect on WT1 mRNA levels. Moreover, induction of WT1 gene expression seemed to be restricted to Top2 inhibitors, including those that did not elicit genotoxic stress. Top2α is mainly expressed in proliferating cells and plays important roles in cell cycle events such as DNA replication (45). However Top2β is present in all cells, including quiescent cells, and recent reports have shown an important role for this enzyme in gene expression regulation (46–48). For instance, Top2β has been described to negatively modulate the expression of retinoic acid receptor-β through a direct interaction with the promoter of this gene (48). However, we were unable to detect the association of Top2β with WT1 promoter by ChIP assay (data not shown). On the other hand, Top2β enzymatic activity, in cooperation with poly(ADP-ribose) polymerase (PARP-1), has been recently shown

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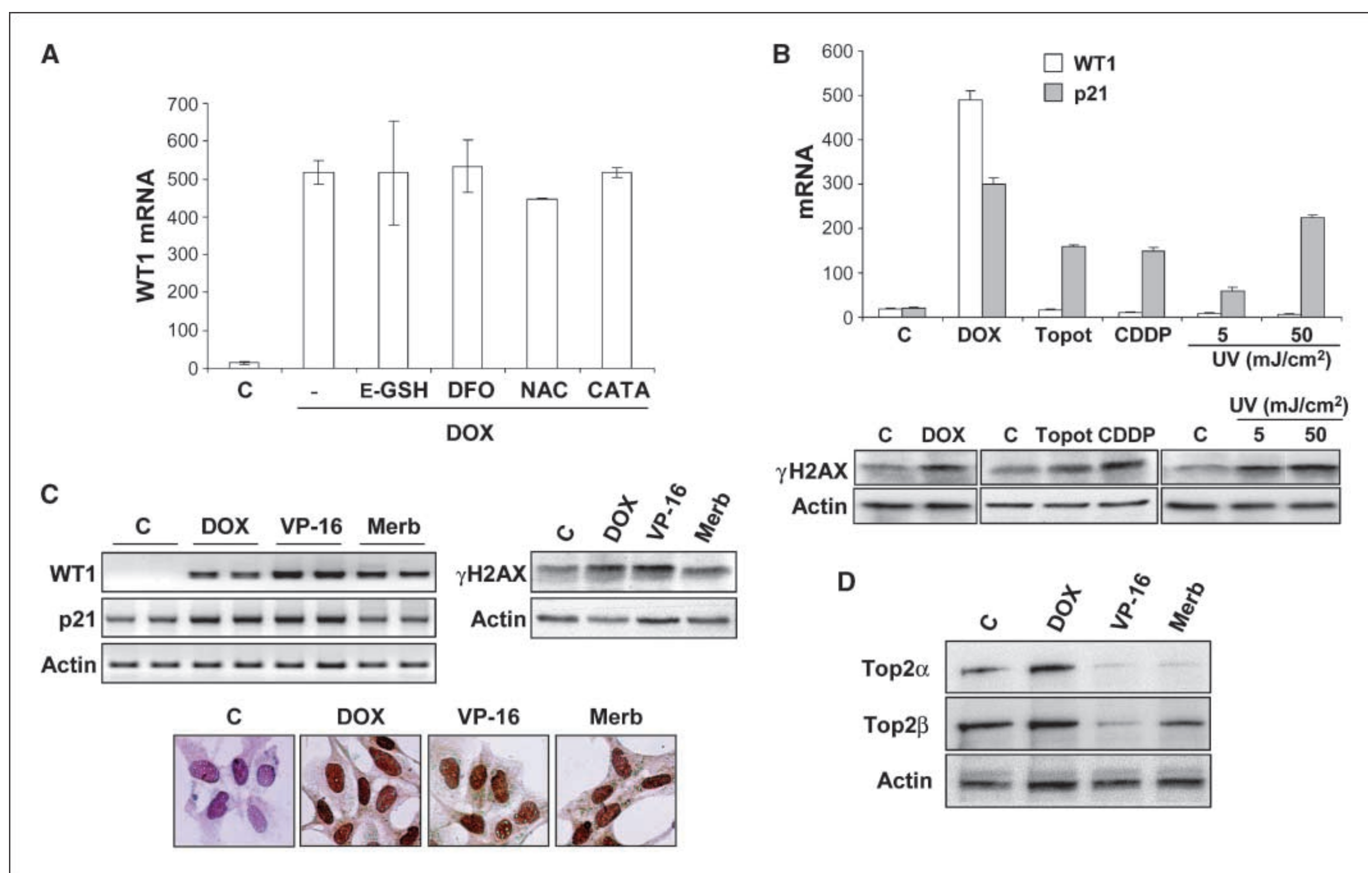


**Figure 6.** Characterization of WT1 up-regulation by doxorubicin in Hep3B cells. *A,* effect of antioxidant compounds on doxorubicin-mediated WT1 induction. Hep3B cells were pretreated for 30 min with E-GSH (2 mmol/L), DFO (180 μmol/L), NAC (1 mmol/L), or CATA (1,000 U/mL), and subsequently with doxorubicin (0.25 μg/mL) for 24 h. WT1 mRNA levels were then measured by real-time PCR. *B,* assessment of WT1 gene expression and genotoxic stress in Hep3B cells treated with cytotoxic drugs or UV radiation. Hep3B cells were treated with doxorubicin (0.25 μg/mL), Topot (5 μmol/L), CDDP (20 μmol/L), or UV radiation at the indicated doses. After 24 h WT1 and p21 gene expression were measured by real-time PCR. *Bottom,* the levels of γH2AX as analyzed by Western blotting in samples of Hep3B lysates treated as described above. *C,* up-regulation of WT1 gene expression by Top2 inhibitors is independent from the generation of genotoxic stress. Hep3B cells were treated for 24 h with doxorubicin (0.25 μg/mL), VP-16 (50 μmol/L), or Merb (100 μmol/L). WT1 expression was analyzed by semiquantitative PCR. Subsequently WT1 and p21 gene expression were assessed by reverse transcription-PCR (*left*), and γH2AX levels were determined by Western blotting (*right*). *Bottom,* representative immunocytochemical analyses of WT1 protein in Hep3B cells treated as described above. There was no staining when the anti-WT1 antibody was omitted (data not shown). *D,* Top2α and Top2β protein levels in Hep3B cells treated with doxorubicin, VP-16, or Merb at the concentrations mentioned above for 24 h. Representative Western blots are shown.

to participate in the activation of gene transcription through the generation of transient site-specific double stranded DNA breaks (47). At this point, we could speculate that WT1 transcription would normally be under the negative control of an intermediary gene, the expression of which is in turn dependent on Top2 activity (or on Top2/PARP-1 enzymatic complexes). Inhibition of Top2 by molecules such as doxorubicin would shut off the expression of that intermediary gene and result in WT1 up-regulation. In support of the existence of an active repressor of WT1 transcription in liver cells is the observation that WT1 gene expression in Hep3B cells can be induced by treatment with the protein synthesis inhibitor cycloheximide (data not shown). Furthermore, we have also observed that the PARP-1 inhibitor 3-aminobenzamide also promotes WT1 gene expression in HCC cells (data not shown).

Of wider implications may be the fact that Top2 inhibitors are able to promote WT1 gene expression in normal human hepatocytes. According to our current and previous findings, up-regulation of WT1 in liver cells promotes apoptosis resistance and cellular dedifferentiation (9). In a transient fashion, such response in a normal cell may have evolved as part of its natural defenses toward noxious stimuli. However, sustained expression of WT1 undoubtedly contributes to malignancy. The up-regulation of WT1 by drugs that target Top2 as shown here may contribute to understand the development of drug-induced secondary malignancies observed in patients receiving Top2-based chemotherapy (49).

In summary, WT1 is overexpressed in a significant number of liver tumors and in specific hepatoma cell lines. WT1 sustains a genetic program, which favors oncogenesis and tumor progression, and may be a novel determinant in the acquisition of resistance toward Top2-targeting chemotherapeutics. WT1 is therefore a potential target for anti-HCC therapies, including novel strategies such as WT1 peptide cancer vaccines, which are showing promising results in hematopoietic malignancies and other solid tumors (50).

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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