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Association of over-expressed *TFDP1* with progression of hepatocellular carcinomas

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Abstract DP-1 is a heterodimerization partner for members of the E2F family of transcription factors; E2F/DP-1 regulates the expression of various cellular promoters, particularly gene products that are involved in the cell cycle. Our earlier studies identified the DP-1 gene (*TFDP1*) as a probable target within a 13q34 amplicon that is frequently detected in hepatocellular carcinomas (HCC) and esophageal squamous-cell carcinomas. The aim of the present study was to investigate the clinicopathological significance of up-regulation of *TFDP1* in HCC. We determined expression levels of *TFDP1* and *E2F1* in 41 primary HCCs by means of quantitative real-time reverse transcription-polymerase chain reactions, and looked for relationships between those data and various clinicopathological parameters. To assess transactivating activity of E2F1/DP-1, we also analyzed expression of ten putative transcriptional targets of this complex in HCCs. Using antisense oligonucleotides, we down-regulated *TFDP1* in Hep3B, an HCC cell line that had shown overexpression of the gene, to examine the role of elevated *TFDP1* expression in the growth of Hep3B cells. Elevated expression of *TFDP1*, but not

E2F1, was associated significantly with large (≥ 5 cm) tumor size ($P=0.021$). Expression levels of *TFDP1* and *E2F1* correlated with those of seven transcriptional targets (*TYMS*, *DHFR*, *PCNA*, *RRM1*, *CCNE1*, *CDC2*, and *MYBL2*) that play important roles in the G1/S transition, and down-regulation of *TFDP1* inhibited growth of Hep3B cells. In conclusion, overexpression of *TFDP1* may contribute to progression of some HCCs by promoting growth of the tumor cells.

Keywords Hepatocellular carcinoma · DP1 · E2F1 · 13q34 · Antisense

Introduction

Hepatocellular carcinoma (HCC) is one of the most common neoplasias among human populations throughout the world (Bosch et al. 1999). Risk factors such as infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), dietary aflatoxin, and alcohol consumption have been associated with the development of HCC. Mutations in p53 gene, *AXIN1*, or *CTNNB1* (encoding beta-catenin) have been reported in HCC (Bressac et al. 1991; Miyoshi et al. 1998; Satoh et al. 2000). However, the genetic events involved in hepatocarcinogenesis are still poorly understood.

Amplification of chromosomal DNA is one of the mechanisms capable of activating genes that contribute to development and progression of cancers. Earlier studies in our laboratory revealed that the 13q34 region was frequently amplified in HCCs (Yasui et al. 2002) and esophageal squamous-cell carcinomas (Shinomiya et al. 1999). Amplification of DNA at 13q34 has been observed in other types of cancer as well (Knuutila et al. 1998), indicating that the region harbors one or more protooncogenes whose amplification can lead to development or progression of a variety of tumors. In a study of primary HCCs, we identified *TFDP1*, which encodes transcriptional factor DP-1, as a probable target within

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this amplicon (Yasui et al. 2002), as *TFDPI* was amplified and consequently overexpressed in some of the tumors examined. Expression levels of *TFDPI* in those HCCs were closely correlated with levels of *CCNE1*, the gene encoding cyclin E, suggesting that overexpression of *TFDPI* would lead to increased expression of this positive regulator of the G1/S transition during the cell cycle (Yasui et al. 2002).

DP-1 is a heterodimerization partner for members of the E2F family of transcription factors (E2F1 through E2F6) (Bandara et al. 1993; Girling et al. 1993; Helin et al. 1993; Krek et al. 1993). Among members of the DP family, DP-1 is likely to be the major component of cellular E2F activity (Bandara et al. 1993; Wu CL et al. 1995). DP-1 possesses DNA-binding and heterodimerization domains that are closely related to those of E2F proteins, but lacks a transcription-activation domain. Although DP-1 by itself has little transcriptional activity, interaction of DP-1 with E2F factors enhances DNA binding and transcriptional activity of E2F molecules (Bandara et al. 1993; Helin et al. 1993; Krek et al. 1993).

The E2F/DP-1 complex regulates expression of various cellular promoters involved in the cell cycle, in apoptosis, and in oncogenic transformation. DP-1 has shown transforming activity in cooperation with activated Ha-ras (Jooss et al. 1995) or ras plus E2F1 (Johnson et al. 1994). In transgenic mice expressing DP-1 under the control of a keratin 5 promoter, deregulated expression of DP-1 induced epidermal proliferation and enhanced carcinogenesis in epidermal tissues (Wang et al. 2001), although another type of DP-1 transgenic mice showed no obvious phenotypic changes (Holmberg et al. 1998). These lines of evidence imply oncogenic potential for DP-1. However, little is known about the actual role of DP-1 in the pathogenesis of HCC.

In the study reported here, we investigated the clinicopathological significance of up-regulated *TFDPI* in HCCs. We determined expression levels of *TFDPI* and *E2F1*, which encodes one of the best-characterized E2F factors, in primary HCCs by means of quantitative real-time reverse transcription-polymerase chain reactions (RT-PCR) and analyzed relationships between those data and various clinicopathological parameters. To assess transactivating activity of E2F1/DP-1 in HCC, we also analyzed expression levels of several known transcriptional targets of the complex. Furthermore, to determine the role of elevated *TFDPI* expression in growth of HCC cells, we used antisense oligonucleotides to reduce *TFDPI* expression.

Materials and methods

Cell lines and tumor samples

Hep3B, a cell line derived from a human HCC, was maintained in RPMI1640 supplemented with 10% fetal calf serum. We obtained 41 primary HCC tumors from patients undergoing surgery at the hospitals of Tokyo Medical and Dental University, and Kyoto University, Japan. All patients gave written informed consent, and

all aspects of these studies were approved by the ethical committees.

Real-time quantitative RT-PCR

We quantified mRNA levels using a real-time fluorescence detection method described previously (Yasui et al. 2002). Briefly, total RNA was isolated from each HCC using Trizol (Invitrogen, Carlsbad, CA, USA), and residual genomic DNA was removed by incubating the RNA samples with RNase-free DNase I (Takara, Tokyo, Japan) prior to RT-PCR. Single-stranded complementary DNA was generated with Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's directions. Real-time quantitative PCR experiments were performed with an ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix according to the manufacturer's protocol, using the following primers: *E2F1* (F, 5'-CACAGATCCCAGCCAGTCTCTA-3' and R, 5'-GAGAAGTCTCCCAGCACATG-3'); *MYBL2* (F, 5'-TGCCAGGGAGGACAGACAAT-3' and R, 5'-CTGTACCGATGGGCTCCTGTT-3'); *CDC2* (F, 5'-AAATATAGTCAGTCTTCAGGATGTGCTTA-3' and R, 5'-AGCCAGTTTAATGTTTCCTTTGTCAT-3'); *RRM1* (F, 5'-GAAAGTGTTTCAGTGTATGTGATGGAA-3' and R, 5'-TAAAGCCGAAGTAATTGTAAGAGAATCT-3'); *TYMS* (F, 5'-TTATCAAGGGATCCACAAATGCTA-3' and R, 5'-GCCCAAGTCCCCTTCTTCTC-3'). We designed the above primers using Primer Express software (Applied Biosystems) on the basis of sequence data obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Primers for *TFDPI* (Yasui et al. 2002), *TK1* (Yasui et al. 2002), *DHFR* (Yasui et al. 2002), *CCNE1* (Muller-Tidow et al. 2001), *CCNA2* (Muller-Tidow et al. 2001), *PCNA* (Muller-Tidow et al. 2001), and *MYC* (Li et al. 2000) had been described previously. *GAPDH* (Applied Biosystems) was used as a reference; i.e., each sample was normalized on the basis of its *GAPDH* content. Each assay was performed in duplicate.

Treatment of Hep3B cells with antisense *TFDPI*

Antisense experiments were performed as described previously (Yasui et al. 2002; Yokoi et al. 2002). Seven antisense oligonucleotides (AS11-AS17) containing phosphorothioate backbones were designed to target different regions of the primary RNA transcript of *TFDPI* (NCBI database, accession no. NT_027140), according to the method proposed by Tu et al. (1998). Scrambled sequences for the antisense oligonucleotides were used as controls (SC11-SC17 respectively). Antisense oligonucleotides (400-nM each) were screened in Hep3B cells by transfection using Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was isolated 24 h after transfection for real-time quantitative RT-PCR experiments to assess expression of *TFDPI* mRNA. AS17, which demonstrated the greatest suppression of target molecules, was selected for further study. For subsequent assays of cell viability, Hep3B cells were seeded in 96-well plates at densities of 5.0×10^3 cells/well the day before transfection. Cells were transfected using 0.2 μ l of Oligofectamine per well in 100 μ l of Opti-MEM I Reduced Serum Medium (Invitrogen). Cells were treated with AS17 or SC17 (5'-ATGTCAGTCCCCAGAGGCCGG-3' and 5'-GGCCGGAGACCCTGACTGTA-3' respectively) at final concentrations of 12.5, 25, 50, or 100 nM, or with Oligofectamine alone. Viable cells were counted by the MTT assay (cell-counting kit-8; Dojindo Laboratories, Kumamoto, Japan) 3 days after transfection, as previously described (Yokoi et al. 2002).

Statistical analysis

Statistical analysis was performed using the StatView statistical package (SAS Institute Inc., Cary, NC, USA). Chi-square tests

(with Fisher's exact probability test when necessary) were used to evaluate any association between a clinicopathological parameter and the expression level of each transcript. Any apparent associations were tested by means of a Pearson's correlation coefficient analysis. For all statistical tests, a probability P value of less than 0.05 was considered significant.

Results

Relationships between expression levels of *TFDP1* or *E2F1* and clinicopathological parameters in primary HCC tumors

The mRNA levels of *TFDP1* and *E2F1* were determined in 41 primary HCCs using quantitative real-time RT-PCR. To clarify potential relationships between expression of those two genes and various clinicopathological parameters, we used HCCs from 37 patients whose clinical data were available. The 37 HCCs were divided into "high-expression" and "low-expression" groups at the average of the mRNA level for each gene (Fig. 1). High expression of *TFDP1* significantly correlated with large (≥ 5 cm) tumor size ($P=0.021$) and with positive HBV ($P=0.043$) and negative HCV ($P=0.016$) infection (Table 1). No significant link was observed between expression level of this gene and any other parameter, such as patient's age and gender, histological differentiation, background liver tissue, or clinical stage (TNM classification). On the other hand, expression levels of *E2F1* were not correlated with any of the clinicopathological parameters examined (Table 1).

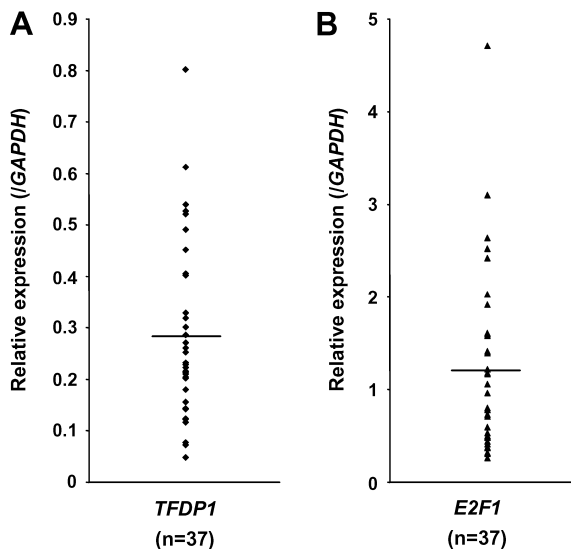


Fig. 1A, B Relative expression levels of *TFDP1* (A) and *E2F1* (B) in 37 primary hepatocellular carcinomas (HCCs). Horizontal lines indicate the average of expression levels for each gene. The average was used to determine the cutoff value for "high" and "low" expression groups

Expression of E2F/DP1 transcriptional targets

We next determined expression levels of ten genes thought to be targets of the E2F1/DP1 transcription complex (*CCNE1*, *CCNA2*, *CDC2*, *PCNA*, *TYMS*, *RRM1*, *MYC*, *MYBL2*, *TK1*, and *DHFR*) in all 41 primary HCCs and analyzed relationships between their expression levels and those of *TFDP1* or *E2F1* in a manner described previously (Yasui et al. 2002). Expression levels of *TFDP1* correlated significantly with those of *CCNE1*, *CDC2*, *PCNA*, *TYMS*, *RRM1*, *MYBL2*, and *DHFR*, but not with *CCNA2*, *MYC*, or *TK1* (Table 2); the closest correlation was observed between *TFDP1* and *CCNE1*. Expression levels of *E2F1* were significantly associated with those of all the presumed target genes, except *MYC* (Table 2).

Inhibition of growth of HCC cells after down-regulation of *TFDP1*

Because elevated expression of *TFDP1* was associated with large tumor size of HCCs, we down-regulated its expression in Hep3B cells, a line that had shown amplification and consequent overexpression of the gene (Yasui et al. 2002). For these experiments, we designed seven antisense oligonucleotides (AS11-AS17) targeting different regions of *TFDP1*. Among them, transfected AS17 exhibited the most striking reduction of *TFDP1* mRNA levels (Fig. 2A). Treatment of Hep3B with AS17, but not with its scrambled control (SC17), resulted in dose-dependent inhibition of cell growth (Fig. 2B).

Discussion

In the work reported here, we investigated expression levels of *TFDP1* and *E2F1* in primary HCCs and demonstrated that elevated expression of *TFDP1*, but not *E2F1*, was associated with large (≥ 5 cm) tumor size (Table 1). This finding suggests that up-regulation of *TFDP1* may be a late event among the genetic alterations that occur during the course of HCC progression. On the other hand, increased expression of *E2F1* may happen earlier, because it is overexpressed in most HCCs compared with their adjacent nontumorous tissues (Yasui K and Inazawa J, unpublished observation). Elevated expression of *TFDP1* was also associated with positive HBV and negative HCV infection in our experiments (Table 1). Our results may be concordant with the observation that HCCs related with HBV were significantly larger in size than those with HCV (Tanabe et al. 1999).

The E2F1/DP-1 complex is thought to be involved in cell-cycle progression by regulating genes in several categories: (1) those whose products are required for DNA synthesis, e.g., thymidine kinase (*TK1*), thymidylate synthetase (*TYMS*), dihydrofolate reductase

Table 1 Relationship between expression levels of *TFDP1* and *E2F1* and clinicopathological parameters

Clinicopathological parameter	<i>TFDP1</i>			<i>E2F1</i>		
	Low (< average) 23 cases	High (≥ average) 14 cases	<i>P</i> ^a	Low (< average) 23 cases	High (≥ average) 14 cases	<i>P</i> ^a
Age						
< 60 years	8	8	0.183	8	8	0.183
≥ 60 years	15	6		15	6	
Gender						
Male	19	8	0.132	16	11	0.709
Female	4	6		7	3	
Tumor size						
< 5 cm	16	4	0.021	10	9	0.313
≥ 5 cm	7	10		13	5	
HBV infection						
Positive	3	6	0.043	5	4	0.451
Negative	19	6		17	8	
Unknown	1	2		1	2	
HCV infection						
Positive	20	6	0.016	18	8	0.347
Negative	2	6		4	4	
Unknown	1	2		1	2	
Tumor differentiation						
Well	3	2	0.697	4	1	0.661
Moderately	14	10		14	10	
Poorly	6	2		5	3	
Background liver tissue						
Normal	0	2	0.206	1	1	0.589
Chronic hepatitis	18	11		19	10	
Liver cirrhosis	4	1		3	2	
Unknown	1	0		0	1	
Stage						
I, II, III	20	8	0.056	18	10	0.704
IV	3	6		5	4	

^aChi-square test**Table 2** Correlation between expression levels of *TFDP1* or *E2F1* and those of their putative target genes in 41 primary hepatocellular carcinomas (HCCs)

	<i>TFDP1</i>		<i>E2F1</i>	
	<i>r</i>	<i>P</i> ^a	<i>r</i>	<i>P</i> ^a
CCNE1	0.713	<0.0001	0.658	<0.0001
CCNA2	0.114	0.48	0.494	0.0008
CDC2	0.502	0.0007	0.772	<0.0001
PCNA	0.605	<0.0001	0.728	<0.0001
TYMS	0.663	<0.0001	0.646	<0.0001
RRM1	0.610	<0.0001	0.499	0.0007
MYC	0.080	0.62	-0.273	0.084
MYBL2	0.541	0.0002	0.680	<0.0001
TK1	0.287	0.069	0.747	<0.0001
DHFR	0.433	0.0043	0.661	<0.0001

^aPearson's correlation coefficient analysis

(*DHFR*), proliferating cell nuclear antigen (*PCNA*), and ribonucleotide reductase M1 polypeptide (*RRM1*); (2) those that encode cell-cycle regulators, e.g., cyclin A (*CCNA2*), cyclin E (*CCNE1*), and cdc 2 (*CDC2*); and (3) those that encode nuclear oncoproteins, e.g., c-Myc

(*MYC*) and b-Myb (*MYBL2*) (DeGregori et al. 1995; Wu et al.1995). Indeed, among the transcriptional targets we examined, expression levels of *TYMS*, *DHFR*, *PCNA*, *RRM1*, *CCNE1*, *CDC2*, and *MYBL2* correlated with those of both *TFDP1* and *E2F1* (Table 2), strongly suggesting that *TFDP1* may be involved in the progression of HCC by activating those seven genes in cooperation with *E2F1* or other members of E2F family.

To clarify the functional role of *TFDP1* in HCC cells, we suppressed its expression by transfecting antisense oligonucleotides into an overexpressing cell line, Hep3B. AS17 reduced expression levels of *TFDP1* more than SC17 (Fig. 2A). SC17 decreased *TFDP1* expression, probably owing to nonspecific toxicity of oligonucleotides. Down-regulation of *TFDP1* inhibited growth of these tumor-derived cells (Fig. 2B) in support of the notion that up-regulation of *TFDP1* promotes growth and leads to progression of some HCCs. A larger and more definitive study will be desirable for assessing this connection, because inactivation of *TFDP1* might lead to regression of HCC tumors. If so, this gene could represent an optimal molecular target for development of a novel therapy for this widespread and often intractable type of cancer.

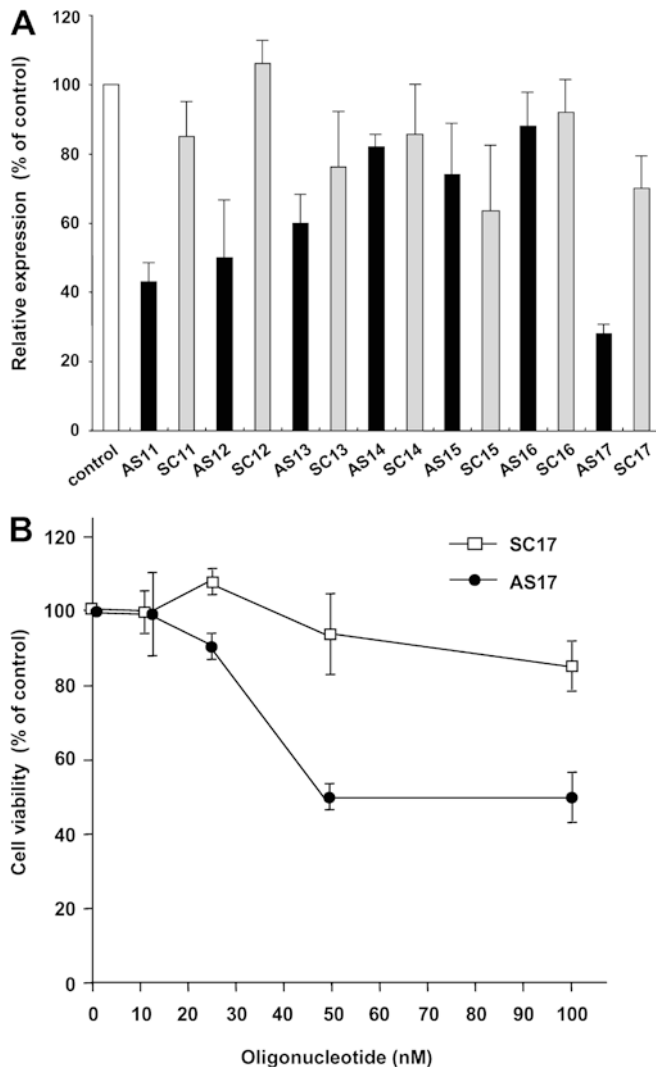


Fig. 2A, B Inhibition of growth in Hep3B cell cultures by transfection of antisense oligonucleotides targeting *TFDPI*. **A** Relative expression levels of *TFDPI* mRNA determined by real-time quantitative RT-PCR. Hep3B cells were treated with 400 nM of each of seven antisense oligonucleotides (AS11-AS17), their scrambled oligonucleotides (SC11-SC17), or untreated (control). Cells were harvested 24 h after transfection. **B** Effect of one antisense oligonucleotide on the viability of Hep3B cells treated with the indicated concentrations of AS17 or SC17. Cell viability was determined by MTT assay 3 days after transfection

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