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Acquired expression of transcriptionally active p73 in hepatocellular carcinoma cells

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p53 and p73 proteins activate similar target genes and induce apoptosis and cell cycle arrest. However, *p53*, but not *p73* is considered a tumour-suppressor gene. Unlike *p53*, *p73* deficiency in mice does not lead to a cancer-prone phenotype, and *p73* gene is not mutated in human cancers, including hepatocellular carcinoma. Here we report that normal liver cells express only ΔN-p73 transcript forms giving rise to the synthesis of N-terminally truncated, transcriptionally inactive and dominant negative p73 proteins. In contrast, most hepatocellular carcinoma cells express TA-p73 transcript forms encoding full-length and transcriptionally active p73 proteins, in addition to ΔN-p73. We also show that together with the acquired expression of TA-p73, the 'retinoblastoma pathway' is inactivated, and E2F1-target genes including *cyclin E* and *p14*^{ARF} are activated in hepatocellular carcinoma. However, there was no full correlation between 'retinoblastoma pathway' inactivation and TA-p73 expression. Most TA-p73-expressing hepatocellular carcinoma cells have also lost p53 function either by lack of expression or missense mutations. The *p73* gene, encoding only ΔN-p73 protein, may function as a tumour promoter rather than a tumour suppressor in liver tissue. This may be one reason why p73 is not a mutation target in hepatocellular carcinoma. *Oncogene* (2001) **20,** 5111–5117.

Keywords: liver cancer; p73; retinoblastoma; p16^{INK4a}; p14^{ARF}; cyclin E

Introduction

The *p53* and its newly discovered homologues, namely *p73* and *p63* form a family of genes that activate similar target genes, and induce apoptosis and cell cycle arrest (Marin and Kaelin, 2000; Lohrum and Vousden, 2000). However, *p53*, but not the other family members, is considered a tumour-suppressor gene. For example, unlike *p53, p73* deficiency in mice does not lead to a cancer-prone phenotype (Yang *et al*., 2000), and *p73* gene is not mutated in human tumours, including hepatocellular carcinoma (HCC) (Marin and Kaelin, 2000; Mihara *et al*., 1999). Presently, the reasons for this discrepancy are not known. One of the major differences between *p53* and the two other family members is the ability of *p63* and *p73* genes to encode multiple transcript isoforms (Marin and Kaelin, 2000; Lohrum and Vousden, 2000). Different *p63* and *p73* C-termini are generated as a result of alternative splicing between exons 10–15 and 10–14, respectively (Marin and Kaelin, 2000). In addition, *p63* utilizes a cryptic promoter located in intron 3 to generate additional transcripts called ΔN-p63 isoforms (Yang *et al*., 1998).

Our knowledge on truncated ΔN-p73 isoforms is scarce, but ΔN-p73 transcripts lacking transactivation domain were proposed to be predominant *p73* gene products in some mouse tissues (Yang *et al*., 2000). Pozniak *et al*. (2000) have recently demonstrated that *p73* is primarily present in developing neurons as a truncated ΔN-p73 isoform in mouse. Like the transcripts encoding ΔN-p63 (Yang *et al*., 1998), murine ΔN-p73 messages were derived from an alternative promoter located in intron 3, and ΔN-p73 failed to activate transcription from a p53-reporter gene, but suppressed the transactivation activity of both TA-p73α and wild-type p53 (Yang *et al*., 2000; Pozniak *et al*., 2000). On the other hand, most, if not all reported studies on the expression of p73 in different cancers did not distinguish between ΔN-p73 and TA-p73 isoforms (reviewed in Marin and Kaelin, 2000; Lohrum and Vousden, 2000).

With regard to p73 implications in liver malignancy, Tannapfel *et al*. (1999a,b) have reported that both p73 transcripts and protein were undetectable in most normal liver cells, but p73 protein was overexpressed in a subset of HCCs, and could serve as an indicator of poor prognosis (Tannapfel *et al*., 1999b). On the other hand, Mihara *et al*. (1999) reported the absence of mutation, as well as absence of overexpression of *p73* gene in HCC. These observations were in apparent contradiction and they did not provide evidence about the mechanisms of p73 involvement in liver malignancy. These reports, together with the identification ΔN-p73 in some mouse tissues (Yang *et al*., 2000; Pozniak *et al*., 2000), led us to test whether p73 expression in normal liver and HCC differs in terms of ΔN-p73 and TA-p73 isoforms. We also compared p73

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expression in HCC cells with the status of 'retinoblastoma pathway' and p53.

Results

By comparison of murine p73 cDNA sequences with that of human *p73* gene, we developed assays for identification and detection of ΔN-p73 and TA-p73 transcript isoforms (Figure 1a). Initial RT–PCR assays and nucleic acid sequencing identified ΔN-p73 isoform in SNU 398 HCC cell line (Figure 1b). The ΔN-p73 was also detected uniformly in mouse, rat (data not shown) and human liver tissues, as well as HCC cell lines (Figure 1c). In contrast, TA-p73 was not detectable in normal liver, but it was present in 14/15 (93%) of HCC cell lines. However, TA-p73 bands displayed various intensities. They were at the limit of detection in cDNAs from SNU 182 and SNU 423 cell lines, and there was no detectable TA-p73 expression in Mahlavu cell line (Figure 1c). p73 transcripts also differ in their 3′ regions because of alternative splicing between exons 10 to 14, giving rise to α, β, γ, δ, ε and φ isoforms (Zaika *et al*., 1999). p73 transcripts expressed in normal liver and HCC cell lines were mainly α, β and φ isoforms (data not shown). To confirm the expression of ΔN-p73 and TA-p73, we tested the presence of these protein isoforms in SNU 398 as compared to COS7 (Figure 1d), using ER 13 monoclonal antibody against amino acids 495–636 of p73-α (Oncogene Research Products, MA, USA). This antibody was shown to react with the α, but not β isoform of p73 protein (Marin *et al*., 1998). As shown in Figure 1d, ER13 antibody reacted with full-length p73-α (i.e. TA-p73-α) in COS7 cell line which was used as a positive control (Marin *et al*., 1998). SNU 398 cell line expressed TA-p73-α similar to COS7, but also a shorter polypeptide with an apparent molecular weight of 62 kD. This polypeptide can not be p73-β because of ER 13 specificity. Its apparent molecular weight correlates with that of mouse ΔN-p73-α described by Pozniak *et al*. (2000). Since, SNU 398 express both TA-p73 and ΔN-p73 transcripts (Figure 1c), the presence of both TA-p73 and ΔN-p73 proteins in this cell line is expected.

Next, p73 expression was studied in primary HCC tumours. As shown in Figure 1e, all tumours, as well as non tumour liver samples expressed ΔN-p73 isoform. In addition, two of seven (29%) HCC tumour samples expressed TA-p73 isoform. An additional tumour (T1) displayed a weakly positive TA-p73 band. There was no detectable TA-p73 in the remaining T3, T4, T6 and T7 tumours, as well as in non tumour liver NT1. Thus, acquired expression of TA-p73 in HCC was demonstrated in both primary HCC tumours and HCC-derived cell lines. However, the presence of TA-p73 was more frequent in cell lines than primary tumours. The TA-p73 transcripts shown in Figure 1 were tested with a primer pair located on exons 3 and 4, respectively. It has recently been described that, in some breast carcinoma cell lines overexpressing TA-p73, a transactivation-deficient splice variant lacking exon 2 (p73Δexon2) is also detected (Fillippovich *et al*., 2001). Since our TA-p73 transcript test system would not distinguish between TA-p73 and p73Δexon2, we performed additional RT–PCR experiments. Using a forward primer located on exon 1 together with a reverse primer located on exon 4 (Fillippovich *et al*., 2001), we detected mainly TA-p73 transcripts in both primary HCC and cell lines (data not shown). This confirms that TA-p73 forms described in Figure 1 are indeed full-length forms, even though one can not rule out a weak expression of p73Δexon2 form in some cell lines.

Induced expression of TA-p73 in some HCCs suggested that its expression is acquired during malignant transformation of hepatocytes. Recently, E2F1 transcription factor has been shown to activate p73 transcription through E2F1-binding motifs around non-coding exon 1 of p73 gene (Stiewe and Putzer 2000; Lissy *et al*., 2000; Irwin *et al*., 2000; Zaika *et al*., 2001). E2F1-responsive *p73* gene products have been identified as full-length p73α and p73β proteins (Stiewe and Putzer, 2000; Lissy *et al*., 2000; Irwin *et al*., 2000) that can be encoded by TA-p73, but not ΔN-p73 transcript isoforms (Figure 1a). Thus, we hypothesized that the selective induction of TA-p73 expression in HCC tumours is due to E2F1 activation. Therefore, we also tested the expression of E2F1 target genes $p14^{ARF}$ and *CCNE1* encoding cyclin E (Dyson, 1998). As shown in Figure 1e, the expression of cyclin E was induced in three tumours (T1, T2, T5), as compared to non tumour liver tissue NT1. The expression of $p14^{ARF}$ was also induced in two tumours (T1 and T2). It was also noteworthy that in TA-p73-positive T2 and T5 tumours, cyclin E expression was also induced, but only T2 showed $p14^{ARF}$ induction. Thus, three E2F1 target genes (i.e. *p73, Cyclin E* and $p14^{ARF}$) were induced in some HCC tumours. However, there was no full correlation between their expression patterns in different tumours. This could be due to the quality of RNA extracted from these archival tumour tissues. (We were not able to test additional samples, because HCC is a rarely operated tumour). Alternatively, the expression of these E2F1 target genes in HCC may be under the influence of additional factors.

As the activity of E2F1 is controlled by the protein product of retinoblastoma (RB1) gene (pRb) (Dyson, 1998), we studied whether the induction of TA-p73 expression is related to the inactivation of 'retinoblastoma pathway' in HCC cells. The 'retinoblastoma pathway' in cancer cells is altered mainly by inactivation of either *RB1* or $p16^{INK4a}$ gene (Dyson, 1998). In HCC, *RB1* mutations are rare, but allelic loss and decreased pRb levels occur frequently (reviewed in Ozturk, 1999), which may be due to overexpression of gankyrin that reduces the stability of pRb, and releases 'free E2F1' (Higashitsuji *et al*., 2000). Loss of $p16^{INK4a}$ expression by gene deletion or promoter methylation is also common in HCC (see Ozturk, 1999; Baek *et al*., 2000). Therefore, we compared the expression of TA-p73 with the expression of pRb protein and $p16^{INK4a}$

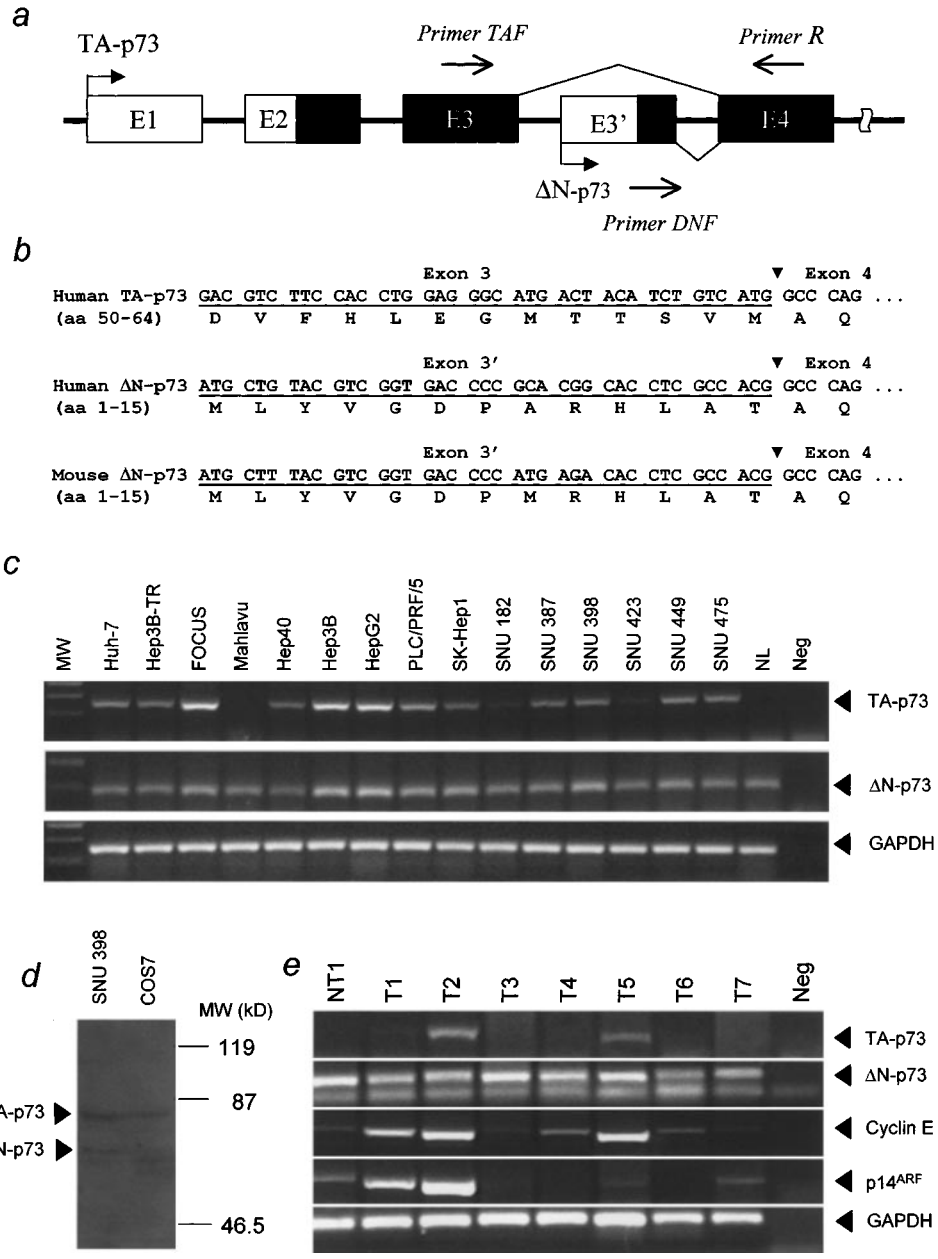

Figure 1 Identification of TA-p73 and ΔN-p73 transcripts and their expression in normal liver, HCC cell lines and primary HCC tumours, as compared to E2F1 target genes cyclin E and $p14^{ARF}$. (**a**) Exon-intron structure of 5′ coding region of human p73 gene showing the transcription start sites and splicing events leading to TA-p73 and ΔN-p73 isoforms. Initiation of transcription in exon 1 produces the TA-isoforms, containing the transactivation (TA) domain (previously called p73), while initiation in exon 3′ gives rise to the ΔN-isoforms without the TA domain. The putative transcriptional start site and exon 3′ for ΔN-p73 were identified by *in silico* analysis of human p73 gene, as compared to mouse ΔN-p73 transcript sequence. Primers TAF and DNF indicate the positions of isoform-specific forward primers used to identify respectively TA-p73 and ΔN-p73 transcripts, in combination with Primer R common to both isoforms. (**b**) Partial nucleotide and amino acid sequences of human TA-p73 and ΔN-p73 transcript isoforms as compared to mouse ΔN-p73 isoform. Data for human ΔN-p73 was obtained from direct sequencing of a 186 bp RT–PCR product obtained from SNU 398 HCC cell line, using DNF and R primer pair. (**c**) As compared to normal liver (NL), the expression of TA-p73, but not ΔN-p73 is induced strongly in 14 out of 15 (93%) HCC cell lines. TA-p73 and ΔN-p73 isoforms were tested as described in **a**. GAPDH was used as a control for equal template loading in PCR. Neg; negative control. (**d**) Western blot analysis of p73 protein in SNU 398 and COS7 reveals the presence of a ~62 kD polypeptide (presumably ΔN-p73 according to Pozniak *et al*., 2000) in SNU 398, in addition to TA-p73 protein present in both cell lines. Five-hundred μg total protein was analysed with anti-p73 ER13 monoclonal antibody which recognizes p73-α, but not p73-β isoforms (Marin *et al*., 1998). (**e**) Expression of TA-p73, ΔN-p73, cyclin E and $p14^{ARF}$ transcripts in primary HCC tumours. RT–PCR analysis using oligonucleotide primers shown in **a**, reveals the expression of ΔN-p73 in all samples tested. TA-p73 is easily detected in two out of seven tumours (T2, T5), T1 shows only weak expression, non tumour liver NT1 (counterpart of T1) is negative. Similarly, cyclin E expression is also induced in T1, T2, and T5, but $p14^{ARF}$ transcripts are induced only in T1 and T2. GAPDH was used as a control for equal template loading in PCR. Neg; negative control

transcripts in HCC cell lines. The expression of either pRb or $p16^{INK4a}$ was lost in six cell lines, and significantly decreased in five others. Thus, 'retinoblastoma pathway' appeared to be inactivated in at least 11 out of 15 (73%) cell lines (Figure 2a). Indeed, this inactivation has previously been reported for eight of these cell lines (Morel *et al*., 2000; Baek *et al*., 2000; Puisieux *et al*., 1993; Suh *et al*., 2000). In support of this conclusion, the expression of E2F1 target genes *cyclin E* and *p14ARF* were also induced in all cell lines, except three for $p14^{ARF}$ (Figure 2b), due to CDKN2A gene deletion in SNU 387 and SNU 449 (Baek *et al*., 2000), and probably in SK-Hep1. These studies showed that both the inactivation of 'retinoblastoma pathway' and the induction of TA-p73 expression are relatively common events in HCC cell lines. However, our observations do not allow establishing a direct correlation between these two events. Additional studies are needed to address this issue.

The overexpression of TA-p73 is not compatible with cellular growth (Marin and Kaelin, 2000; Fang *et al*., 1999), but HCC cells can apparently tolerate TA-p73 expression (Figure 1c). The ΔN-p73, co-expressed in these cells, is known to suppress both TA-p73 and p53 activities (Yang *et al*., 2000; Pozniak *et al*., 2000). In addition, some tumour-derived p53 mutant proteins were shown to bind and inactivate TA-p73 protein (Marin and Kaelin, 2000; Di Como *et al*., 1999; Strano

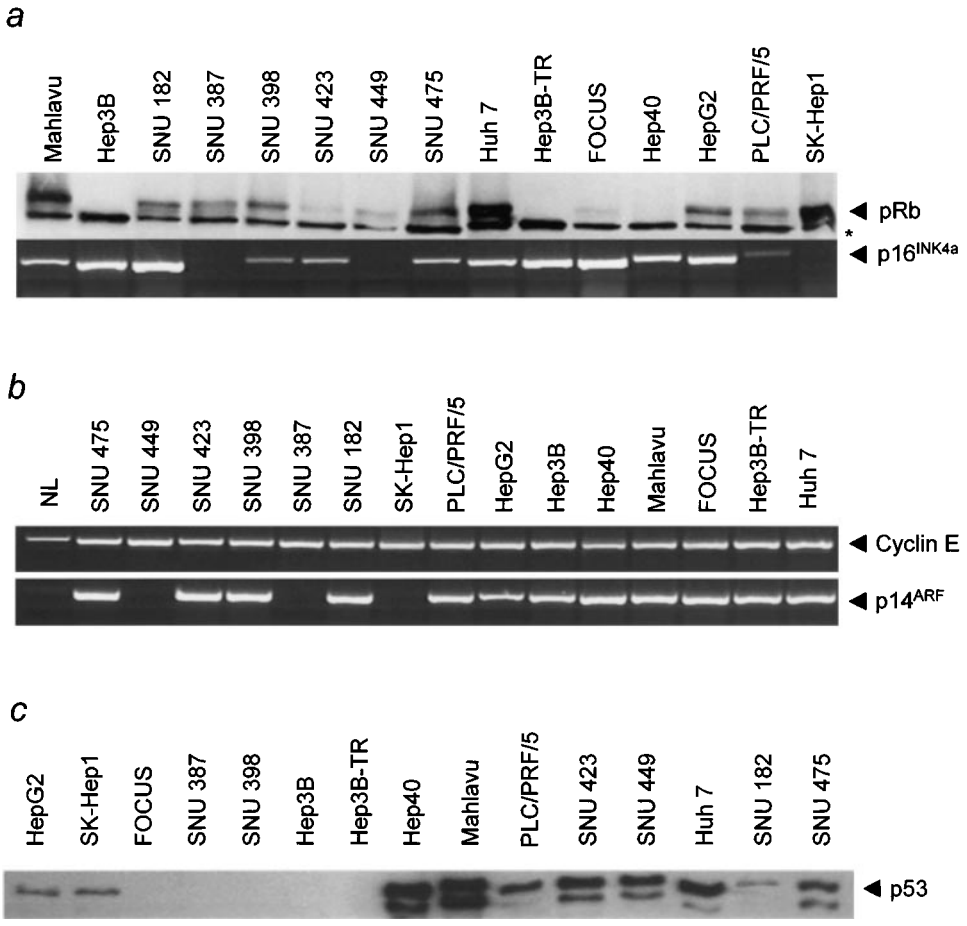

Figure 2 Comparative analysis of the status of retinoblastoma pathway (pRb and $p16^{INK4a}$), E2F1 target (cyclin E and $p14^{ARF}$) and p53 genes in HCC cell lines. (**a**) Either pRb or $p16^{INK4a}$ expression is totally lost (Hep3B, SNU 387, SNU 449, Hep3B-TR, Hep40, SK-Hep1), or significantly decreased (SNU 398, SNU 423, SNU 475, FOCUS, PLC/PRF/5) in 11 out of 15 (73%) cell lines tested. Western blot analysis using antibody IF8 reveals that pRb protein is not detectable in HepB3, Hep3B-TR and Hep40, or weakly positive in SNU 423, SNU 449 and FOCUS. The asterix (*) denotes a non-pRb cross-reactive antigen (Morel *et al*., 2000), serving as a loading control for Western blot assay. RT–PCR analysis shows that $p16^{INK4a}$ is not detectable in SNU 387, SNU 449 and SK-Hep1, or weakly positive in SNU 398, SNU 423, SNU 475 and PLC/PRF/5. See Figure 1c for template loading control using GAPDH. Negative control for $p16^{INK4a}$ RT–PCR is not shown. (**b**) The expression of E2F1 target cyclin E and $p14^{ARF}$ genes is induced in HCC cell lines. RT–PCR analysis of cyclin E transcripts reveals that their expression is uniformly induced in all cell lines tested, as compared to normal liver (NL). The induction of $p14^{ARF}$ expression is detectable in all but three cell lines. The lack of expression in SNU 449 and SNU 387 (see Baek *et al*., 2000), and probably SK-Hep1 (notice the lack of both $p16^{INK4a}$ and $p14^{ARF}$ expression) is due to CDKN2A gene deletion. See Figure 1c for the use of GAPDH as template loading control. Negative control is not shown. (**c**) Western blot assay for p53 shows no expression in FOCUS, SNU 387, SNU 398, Hep3B, Hep3B-TR, and high levels in Hep40, Mahlavu, PLC/PRF/5, SNU 423, SNU 449, Huh7, SNU 475 cell lines. Mahlavu and PLC/PRF/5 display Arg249Ser, and Huh7 Tyr220Cys mutations. HepG2 and SK-Hep1 express wild-type p53 (Puisieux *et al*., 1993; Hsu *et al*., 1993). pRb Western blot data was used as a loading control (as in **a**)

et al., 2000; Marin *et al*., 2000; Gaiddon *et al*., 2001). Therefore, we tested whether there was a correlation between TA-p73 expression and *p53* gene status in HCC cells. As shown in Figure 2c, p53 protein was either lost or mutant in at least eight out of 15 (53%) cell lines tested.

Discussion

Our observations demonstrate that *p73* gene in normal liver cells encodes only truncated ΔN-p73 transcript isoform. In mouse, this isoform was shown to code for an inactive p73 protein, acting as a dominant negative form on the activities of full-length TA-p73 as well as wild-type p53 proteins (Yang *et al*., 2000; Pozniak *et al*., 2000). The dominant expression of ΔN-p73 transcript isoform was demonstrated in normal liver tissues from human, mouse as well as rat organisms (see Figure 1c,e for human liver, other data not shown). In contrast, *p73* gene encodes both TA-p73 and ΔN-p73 isoforms in most HCC cell lines and some primary tumours. The acquired expression of TA-p73 in HCC cells appears to be transformation-related rather than proliferation-related, since Mahlavu cells did not express detectable TA-p73 transcripts, while expressing ΔN-p73 isoform (Figure 1c). We also detected ΔN-p73, but not TA-p73 transcripts in proliferating normal human fibroblast cell line MRC5 (data not shown).

Separate analysis of ΔN-p73 and TA-p73 transcripts in liver and HCC has not been reported yet. However, Tannapfel *et al*. (1999a) reported the absence of p73 expression in normal hepatocytes, using an antibody directed against amino acid residues encoded by exon 3 of p73, which is absent in ΔN-p73, but present in TA-p73 transcripts (see Figure 1b). Studies with this antibody (together with another anti-p73 antibody directed against a region common to both TA-p73 and ΔN-p73 proteins) also demonstrated that the p73 protein was negative in non tumour liver tissues, but positive in 32% of HCCs (Tannapfel *et al*., 1999b). Based on observations described here, it now appears that p73 detected in these HCCs is the TA-p73 form, although this requires a separate confirmation analysis with the TA-p73-specific antibody alone.

The induction of TA-p73 and the 'retinoblastoma pathway' inactivation was common in HCC cell lines (Table 1). However, comparative studies did not allow us to establish a link between the induction of TA-p73 expression and the inactivation of 'retinoblastoma pathway'. For example, the expression of E2F1-target genes was also induced in cell lines with apparently normal pRb and $p16^{INK4a}$ expression. Nevertheless, our results indicate that 'retinoblastoma pathway' inactivation is common in HCC cell lines and it is accompanied by an induction of E2F1 target genes (i.e. *p73*, *cyclin E* and $p14^{ARF}$) in most HCCs. This may suggest that the acquired expression of TA-p73 in some HCC cell lines is due to the inactivation of 'retinoblastoma pathway'. However, this remains to be demonstrated with further studies.

The expression of ΔN-p73 in normal liver, and the acquired expression of TA-p73 in HCC reveal new implications for p73 in tumour biology. The *p73* gene, encoding only ΔN-p73 transcripts in normal liver, may function as a tumour promoter rather than a tumour suppressor in this tissue, by interfering with wild-type p53 function (Yang *et al*., 2000; Pozniak *et al*., 2000). This could explain why *p73* is not mutated in HCC (Mihara *et al*., 1999). However, *p53* gene is frequently mutated in HCC (Ozturk, 1999), as also demonstrated here in HCC-derived cell lines (Figure 2c). Thus, ΔN-p73 protein may not completely neutralize wild-type p53 activity. Alternatively, the induction of TA-p73 transcription in tumour cells may redirect ΔN-p73 protein from wild-type p53 to newly expressed TA-p73 proteins.

On the other hand, the expression of TA-p73 transcripts in HCC cells is puzzling, based on the fact that TA-p73 overexpression is known to induce growth arrest and apoptosis (Marin and Kaelin, 2000; Lohrum and Vousden, 2000). Therefore, HCC cells expressing TA-p73 need to equip themselves with mechanism(s) to tolerate TA-p73 protein expression. In addition to a possible role of ΔN-p73 protein for TA-p73 inactivation (Yang *et al*., 2000; Pozniak *et al*., 2000), mutant p53 proteins may also inactivate TA-p73, as reported previously (Di Como *et al*., 1999; Strano *et al*., 2000; Marin *et al*., 2000; Gaiddon *et al*., 2001). Accordingly, several HCC cell lines reported here (Mahlavu, PLC/PRF/5, Huh7 etc.) express mutant p53 proteins that may inactivate TA-p73 protein. However, some other cell lines (FOCUS, Hep3B, SNU 387, SNU 398 etc.) have lost p53 expression. In this later group of cell lines, the tolerance of TA-p73 expression may require mutant p53-independent mechanisms. One additional hypothesis is that TA-p73 protein levels and/or its growth suppressive activities are regulated by post-translational mechanisms, independent of both ΔN-p73 and mutant p53. For example, endogenous p73 protein in a tumour cell line was shown to be stabilized by cisplatin and by c-Abl kinase, with no change in p73 transcript levels (Gong *et al*., 1999). Moreover, the apoptosis-inducing function of p73 was demonstrated to be dependent to or enhanced by the c-Abl kinase in different cell types (Yuan *et al*., 1999; Agami *et al*., 1999; Gong *et al*., 1999). Thus, some HCC cell lines may tolerate TA-p73 expression, if, for example, they are deficient in c-Abl kinase activity.

Materials and methods

Tissues and cell lines

Normal liver tissues from Balb/c mice and Sprague-Dawley rats were obtained after ether anaesthesia. Normal human liver tissue was obtained from discarded surgical material from a patient operated for hepatic hydatid cyst. Primary HCC tumours and non tumour liver were freshly frozen

Table 1 Comparative analysis of TA-p73 induction, E2F1 target gene activation and the retinoblastoma (*RB1*) pathway inactivation in HCC cell lines

	RB1 pathway genes		*EF21 target genes*		
Cell lines	*pRb*	*$p16^{INK4a}$*	*TA-p73*	*$p14^{ARF}$*	*Cyclin E*
Group I: Inactivated RB1 pathway					
hep3B	ND	Positive	Positive	Positive	Positive
Hep3B-TR	ND	Positive	Positive	Positive	Positive
hep40	ND	Positive	Positive	Positive	Positive
FOCUS	Traces	Positive	Positive	Positive	Positive
SNU-449	Positive	ND	Positive	ND	Positive
SNU-387	Positive	ND	Positive	ND	Positive
SK-hep1	Positive	ND	Positive	ND	Positive
PLC/PRF/5	Positive	Traces	Positive	Positive	Positive
SNU-475	Positive	Traces	Positive	Positive	Positive
SNU-398	Positive	Traces	Positive	Positive	Positive
SNU-423	Traces	Traces	Traces	Positive	Positive
Group II: Normal RB1 pathway					
Mahlavu	Positive	Positive	ND	Positive	Positive
SNU-182	Positive	Positive	Traces	Positive	Positive
HepG2	Positive	Positive	Positive	Positive	Positive
Huh-7	Positive	Positive	Positive	Positive	Positive

ND: not detected

archival materials. Fifteen human hepatoma-derived cell lines (14 HCC and one hepatoblastoma), MRC5 and COS7 were grown in culture as described (Morel *et al*., 2000). Most cell lines were from ATCC, the others have been previously described (Morel *et al*., 2000; Baek *et al*., 2000; Puisieux *et al*., 1993; Bouzahzah *et al*., 1995).

RNA and cDNA preparations

Total RNA from cell lines and tissues was extracted using NucleoSpin RNA II Kit (MN Macherey-Nagel, Duren, Germany) and TriPure reagent (Boehringer Mannheim, Indianapolis, IN, USA), respectively. The cDNAs were prepared from total RNA (5 μg) using RevertAid First Strand cDNA Synthesis Kit (MBI-Fermentas, Vilnius, Lithuania).

Transcript analysis by RT–PCR

For the identification of TA-p73 and ΔN-p73 forms, we used the available human p73 genomic sequence and mouse ΔN-p73 cDNA sequence data and designed two forward and one reverse (Primer R; 5′-GCGACATGGTGTCGAAGGTG-GAGC-3′) primers to specifically amplify these forms from human, mouse and rat tissues. These sequences were selected on the basis of highest homology between mouse and human DNA sequences (maximum one mismatch, data not shown). The forward primers TAF (5′- AACCAGACAGCACCT-ACTTCGACC-3′) and DNF (5′-ACCATGCTG TAC-GTCGGTGACCCC-3′) were used for specific amplification of TA-p73 and ΔN-p73 forms, respectively. For the detection of p73 isoforms generated by differential splicing between exons 10 to 14, a forward primer from exon 10 (5′-CGGCCATATT GGTGCCGCAGCCACTGGTG-3′) and two different reverse primers were used. A reverse primer from exon 13 (5′-GTTTGGCACCCCCAATCCTGT-3′) was used for specific amplification of transcripts containing this exon, i.e. p73α, p73ε, and p73γ (see Zaika *et al*., 1999 for terminology). Another reverse primer, encompassing sequences from exon 12 followed by exon 14 (5′-AGGGCCCC-CAGGTCCTGAGC-3′), was used for specific amplification of p73β and p73ϕ isoforms (Zaika *et al*., 1999) that contain this particular sequence. $p16^{INK4a}$ and $p14^{ARF}$ RT–PCRs were done using a specific forward primer for each transcript (5′-CGGAGAGGGGGAGAACAGAC-3′ for $p16^{INK4a}$ and 5′-TCACCTCTGGTGCCAAAGGG-3′ for $p14^{ARF}$) and a common reverse primer (5′-GGCAGTTGTGGCCCTG-TAGG-3′). For the detection of cyclin E mRNA levels, cyclin E-F (5′-TTGACCGGTATATGGCGACACAAG-3′) and cyclin E-R (5′-ATGATACAAGGCCGAAGCAG-CAAG-3′) primers were used. All RT–PCR reactions were done using appropriate annealing and extension conditions (additional information is available upon request). Equal amount of RNA was used in cDNA synthesis and the quality of cDNA was initially tested by GAPDH RT–PCR amplification with primer pair F (5′-GGCTGAGAACGG-GAAGCTTGTCAT-3′) and R (5′-CAGCCTTCTCCAT-GGTGGTGAAGA-3′), using 1/40 vol-ume of cDNA preparation. Further PCR studies were performed with cDNA preparations yielding equal amounts of GAPDH amplification products. Total PCR cycle numbers have been defined following an initial study at 22, 26, 30 and 34 cycles, in order to remain in the logarithmic phase of amplification. All RT–PCR results have been repeated several times from different batches of RNA preparations except primary tumours for which single RNA preparations were used. The expression of TA-p73 isoform was confirmed with an additional pair of primers, as described by Fillippovich *et al*. (2001). The identity of different p73 isoforms (TA-p73, ΔN-p73, p73α, p73β, p73γ, p73ϕ) has been confirmed by restriction enzyme mapping and automated sequencing (PE, ABI PRISM 377 automated sequencer) techniques.

Western blotting

The expression of retinoblastoma (pRb) and p53 proteins in different cell lines was studied by Western blotting, using anti-pRb IF8 (SC102, Santa Cruz Biotechnology) and anti-p53 6B10 (Yolcu *et al*., 2001) monoclonal antibodies, respectively, as described previously (Morel *et al*., 2000). Equal protein loading was confirmed by blotting with control antibody against cytokeratin 18 (JAR13 clone, gift from D Bellet, Institut Gustave Roussy, France). p73 protein Western

blot assays were done with ER13 (Ab-1, Oncogene Research Products, MA, USA) using the experimental procedure described by Marin *et al*. (1998).

Abbreviations

HCC, hepatocellular carcinoma; RB1, retinoblastoma gene; pRb, retinoblastoma protein.

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Accession numbers

The following sequences were deposited in the GenBank database. Human DNA sequence from clone RP5-1092A11 on chromosome 1p36.2-36.33 containing p73 gene: AL136528; Mouse mRNA for p73 delta-N protein: Y19235; Homo sapiens p73 gene: AH007820.

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