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ARTICLE

Analysis of alterations of *WFDC1*, a new putative tumour suppressor gene, in hepatocellular carcinoma

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***WFDC1* is a recently isolated human gene identified as a tumour suppressor gene candidate. It is not known whether alterations in this gene are associated with human cancers. The *WFDC1* gene maps in human chromosome 16q24, an area of frequent loss of heterozygosity (LOH) in several tumour types, in particular in hepatocellular carcinoma (HCC). We investigated its role in 46 European HCC by means of the detection of LOH at the *WFDC1* locus. We describe here an assay for the detection of loss of heterozygosity at this locus using two dinucleotide repeat polymorphisms identified in *WFDC1* introns, with a combined informativity of 86%. LOH was observed in 4/40 informative HCC samples. We further investigated the role of WFDC1 as a tumour suppressor gene candidate in five hepatocellular cell lines and in tumours exhibiting LOH by means of mutation, promoter methylation and gene expression analysis. In HCC samples showing LOH, no mutation of the remaining allele was observed. No significant up or down gene expression was observed in tumour samples comparatively to normal liver and gene expression did not seem related to promoter methylation. These results suggest a minor role, if any, of *WFDC1* in hepatocarcinogenesis. However, this approach might be useful for investigating the role of this candidate tumour suppressor gene in other tumour types.**
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Introduction

Recently, a new human gene involved in carcinogenesis, *WFDC1*, was cloned and characterized.[1] The *WFDC1* gene encodes a protein named ps20. The rat homologue of ps20 was originally identified as a secreted growth inhibitor[2,3] and was purified from the conditioned medium of a foetal rat urogenital sinus mesenchymal cell line.[4] Amino acid sequence analysis shows that ps20 contains a WAP-type four-disulphide core motif suggestive of a protease inhibitor.[5] The rat ps20 exhibited growth inhibitory effects on tumour cell lines.[5] These growth regulatory effects and the cell phenotypic properties in vitro, suggest that ps20 may function as a mediator of stromal-epithelial interactions and contribute to the maintenance of tissue homeostasis.[6] The ps20 gene, *WFDC1*, is thus a putative tumour suppressor gene.

The human *WFDC1* gene consists of seven exons and was mapped by fluorescent *in situ* hybridization (FISH) to Chr 16q24, less than 2 Mb from the telomere.[1] A BLASTN search against nr (the non redundant set of GenBank, EMBL and DDBJ database sequences) revealed that the chromosome 16 clone RP11-486L19 (GenBank accession no AC009123),

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contains *WFDC1* cDNA sequences and confirmed that the *WFDC1* gene maps to 16q24.3.

Chromosomal deletions at 16q24 have been associated with several human cancers, including hepatocellular carcinoma,[7,8] prostate cancer,[9] Wilms' tumour,[10] breast cancer[11,12] and ovarian cancer.[13] It is therefore likely that there are one or more tumour suppressor genes in the 16q24 area. *WFDC1* may be one of these genes. There have been no studies of alterations at the *WFDC1* locus in human cancers.

The inactivation of a tumour suppressor gene requires complete loss or inactivation of both alleles. This occurs generally by mutation of one allele and deletion of the other. This mechanism, initially proposed by Knudson for the retinoblastoma gene,[14] has been confirmed for a large variety of genes. As a consequence, recurrent allelic losses of specific chromosomal regions in tumour cells have been taken as evidence for the presence in these regions of tumour suppressor genes that may be functionally inactivated by a two-hit process. Detection of loss of heterozygosity (LOH) at the *WFDC1* locus may be very informative for investigating its role, if any, in tumour suppression. As a consequence, data on LOH for the *WFDC1* locus may be more easily interpreted if a polymorphism at the *WFDC1* locus is detected. In this paper we have identified two dinucleotide repeat polymorphisms at the human *WFDC1* locus (WFDC1-GT20 and WFDC1-GT12) and used them to detect LOH in hepatocellular carcinomas. Furthermore, to precise the role of *WFDC1* in hepatocarcinogenesis, we also screened the *WFDC1* gene for mutations and the level of promoter methylation was assessed. Finally, we studied its expression in normal and tumoral liver samples, in isolated hepatocytes and in five hepatocellular carcinoma cell lines.

Materials and methods

Tumour samples and cell lines

Liver tissues were harvested during the course of surgery and kept embedded in paraffin. Histology was re-examined by the pathologist and samples containing more than 70% tumourous cells were selected. Five human liver tumor cell lines HepG2, PLC/PRF/C, TONG, HA22TNGH and MAHLAVU were also obtained from the American Type Culture Collection. Cells were grown in Dulbecco F12 RPMI–1640 medium (Life Technologies, Cergy Pontoise, France), containing 10% FCS and gentamycine, and harvested at 70% confluence. Human hepatocytes were isolated by collagenase digestion from normal livers from three cadaveric multiple organ donors, as previously reported.[15]

LOH analysis

Polymorphisms in the *WFDC1* cDNA sequences were located by analysis of the sequence of the RP11-486L19 clone. WFDC1-GT20 was located between the fourth and fifth exons and WFDC1-GT12 between the third and fourth exons. To study WFDC1-GT20, primers were synthesized to give a final amplification product of 181 bp (GT strand primer: 5′-CCTGTCTTCGTAAAGGGAGG-3′; AC strand primer: 5′-TCAAATCGTTCATTTGGGAG-3′). The GT strand PCR primer was labelled with HEX fluorescent dye (Eurogentec, Seraing, Belgium). WFDC1-GT12 was analysed by means of primers synthesized to give a final amplification product of 206 bp (GT strand primer: 5′-TGACTGTGTCCGCTAGAGTG-3′; AC strand primer: 5′-TACGCACGCATCCCC-3′). The GT strand PCR primer was labelled with 6-FAM fluorescent dye. We conducted a BLASTN search to confirm the specificity of the nucleotide sequences chosen as amplification primers. PCR was performed in multiplex for the two polymorphisms in a 25 μl volume containing 5 pmol of each primer, 40 μM each of deoxynucleotide triphosphate, standard PCR reaction buffer containing 1.5 mM $MgCl_2$, and 1 U Taq DNA polymerase (Q.biogene, Illkirch, France) using a GeneAmp PCR system 2400 (Applied Biosystems, Courtaboeuf, France). One μl of DNA extracted from formalin-fixed paraffin-embedded specimens of tumour tissue or 50 ng of DNA extracted from paired peripheral blood samples obtained at distance from surgery was amplified. Amplification consisted of one cycle of 2 min at 94°C, 35 cycles of 1 min at 92°C, 30 s at 55°C, 45 s at 72°C plus a final extension step of 7 min at 72°C. One μl of the PCR products diluted in 25 μl final formamide were separated by capillary electrophoresis using an ABI PRISM 310 sequencer (Applied Biosystems, Courtaboeuf, France). LOH was quantitatively assessed by calculating the LOH index, which was defined as the allele ratio in the tumour tissue in comparison to the allele ratio in the normal control. The allele ratio was calculated as the peak height of the smaller allele divided by the peak height of the larger allele. If the LOH index was less than 0.5 or more than 2.0, we defined the case as showing LOH.

Table 1 Primer sequence for exon-amplification

Target region	*Forward* (5′→3′)	*Reverse* (5′→3′)	*Size of product*
Exon 1	GGACACATGATCCGAGGGAC	CCTCTCCTCAAGGCTGACTCC	351bp
Exon 2a	AGGTGGCCCAGCTTTAAGCC	CTGCTGACACCGCCCTCTC	322bp
Exon 2b	GCGGGCAGCGGTCTG	GGCTCTGCCTGCTTGCTGTT	201bp
Exon 3	GGAGCCTCTGTGCTGTCATGA	CCTGGTGCTGCCAGCTTT	271bp
Exon 4	CGTTCCCTGCACCCGTC	TCAGTCCCAGGTGCTGGG	360bp

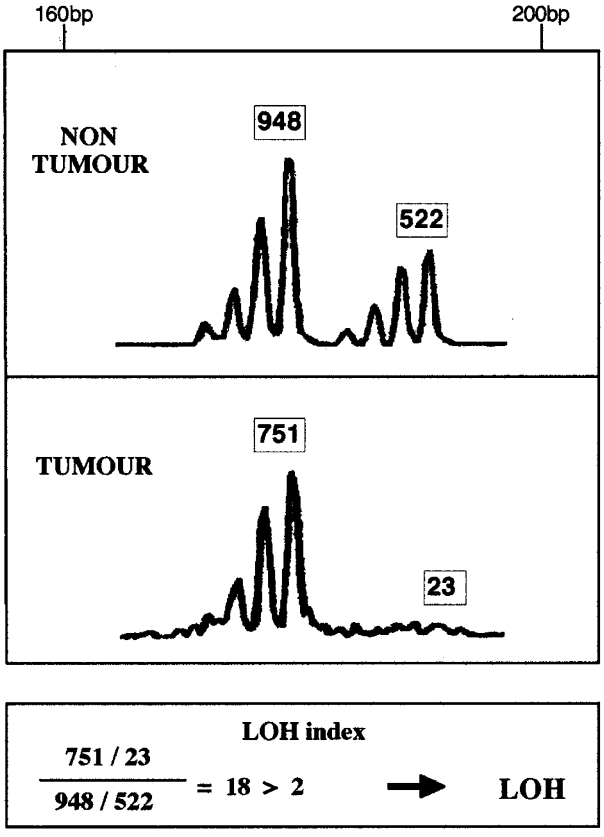

Figure 1 Example of LOH assessment at locus WFDC1-GT20. Upper trace, amplification from normal tissue; lower trace, amplification from tumour tissue. Alleles are automatically labelled with their peak height (in arbitrary units, in boxes). The LOH index is calculated as indicated.

Mutation analysis

PCR was performed in coding exons (exons 1 to 4) and their surrounding regions. Details of oligonucleotide sequences are summarized in Table 1. PCR reactions were carried out in a 50 μl volume containing 0.5 μM of each primer; 50 μM of each dNTP; 2.5 μl of formamide and 2 U of Taq (Q.biogene, Illkirch, France) using a gene Amp PCR system 2400 (Applied Biosystems, Foster City, USA) and using the following conditions: (a) 94°C (4 min); (b) 40 cycles of 92°C (1 min), 58°C (1 min), 72°C (1 min); and (c) a final extension step at 72°C (7 min). Sequencing was performed on both strands using the ABI Prism dichloro-rhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) after purification of the PCR products using the Qiaquick PCR purification kit (Qiagen, Courtaboeuf, France). The sequences were analysed on an ABI 310 automated sequencer unit (Applied Biosystems, Foster City, USA).

Analysis of methylation status

To determine whether the *WFDC1* presumptive promoter regions were hypermethylated, a PCR-based *Hpa*II restriction enzyme assay was used. Two μg of DNA was digested separately in a total volume of 20 μl with either 20 units of *Rsa*I or 20 units of *Rsa*I and *Hpa*II (Roche Diagnostics, Meylan, France) at 37°C for 16 h. Another 2 units of each enzyme were added for an additional 1 h at 37°C to ensure complete digestion with the methylation-sensitive restriction endonucleases. Two μl of restricted DNA was then included in 2 PCR directed to the *WFDC1* promoter containing two *Hpa*II sites (sense primer: 5′-CGAAATCTGAACAAGGTAGCAG-3′; antisense primer: 5′-CTTGCTTGGAGACGTGGC-3′) and to a reference sequence devoid of *Rsa*I and *Hpa*II sites located in exon 6 of the *WFDC1* gene (sense primer: 5′-ACCAGGCTTCATTTGGCAGT-3′; antisense primer: 5′-TCCTCAACCTGGTGGTGAAAG-3′). PCR was performed by using an ABI PRISM 7700 Sequence Detector and SYBR Green reagents in the standard conditions recommended by the manufacturer (Applied Biosystems, Foster City, USA). For each PCR, a same standard curve was produced, using four 1 in 10 dilutions of a same non digest DNA. All samples were run in triplicate. The methylation index (%) in a sample was calculated using the following equation:

$$\text{Methylation index} = \text{RH (RsaI+HpaII)}/\text{R (RsaI)} \times 100\%$$

where RH is the quantity of methylated *WFDC1* sequences measured following digestion by *Rsa*I+*Hpa*II and R the results from the 'mock' digest without *Hpa*II. The reference sequence in exon 6 was used to normalize the RH and R results. For each sample, a control where *Msp*I, an enzyme insensitive to the methylation, replaced *Hpa*II, showed full digestion.

Expression of the *WFDC1* gene

WFDC1 gene expression was studied on total RNA extracted from 20 mg tissue or 10^6 cells from three independant primary culture of hepatocytes or cell lines with a QIAamp Tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. cDNA synthesis was performed in a volume of 50 μl using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, USA). RT buffer 10 × (5 μl), 5 mM $MgCl_2$, 500 μM each dNTP, 2.5 μM random hexamers, 20 U Rnase inhibitor and 75 U Multiscribe reverse transcriptase were mixed with 2.5 μg of total RNA. The cycling conditions were 10 min at 25°C, 30 min at 48°C, and 5 min incubation at 95°C. PCR was performed by using an ABI PRISM 7700 Sequence Detector and SYBR Green reagents in the standard condition recommended by the manufacturer (Applied Biosystems, Foster City, USA) with two primers located on different exons (forward primer 5′-GCTATGAGTGCCACATCCTGAG-3′ in exon 4; reverse primer 5′-GTTGTCCCCTTCCAGGTTCTG-3′ in exon 6). Taqman Ribosomal RNA Control Reagents (Applied Biosystems, Foster City, USA) designed to the Ribosomal *S18* RNA amplification and primers designed to the *β-actin* gene amplification (forward primer 5′-AGCCTCGCCTTTGCCGA-3′; reverse primer 5′-GCGCGGCGATATCATCATC-3′) were used as reference to normalize the results.

Results

LOH analysis

Allele sizes in 50 DNA samples extracted from blood from unrelated European patients with hepatocellular carcinomas varied from 151 to 197 bp for WFDC1-GT20 and from 204 to 212 bp for WFDC1-GT12. Heterozygosity was observed in 36 of 50 individuals (72%) for WFDC1-GT20 and 21 of 50 individuals (42%) for WFDC1-GT12. For the two polymorphisms combined, informativity was 86% among the 50 individuals analysed. DNAs from 46 hepatocellular carcinoma samples and from paired peripheral blood samples were subjected to PCR. Forty were informative. An example of LOH assessment is shown in Figure 1. We unequivocally identified LOH in the *WFDC1* gene in 4/40 (10%) of the informative hepatocellular tumour samples.

Of the five cell lines analysed, only the HepG2 cell line showed two distinct alleles indicative of the absence of LOH.

Mutation analysis

In the aim to precise the role of *WFDC1* in hepatocarcinogenesis, tumour samples from patients with LOH and cell lines were screened for mutation in the coding exons of the *WFDC1* gene (exons 1 to 4). No nucleotide change resulting in amino acid change was detected.

Promoter methylation analysis

We further analysed the methylation status of the 5′ promoter region of the *WFDC1* gene. Methylation status was classified as not significant (<20%), partial (20–80%) or full (>80%). Of the five cell lines analysed, two showed no significant *WFDC1* promoter methylation and three exhibited partial methylation. In isolated hepatocytes the promoter was not significantly methylated (Table 2a). No significant difference in promoter methylation was neither observed between tumour samples and the corresponding non tumorous liver from patients exhibiting *WFDC1* LOH in their tumour (Table 2b). In eight normal liver samples (obtained from donors during graft harvesting), promoter methylation was low (9%, SD 8%) (data not shown).

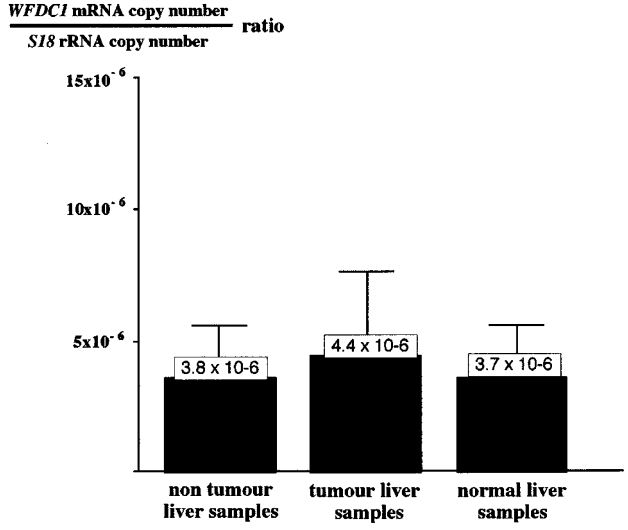

Figure 2 *WFDC1* gene expression in 4 HCC with LOH, the corresponding non tumour samples and in eight normal livers.

Table 2a *WFDC1* promoter methylation and gene expression in cell lines and isolated hepatocytes

	Methylation		*Gene expression*	
Cell line	%	(SD)	($\times 10^{-6}$)	(SD)
HA22TNGH	7	(10)	0.03	(0.01)
HepG2	60	(12)	0	(0)
PLC/PRF/C	9	(4)	0.06	(0.03)
MAHLAVU	52	(5)	0.33	(0.20)
TONG	32	(4)	0.05	(0.01)
Isolated hepatocytes	2	(2)	0.18	(0.15)

Table 2b *WFDC1* promoter methylation and gene expression (normalized to S18 RNA) in tumour and non-tumour samples from patients exhibiting LOH on the *WFDC1* locus

	Non-tumour sample					*Tumour sample*				
	Methylation		*Gene expression*			*Methylation*		*Gene expression*		
Patient	%	(SD)	($\times 10^{-6}$)	(SD)	*LOH*	%	(SD)	($\times 10^{-6}$)	(SD)	*LOH*
Patient 1	1	(2)	5.7	(1.2)	(−)	5	(3)	3.8	(1.5)	(+)
Patient 2	7	(2)	3.0	(1.5)	(−)	2	(2)	0.8	(0.5)	(+)
Patient 3	53	(10)	4.5	(3.0)	(−)	38	(9)	8.2	(2.6)	(+)
Patient 4	28	(5)	2.0	(1.3)	(−)	25	(7)	4.9	(2.1)	(+)

Analysis of *WFDC1* gene expression in patients' samples and cell lines

Gene expression was quantified in the four tumour samples exhibiting LOH comparatively to the four corresponding non tumour liver samples which were exempt of LOH (Table 2b). No significant up or down variation was observed between tumour, corresponding non tumour and normal liver samples (n=8) (Figure 2). *WFDC1* mRNA/*β-actin* mRNA copy number ratio was 2.6×10^{-4} (SD 3×10^{-4}) in normal liver samples. No significant difference was observed when *β-actin* was used to normalize the results (data not shown). In contrast, low level of gene expression was found in isolated hepatocytes preparations. Little or no gene expression was also found in hepatocellular carcinoma cell lines (Table 2a). Paradoxically, *WFDC1* gene expression in MAHLAVU cell line was higher than that found in isolated hepatocytes although the level of its *WFDC1* promoter methylation was higher. In HepG2 cell line where no expression of *WFDC1* was observed, the two alleles of the genes were present and methylation of the promoter was similar to that of MAHLAVU.

Discussion

A high frequency of LOH has been observed on chromosome 16q (33 – 70%) in hepatocellular carcinoma.[16] We report here LOH in the *WFDC1* gene in 10% of informative hepatocellular tumour samples. This low percentage can not be attributed to contamination of tumour DNA by DNA from non-tumourous cells since we selected samples containing more than 70% tumourous cells. These results suggest that LOH at the *WFDC1* locus does not play a major part in European HCC.

Furthermore, absence of inactivating mutation of the *WFDC1* gene in patients exhibiting LOH does not support a role of *WFDC1* as a tumour suppressor gene.

Gene expression was observed in liver samples but no significant variation was observed between normal, non tumour and tumour samples. In contrast, little or no gene expression was found in isolated hepatocytes preparations and in hepatocellular carcinoma cell lines. These observations show that hepatocytes are not the main source of *WFDC1* mRNA in liver.

DNA hypermethylation on 16q may be associated with LOH in HCC.[17,18] In order to assess whether inactivation of *WFDC1* could occur in hepatocytes via DNA methylation of the promoter region, DNA methylation of the 5′ sequences was measured. Absence or low level of DNA methylation was observed in tumour samples exhibiting LOH at the *WFDC1* locus and partial DNA methylation (32 – 60%) was observed in three hepatocellular carcinoma cell lines (HepG2, MAHLAVU and TONG) analysed. In HepG2, promoter methylation might contribute to the absence of expression of the gene although LOH is not observed. In MAHLAVU and TONG, mRNA expression did not seem related to promoter methylation.

In summary, we did not find frequent alterations of the *WFDC1* gene in HCC. Taken together, these results are not in favour of a major role of *WFDC1* as a tumour suppressor gene in this tumour type. However, we have described here a technique using two new microsatellite markers to identify LOH that is rapid and also informative for a large percentage of individuals. DNA promoter methylation analysis and sequencing of the *WFDC1* gene are also described. As these assays are PCR-based, analysis of archival DNA is possible. Therefore, it may be used for retrospective analysis at the *WFDC1* gene in paraffin-embedded tissue allowing investigation of both its tumour suppressor role and its prognostic value in other tumour types, preferentially in those known to show frequent 16q24 alterations.

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