Expression of WISPs and of Their Novel Alternative Variants in Human Hepatocellular Carcinoma Cells

MELCHIORRE CERVELLO,[a] LYDIA GIANNITRAPANI,[b]
MANUELA LABBOZZETTA,[b] MONICA NOTARBARTOLO,[c]
NATALE D'ALESSANDRO,[c] NADIA LAMPIASI,[a] ANTONINA AZZOLINA,[a]
AND GIUSEPPE MONTALTO[b]

[a]*Istituto di Biomedicina e Immunologia Molecolare, CNR, Palermo, Italy*

[b]*Dipartimento di Clinica Medica e Patologie Emergenti, and* [c]*Dipartimento di Scienze Farmacologiche, Università di Palermo, Palermo, Italy*

ABSTRACT: WISPs (Wnt-induced secreted proteins) are members of the CCN (CTGF/Cyr61/Nov) family involved in fibrotic disorders and tumorigenesis. They have a typical structure composed of four conserved cysteine-rich modular domains, but variants of CCN members lacking one or more modules, generated by alternative splicing or gene mutations, have been described in various pathological conditions. WISP genes were first described as downstream targets of the Wnt signaling pathway, which is frequently altered in human hepatocellular carcinoma (HCC). In the present study, WISP mRNA expression was analyzed by RT-PCR in four human HCC cell lines (HepG2, HuH-6, HuH-7, HA22T/VGH). Our results show for the first time that WISP1, WISP1v, and WISP3 are expressed in HCC cell lines. Moreover, we identified two novel variants, generated by alternative splicing of WISP1 and WISP3, respectively, named WISP1Δex3-4 and WISP3v$_L$. Overall, our study suggests that WISP transcripts may have a role in the development of HCC, although further studies are necessary to clarify the relative importance of the expression of wild-type WISPs, as well as of their novel variants, in this tumor type.

KEYWORDS: hepatocellular carcinoma; WISP; CCN; Wnt; alternative splicing

INTRODUCTION

WISP proteins are members of the CCN family, which includes connective tissue growth factor (CTGF/CCN2), cysteine-rich 61 (Cyr61/CCN1), and nephroblastoma overexpressed (Nov/CCN3) proteins. They have been described as being involved in several processes, such as apoptosis, mitosis, cell adhesion, the production of extracellular matrix, and angiogenesis. Most important, they seem to have an unfavorable role in human fibrotic and cancer disorders.[1] WISPs have a typical structure com-

Address for correspondence: Dr. Melchiorre Cervello, Istituto di Biomedicina e Immunologia Molecolare, CNR, Via Ugo La Malfa 153, 90146 Palermo, Italy. Voice: +39 091-6809-534; fax: +39 091-6809-548.
cervello@ibim.cnr.it

Ann. N.Y. Acad. Sci. 1028: 432–439 (2004).
doi: 10.1196/annals.1322.051

posed of four conserved cysteine-rich modular domains, encoded by separate exons, which share sequence similarities with the insulin-like growth factor binding proteins (IGF-BPs), Von Willebrand factor type C (VWC), thrombospondin (THBS), and the cysteine knots (CKs). The multimodular architecture of the CCN proteins, along with the production of some of their truncated isoforms in tumors, raises interesting questions regarding the participation of each individual module in the various biological properties of these factors. Their roles can in fact be different, depending on the tumor and on the cell type.[1]

Accordingly, it has been shown that while the expression of CTGF/CCN2 induces cell death,[2] WISP1/CCN4 expression inhibits apoptosis induced by p53[3] and c-myc.[4] WISP3/CCN6 acts as an oncosuppressor gene in breast cancer,[5] but it is oncogenic in colorectal cancer.[6] In hormone-related tumors, such as carcinomas of the uterus or breast, the activation by sex steroids of the Cyr61/CCN1, CTGF/CCN2, and WISP2/CCN5 genes promotes cancer progression.[7–9] However, in colorectal cancer WISP2/CCN5 acts as an oncosuppressor gene.[6] Importantly, the presence of variants of the CCN proteins, lacking one or more modules and generated by alternative splicing or gene mutations, has been described in different pathological conditions, including the development and progression of some tumor types.[10–12] It must also be said that among the members of the CCN family, the WISPs arouse particular interest in liver cancer, because their coding genes were first described as downstream targets of the Wnt signaling pathway,[6] which is one of the pathways most frequently altered in human hepatocellular carcinoma (HCC).[1]

The aim of the present study was to shed more light on the role of WISPs in HCC, starting from the examination, by reverse transcriptase–polymerase chain reaction (RT-PCR), of the expression of their transcripts in four human HCC cell lines with different origins, grades of differentiation, and genetic profiles.

MATERIALS AND METHODS

Cell Cultures

Four different human hepatoma cell lines (HepG2, HuH-6, HuH-7 and HA22T/VGH) were used in this study. All cell lines were maintained in MEM (Sigma, Milan, Italy) supplemented with 10% heat-inactivated fetal calf serum (HyClone Europe), 1% L-glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin solution (all from Sigma, Milan, Italy). Cells were grown as adherent cells in a humidified atmosphere at 37°C in 5% CO_2. They were subcultured after trypsinization (with 0.05% trypsin/0.02% EDTA in phosphate-buffered saline solution from HyClone Europe) for 5 min at 37°C, washing, and resuspension in complete medium. Cells with a narrow range of passage number were used for all experiments.

Extraction of Cellular RNA and RT-PCR

Total RNA was extracted from all cell lines in the presence of TRIzol reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. Total RNA was then used for RT-PCR analyses using WISP-specific primers and β-actin as a control for mRNA quality. Both cDNA synthesis and PCR were performed using the SU-

PERSCRIPT One-Step RT-PCR method (Invitrogen, Milan, Italy). First-strand cDNAs were obtained after 30 min at 55°C. Following inactivation at 94°C for 2 min, PCR amplification was performed under the following reaction conditions: 94°C for 45 s, 60°C for 45 s, 72°C for 1 min, for a total of 35 cycles, and a final extension at 72°C for 10 min. All PCR products (10 μL) were analyzed by electrophoresis on 1.5% agarose gel stained with ethidium bromide and photographed.

The sequences of primers used in the RT-PCR were as follows:

WISP1-31F (forward): 5′-AGAGGTGGTCGGATCCTCTG-3′;
WISP1-1234R (reverse): 5′-CAGGCATTGGGTTAGTCCCC-3′;
WISP1-717R: 5′-TCCACCTCACCCACAGCATGTGC-3′;
WISP1-912R: 5′-GCCAGACACTTCTTCCCTGCGTGC-3′;

WISP2-70F: 5′-CCAACTGCACCTCGGTTCTATC-3′;
WISP2-1037R: 5′-TGGACCCAAGCTAAAGTGTTGC-3′;

WISP3-1F: 5′-CACGGTCCCAGCGACATGCA-3′;
WISP3-559F: 5′-AACCATTACTACAGCAGCTTTCAAC-3′;
WISP3-943R: 5′-GATACAGCATCTCTTATCCAAGCAT-3′;
WISP3-1095R: 5′-CCCATTTGCTTGGTTTTACAG-3′.

Sequence of RT-PCR Products

The RT-PCR products were purified by chromatography and sequenced using the dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA) and an ABI 373 sequencer (Perkin Elmer). Analysis of the nucleotide sequences was performed with Chromas version 1.6 software (Technelysium Pty. Ltd., Halensvale, Australia).

RESULTS

To characterize the types of WISP transcripts expressed by the HCC (HepG2, HuH-6, HuH-7 and HA22T/VGH) cell lines, we first isolated total RNA and then analyzed the expression of WISP mRNA by a specific RT-PCR procedure. As an initial screening, we used different forward and reverse primers specific for the coding regions of WISP1/CCN4 and WISP3/CCN6 (FIGS. 1 and 2) or WISP2/CCN5 (not shown). As shown in FIGURE 3A, we observed the expression of the expected normal transcript of WISP1/CCN4 (1204 bp) only in the HuH-6 and HA22T/VGH cell lines. In these cells there were also two shorter transcripts of 943 and 750 bp, respectively. Sequence analysis of the purified 943-bp fragment revealed that this variant lacks exon 3 and consequently is identical to the splicing variant that had already been described in scirrhous gastric carcinoma and cholangiocarcinoma and referred to as *WISP1variant* (WISP1v).[10,11] The 750-bp fragment appeared to be a novel shorter variant, which we named *WISP1Δex3-4*. In fact, the sequence analysis of its amplified product revealed that this transcript lacks both exons 3 and 4. Furthermore, the presence of WISP1v and of the novel WISP1Δex3-4 transcript was confirmed using a RT-PCR assay, in which the reverse primers (WISP1-717R and WISP1-912R, respectively, FIG. 1) were based on the sequences resulting from the joining of exons 2 and 4 for WISP1v and of exons 2 and 5 for WISP1Δex3-4 (data not shown).

With regard to the possible translation of the mRNAs into proteins, in the case of WISP1v the joining of exons 2 and 4 did not cause any shift in the reading frame.

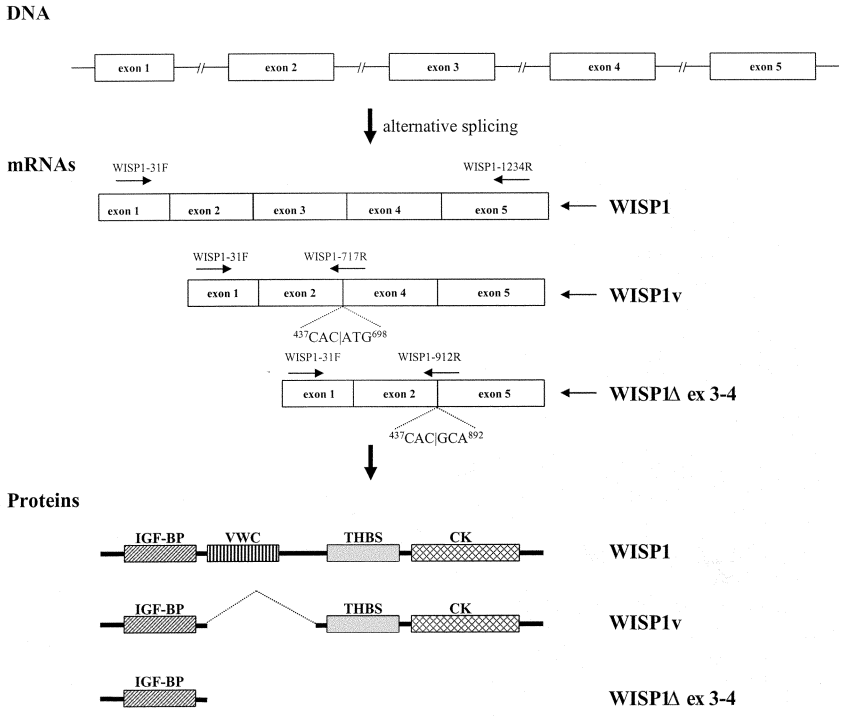

FIGURE 1. Schematic representation of WISP1 gene, mRNA transcripts, and predicted proteins. The positions of the primers are indicated.

Therefore, the predicted truncated protein lost only the second VWC module (FIG. 1). On the other hand, the joining of exons 2 and 5 in the WISP1Δex3-4 splice variant caused a frameshift at residue 117 that led to a premature stop 38 residues downstream. Consequently, the predicted protein retained only the first module, IGF-BP (FIG. 1).

We also analyzed the four HCC cell lines for the expression of WISP2/CCN5 mRNA using a specific pair of primers, but none of the cell lines expressed such a transcript (data not shown).

In the case of WISP3/CCN6, using primers WISP3-1F and WISP3-1095R (FIG. 2), only HuH-6 and HuH-7 cells expressed the expected regular form of WISP3 mRNA (1095 bp), together with a longer transcript, which we named *WISPvariant long* ($WISP3v_L$) (data not shown). Thereafter, to better define the position where the insertion occurs we used in the RT-PCR experiments a different pair of primers (WISP3-559F and WISP3-943R respectively, FIG. 2). Also in this case, together with the expected fragment of 384 bp we found a longer transcript of 448 bp (FIG. 3B). The sequence analysis of the amplified 448-bp fragment revealed that it results from the insertion of a new exon of 64 bp between exons 4 and 5 (FIG. 2). This insertion caused a frameshift at residue 197, leading to a premature stop 22 residues down-

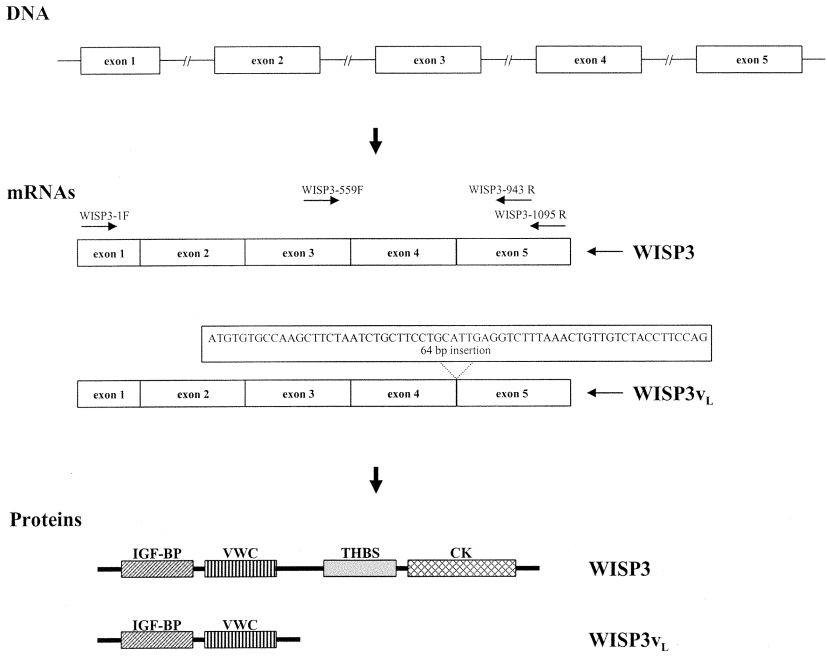

FIGURE 2. Schematic representation of WISP3 gene, mRNA transcripts, and predicted proteins. The positions of the primers are indicated.

stream. Therefore, the predicted truncated protein lacked both the third module (THBS) and the fourth module (CK) (FIG. 2). Deletion of one nucleotide in the repetitive sequence (A)9 in exon 4 ($WISP3_{(A)8}$) has been reported to be present in 31% of MSI-H colorectal carcinomas.[12] In our study, sequence analyses of both WISP3 and $WISP3v_L$ fragments revealed the presence of the normal (A)9 sequence in HCC cells (data not shown).

DISCUSSION

Hepatocarcinogenesis is considered a typical "multistep" process in which different genomic alterations occur, accompanied by the aberrant expression of growth and survival factors. Among the several oncogenic pathways that have been implicated in the malignant transformation of liver cells, one of the most frequently altered is the Wnt/β-catenin pathway, owing to mutations of the *β-catenin* or *Axin* genes.[13]

The CCN (CTGF/Cyr61/Nov) proteins constitute a family of regulatory factors involved in many processes related to cell proliferation and differentiation. An increasing body of evidence indicates that abnormal expression of the CCN proteins is associated with tumorigenesis.[1] Among the CCN members, the WISPs are of par-

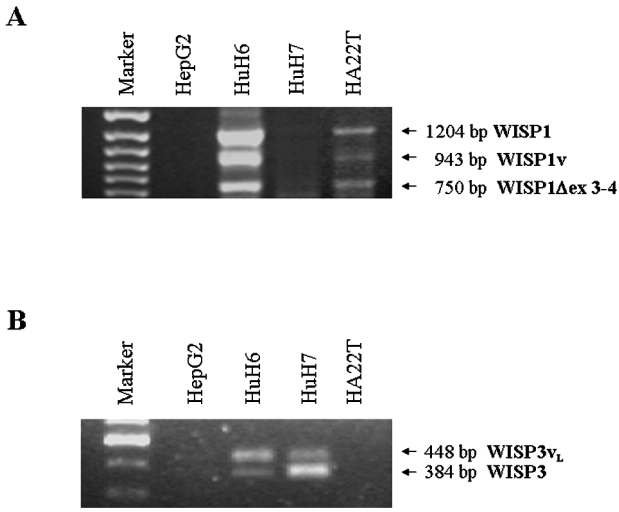

FIGURE 3. RT-PCR analysis of WISP1 and WISP3 mRNAs expression in human HCC cells. Total RNA was isolated, and RT-PCR was performed as indicated in MATERIALS AND METHODS using a specific pair of primers for WISP1 and WISP3 transcripts. Marker = 100 bp.

ticular interest in liver cancer, because their coding genes were first described as downstream targets of the Wnt/β-catenin signaling pathway.[6]

Previous studies have shown that the wild-type forms of WISP1 and WISP3, together with their variant forms (WISP1v and WISP3$_{(A)8}$, respectively) can be expressed in various human tissues and cell types.[6,10–12] The alternative transcripts lead to the production of truncated proteins, which retain different modules of the original structure.

In an attempt to identify genetic defects that could contribute to HCC development, we studied the expression of WISP mRNAs in four HCC cell lines. They had different differentiation grades and origins. HuH-7, a well-differentiated cell line, is derived from an HBV-negative HCC. HA22T/VGH is a poorly differentiated hepatoma cell line, which contains HBV integrants. HuH-6 and HepG2 are two well-differentiated hepatoblastoma-derived cell lines with activating mutations of the *β-catenin* gene.[14,15] In particular, HepG2 cells express high levels of a mutated β-catenin molecule of about 73 kDa, as well as a 92-kDa wild-type form; while HuH-6 cells have a point mutation in exon 3 of the *β-catenin* gene, which results in the substitution of glycine for valine at codon 34. HA22T/VGH (Cervello *et al.*, unpublished data) and HuH-7[15] cells express only the wild-type form of β-catenin.

The human HCC cell lines of this study expressed different WISP transcripts. Expression of WISP1/CCN4 was observed only in HuH-6 and HA22T/VGH. In these cells there was also the already-described variant WISP1v,[10,11] together with a novel shorter variant, WISP1Δex3-4. Sequence analysis revealed that WISP1v lacks exon 3, while WISP1Δex3-4 lacks both exons 3 and 4. With regard to WISP3/CCN6, only

HuH-6 and HuH-7 expressed wild-type WISP3 mRNA, together with a longer transcript, WISP3v$_L$. Sequence analysis of WISP3v$_L$ revealed that it results from the insertion of a new 64-bp exon between exons 4 and 5. Overall, these results suggested that WISP mRNA expression in HCC is not related to the grades of cell differentiation or to the origin of the cells. Moreover, WISP expression was independent of the presence of an activated Wnt/β-catenin signaling, as in HA22T/VGH and HuH-7, suggesting that other pathways may also be involved in the control of WISP gene transcription in HCC cells.

In the case of WISP2/CCN6, none of the cell lines expressed its mRNA. This result may be in agreement with a recent study on the rat orthologue of WISP2/CCN6, which described the loss of expression of this gene after cell transformation and suggested that it may act as a negative regulator of growth or even as a tumor suppressor.[16]

Overall, we suggest that WISP transcripts, like other CCNs, may play a role in hepatocarcinogenesis. Further investigation should confirm the expression of these molecules in primary human HCC tissues as well as providing a better understanding of their differential functions and modes of regulation. Further analyses of the novel WISP alternative splicing variants described here would be helpful in this respect.

REFERENCES

1. PLANQUE, N. & B. PERBAL. 2003. A structural approach to the role of CCN (CYR61/CTGF/NOV) proteins in tumourigenesis. Cancer Cell Int. **3:** 15.
2. HISHIKAWA, K., B.S. OEMAR, F.C. TANNER, *et al*. 1999. Connective tissue growth factor induces apoptosis in human breast cancer cell line MCF-7. J. Biol. Chem. **274:** 37461–37466.
3. SU, F., M. OVERHOLTZER, D. BESSER & A.J. LEVINE. 2002. Wisp-1 attenuates p53-mediated apoptosis in response to DNA damage through activation of the Akt kinase. Genes Dev. **16:** 46–57.
4. YOU, Z., D. SAIMS, S. CHEN, *et al*. 2002. Wnt signaling promotes oncogenic transformation by inhibiting c-Myc-induced apoptosis. J. Cell Biol. **157:** 429–440.
5. KLEER, C.G., Y. ZHANG, Q. PAN, *et al*. 2002. WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. Oncogene **21:** 3172–3180.
6. PENNICA, D., T.A. SWANSON, J.W. WELSH, *et al*. 1998. WISP genes are members of the connective tissue growth factor family that are up-regulated in Wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc. Natl. Acad. Sci. USA **95:** 14717–14722.
7. SAMPATH, D., R.C. WINNEKER & Z. ZHANG. 2001. Cyr61, a member of the CCN family, is required for MCF-7 cell proliferation: regulation by 17 beta-estradiol and overexpression in human breast cancer. Endocrinology **142:** 2540–2548.
8. XIE, D., K. NAKACHI, H. WANG, *et al*. 2001. Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. Cancer Res. **61:** 8917–8923.
9. BANERJEE, S., N. SAXENA, K. SENGUPTA, *et al*. 2003. WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. Neoplasia **5:** 63–73.
10. TANAKA, S., K. SUGIMACHI, H. SAEKI, *et al*. 2001. A novel variant of WISP1 lacking a Von Willebrand type C module overexpressed in scirrhous gastric carcinoma. Oncogene **20:** 5525–5532.
11. TANAKA, S., K. SUGIMACHI, T. KAMEYAMA, *et al*. 2003. Human WISP1v, a member of the CCN family, is associated with invasive cholangiocarcinoma. Hepatology **37:** 1122–1129.

12. THORSTENSEN, L., C.B. DIEP, G.I. MELING, *et al*. 2001. Wnt1 inducible signaling pathway protein 3, WISP-3, a novel target in colorectal carcinoma with microsatellite instability. Gastroenterology **121:** 1275–1280.
13. BUENDIA, M.A. 2002. Genetic alterations in hepatoblastoma and hepatocellular carcinoma: common and distinctive aspects. Med. Pediatr. Oncol. **39:** 530–535.
14. CARRUBA, G., M. CERVELLO, M.D. MICELI, *et al*. 1999. Truncated form of β-catenin and reduced expression of wild-type catenins feature HepG2 human liver cancer cells. Ann. N.Y. Acad. Sci. **886:** 212–216.
15. DE LA COSTE, A., B. ROMAGNOLO, P. BILLUART, *et al*. 1998. Somatic mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc. Natl. Acad. Sci. USA **95:** 8847–8851.
16. ZHANG, R., L. AVERBOUKH, W. KHU, *et al*. 1998. Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. Mol. Cell. Biol. **18:** 6131–6141.