Truncated Form of β-Catenin and Reduced Expression of Wild-Type Catenins Feature HepG2 Human Liver Cancer Cells

GIUSEPPE CARRUBA, [a,e] MELCHIORRE CERVELLO,[b] MARIA D. MICELI,[a] ROSARIA FARRUGGIO,[a] MONICA NOTARBARTOLO,[c] LUCREZIA VIRRUSO,[b] LYDIA GIANNITRAPANI,[d] ROBERTO GAMBINO,[b] GIUSEPPE MONTALTO,[d] AND LUIGI CASTAGNETTA[a,c]

[a]*Institute of Oncology, and* [d]*Institute of Internal Medicine, University of Palermo, Palermo, Italy*

[b]*Institute of Developmental Biology, C.N.R., Palermo, Italy*

[c]*Experimental Oncology, Palermo Branch of IST-Genoa, c/o M. Ascoli Cancer Hospital Center, Palermo, Italy*

The cadherins are a family of calcium-dependent cell to cell adhesion molecules that are primarily involved in both embryonic morphogenesis and structural and functional preservation of adult tissues.[1] Cadherin function is mediated by a group of cytoplasmic proteins, referred to as α-, β- and γ-catenin (plakoglobin). Assembly of the cadherin/catenin complex is accomplished by linkage of E-cadherin to the actin microfilaments of cytoskeleton through both γ- and β-catenin.[2] Therefore, altered expression of the catenin molecules may be responsible for disturbance of the cadherin-mediated adhesion system, eventually leading to the onset of an invasive cell phenotype.[3,4] Reports on the cadherin/catenin system and its regulation in human liver tumor tissues remain sparse and occasionally controversial. In the present work, we compared expression of the various catenins in a human liver cancer cell line (HepG2) with that in a nonneoplastic human hepatocytic cell line (Chang liver) using Western blotting analysis. We also used reverse transcriptase-polymerase chain reaction (RT-PCR) and DNA sequencing analyses to inspect the structure and expression of the β-catenin gene.

MATERIALS AND METHODS

Cell Cultures. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Both culture medium and cells were periodically tested for mycoplasma contamination. Cells with a narrow range of passage number were used for all experiments.

Gel Electrophoresis and Immunoblotting. Chang liver and HepG2 cells were lysed for 20 minutes in a lysis buffer containing 20 mM TRIS, pH 7.4, 1% Triton, 1%

[e]Address for correspondence: Institute of Oncology, Via Marchese Ugo 56, 90141 Palermo, Italy. Phone +39 091 666-4346; fax, +39 091 666-4352.
e-mail, lucashbl@unipa.it

Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Missouri). Cell lysates were centrifuged at 12,000 × *g* for 10 minutes at 4°C and the resulting supernatants recovered. Sample protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories SrL, Milan, Italy). Fifty microgram protein aliquots of each supernatant were mixed with reducing 3x Laemmli sample buffer and subjected to SDS-PAGE, as originally described by Laemmli.[5] Proteins were electrophoresed onto a nitrocellulose membrane (Amersham) and membranes probed with a 1:200 to 1:1500 dilution of anti-α, anti-β, or anti-γ-catenin antibody. Hybridization was visualized using an ECL detection kit (Amersham Italia SrL, Milan, Italy), according to the manufacturer's instructions. Relative estimates of hybridized bands were carried out with a GS-670 Bio-Rad Densitometer using the Molecular Analyst/PC image analysis software (version 1.1).

Extraction of Cellular RNA and Reverse-Transcriptase-PCR (RT-PCR). Total RNA was extracted from Chang liver and HepG2 cells by the guanidinium-thiocyanate method according to Chomczynski and Sacchi.[6] Prior to cDNA preparation, all RNA was treated with 5 U of DNAse I (Boehringer). cDNA was made from 5 μg of total RNA using a First-Strand Synthesis Kit (Amersham). PCR was then performed using 1–2 μl of cDNA solution as template. Two pair of primers were designed to examine the entire coding sequence of β-catenin gene into two separate regions. The sequences of the primers used were as follows: S1 (sense), corresponding to nucleotides +192 to +211; A1 (antisense), corresponding to nucleotides +1101 to +1120; S2, corresponding to nucleotides +763 to +782; and A2, corresponding to nucleotides +2590 to +2609. The primers were designed according to the EMBL/GenBank cDNA sequence (accession No. X87838). PCR fragments were analyzed by 1.5% agarose gel electrophoresis.

DNA Sequencing. Primer A888 (corresponding to nucleotides +868 to +888) was designed to amplify a short N-terminal region of β-catenin mRNA. The PCR products obtained using primers S1 and A888 were separated by agarose gel electrophoresis, and the fragment of interest was extracted. Each fragment was cloned using the TOPO-TA cloning kit (Invitrogen). Purified DNA containing β-catenin cDNA was used in a thermocycle sequencing reaction with the Thermo sequenase-radiolabeled terminator cycle sequencing kit (Amersham). All the sequencing reactions were performed using the S1 and A888 primers.

RESULTS

Western blotting experiments revealed expression of normal α-catenin in the two cell lines studied, although HepG2 cells expressed significantly smaller amounts of this 102-kD component with respect to Chang liver cells (FIG. 1A). Analysis of β-catenin expression showed that whereas both cell lines contained a normal 92-kD protein, markedly reduced levels were found in HepG2 cells. In the latter cell line a smaller component of about 75 kD was coexpressed with the wild-type β-catenin protein (FIG. 1B); this resulted in an overall increase of β-catenin expression levels. Greater amounts of γ-catenin were found in Chang liver cells, while again HepG2 cells exhibited a pronounced reduction in γ-catenin expression (FIG. 1C). Relative

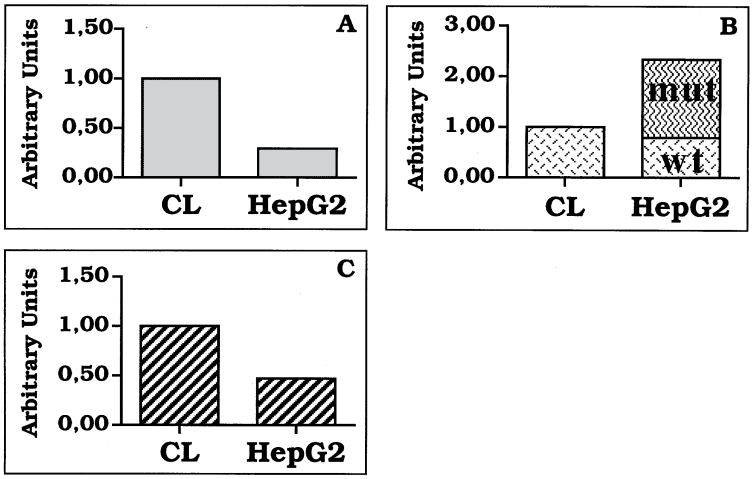

FIGURE 1. Quantification of α-catenin **(A)**, β-catenin **(B),** and γ-catenin **(C)** expression in Chang liver and HepG2 cells. Histograms illustrate results obtained by densitometric analysis of Western blotting experiments. mut, mutated form; wt, wild type.

estimates of catenin levels in Chang liver and HepG2 cells revealed a clear increase (2.3-fold) of total β-catenin amounts in HepG2 cells, whereas expression of wild-type α-, β-, and γ-catenins was reduced in this cell line, respectively, being 29%, 78%, and 47% of that seen in Chang liver cells. The potential deletion of β-catenin mRNA in HepG2 cells was investigated by means of RT-PCR. Agarose gel electrophoresis showed that the S1-A1 PCR product from HepG2 cells gave rise to two separate bands, one corresponding to the full-length β-catenin message and one additional band about 350-bases shorter (not shown). Sequencing analysis of the PCR-amplified 5'-terminal S1-A888 fragments from HepG2 revealed a 348-base in-frame deletion from position 287 to 634 of the shorter fragment, resulting in a 116 amino acid deletion (from Trp^{25} to Ile^{140}) (FIG. 2). As a consequence, the mutated β-catenin molecule consisted of 665 amino acids, with an expected molecular mass of about 73 kD. No sequence mutation was detected in the full-length fragment (not shown).

DISCUSSION

In the present work we report evidence of a truncated form of β-catenin in HepG2 human liver cancer cells, accompanied by a significant decrease in the expression of wild-type α-, β- and γ-catenins. Because both γ- and β-catenins represent an essential requirement for formation of the cadherin/catenin complex, it seems likely that loss of catenin results in alteration of the E-cadherin–mediated cell-cell adhesion system in HepG2 cells. RT-PCR and DNA sequencing analyses showed that HepG2 cells contain a deletion of β-catenin at the NH_2-terminal region of the protein, namely at the potential phosphorylation site of glycogen synthase kinase-3β (GSK-3β).

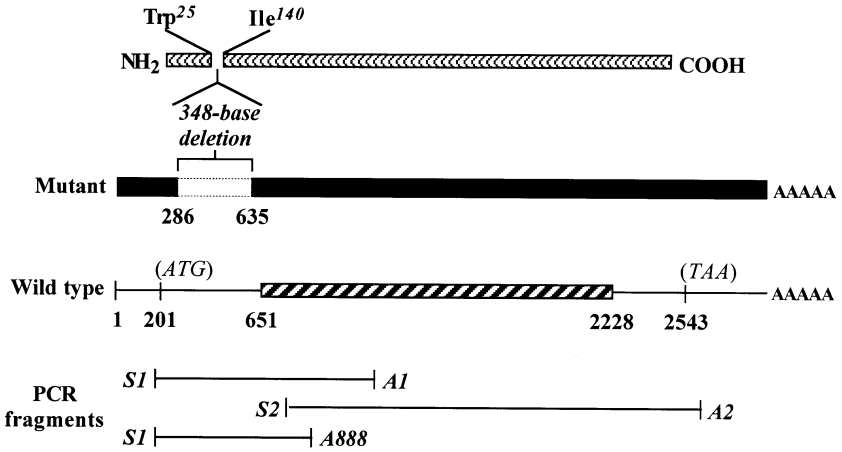

FIGURE 2. Sequencing analysis for mutant β-catenin protein and mRNA in HepG2 cells. The 348-base deletion and resulting 116 amino acid deletion are indicated. Cross hatching, the 13-cycle repeat sequence domain. *ATG*, initiation codon; *TAA*, stop codon. The extension of both sense (S1, S2) and antisense (A1, A2) primers used for RT-PCR as well as that of the primer set (S1, A888) used for DNA sequencing is reported. Numbers indicate the nucleotide positions at boundaries.

This may eventually lead to accumulation of a hypophosphorylated form of β-catenin, because the GSK-3β–induced phosphorylation of β-catenin promotes its turnover.[7] Recently, de La Coste and colleagues detected point mutations or deletions of the β-catenin gene in both human liver tumors and hepatoma cells, leading to accumulation of the β-catenin in the nucleus.[8] All this strongly supports the hypothesis that β-catenin mutations and/or hindrance of its turnover may be involved in human liver carcinogenesis. Further investigation should define the potential biologic role of β-catenin derangements during development of human hepatocellular carcinoma and ascertain whether similar variations are present in both precancerous lesions and primary tumors of the liver.

ACKNOWLEDGMENTS

This study was funded in part by the Italian Association for Cancer Research (AIRC, to L.C.) and by research grants from Italian MURST 60% (to L.C. and G.M.). The authors are grateful to Dr. De Pasqua (Zeneca Pharmaceuticals, Milan, Italy) for financial support of the present study. The authors wish to thank Dr. A. Giallongo (Palermo, Italy) for fruitful discussion and continuous support.

REFERENCES

1. TAKEICHI, M. 1991. Cadherin cell adhesion receptor as a morphogenetic regulator. Science (Washington, DC) **251:** 1451–1455.

2. KEMLER, R. 1993. From cadherins to catenins: Cytoplasmic protein interactions and regulation of cell adhesion. Trends Genet. **9:** 317–321.
3. SHIMOYAMA, Y., A. NAGAFUCHI, S. FUJITA, M. GOTOH, M. TAKEICHI, S. TSUKITA & S. HIROHASHI. 1992. Cadherin dysfunction in a human cancer cell line: Possible involvement of loss of α-catenin expression in reduced cell-cell adhesiveness. Cancer Res. **52:** 5770–5774.
4. NAKANISHI, Y., A. OCHIAI, S. AKIMOTO, H. KATO, H. WATANABE, Y. TACHIMORI, S. YAMAMOTO & S. HIROHASHI. 1997. Expression of E-cadherin, α-catenin, β-catenin and plakoglobin in esophageal carcinomas and its prognostic significance. Oncology **54:** 158–165.
5. LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685.
6. CHOMCZYNSKI, P. & N. SACCHI. 1987. Single-step method of RNA isolation by acidic guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.
7. YOST, C., M. TORRES, J.R. MILLER, E. HUANG, D. KIMELMAN & R.T. MOON. 1996. The axis-inducing activity, stability and subcellular distribution of β-catenin is regulated in *Xenopus* embryo by glycogen synthase kinase 3. Genes Dev. **10:** 1443–1454.
8. DE LA COSTE, A., B. ROMAGNOLO, P. BILLUART, C.A. RENARD, M.-A. BUENDIA, O. SOUBRANE, M. FABRE, J. CHELLY, C. BELDIORD, A. KAHN & C. PERRET. 1998. Somatic mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **95:** 8847–8851.