Short Communication

Frequent Nuclear/Cytoplasmic Localization of β -Catenin without Exon 3 Mutations in Malignant Melanoma

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β -Catenin has a critical role in E-cadherin-mediated cell-cell adhesion, and it also functions as a downstream signaling molecule in the *wnt* pathway. Mutations in the putative glycogen synthase kinase 3 β phosphorylation sites near the β -catenin amino terminus have been found in some cancers and cancer cell lines. The mutations render β -catenin resistant to regulation by a complex containing the glycogen synthase kinase 3 β , adenomatous polyposis coli, and axin proteins. As a result, β -catenin accumulates in the cytosol and nucleus and activates T-cell factor/lymphoid enhancing factor transcription factors. Previously, 6 of 27 melanoma cell lines were found to have β -catenin exon 3 mutations affecting the N-terminal phosphorylation sites (Rubinfeld B, Robbins P, Elgamil M, Albert I, Porfiri E, Polakis P: Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* 1997, 275:1790–1792). To assess the role of β -catenin defects in primary melanomas, we undertook immunohistochemical and DNA sequencing studies in 65 melanoma specimens. Nuclear and/or cytoplasmic localization of β -catenin, a potential indicator of *wnt* pathway activation, was seen focally within roughly one third of the tumors, though a clonal somatic mutation in β -catenin was found in only one case (codon 45 Ser→Pro). Our findings demonstrate that β -catenin mutations are rare in primary melanoma, in contrast to the situation in melanoma cell lines. Nonetheless, activation of β -catenin, as indicated by its nuclear and/or cytoplasmic localization, appears to be frequent in melanoma, and in some cases, it may reflect focal and

transient activation of the *wnt* pathway within the tumor. (*Am J Pathol* 1999, 154:325–329)

β -catenin is a 92-kd protein, initially identified as a co-precipitating species with the E-cadherin cell-cell adhesive complex.¹ β -catenin was subsequently shown to link E-cadherin to α -catenin, a vinculin-like protein that, in turn, links the E-cadherin/catenin complex to the cortical cytoskeleton.² Molecular cloning revealed that β -catenin is a member of the armadillo (*arm*) family of proteins, whose prototype molecule, Arm, functions as a downstream component of the wingless (*wnt*) signaling pathway in *Drosophila*.³

Although the role of β -catenin in mammalian cells is not completely understood, like the Arm protein, it has been shown to function in the *wnt*-signaling pathway. The present model relating β -catenin to the *wnt* pathway is the following. 1) Binding of the Wnt protein to the Frizzled transmembrane receptor activates the Disheveled protein, which, in turn, inhibits the activity of glycogen synthase kinase 3 β (GSK3 β). 2) GSK3 β , when complexed with the adenomatous polyposis coli (APC) tumor suppressor protein and the axin protein, appears to phosphorylate specific serine and threonine residues in the amino N-terminal region of β -catenin. 3) Phosphorylated β -catenin, but not the unphosphorylated form, is rapidly degraded by the ubiquitin-proteasome pathway. Hence, because *wnt* activation inhibits GSK3 β , *wnt* activation promotes accumulation of unphosphorylated β -catenin in the cell. 4) β -catenin can then complex with members of the T-cell factor (Tcf) or lymphoid enhancer factor (LEF) transcription factor family and activate expression of downstream Tcf/LEF-regulated target genes.⁴

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Mutational inactivation of the *APC* gene has been found in the majority of colorectal cancers, and, as might be predicted from the model above, constitutive activation of Tcf/LEF transcription activity is seen in such cases. In a subset of the colorectal cancers lacking *APC* mutations, mutations in the putative GSK3 β phosphorylation sites of β -catenin result in constitutive activation of Tcf/LEF transcriptional activity.⁵ Similar mutations in β -catenin exon 3 affecting its N-terminal phosphorylation sites have been described in other cancers and cancer cell lines, including melanoma cell lines,⁶ medulloblastoma,⁷ ovarian carcinoma,⁸ endometrial carcinoma,⁹ hepatocellular carcinoma,¹⁰ and prostatic adenocarcinoma.¹¹ In a few cancers, in-frame deletions of the N-terminal region of β -catenin have been detected.¹²

In this work, we sought to determine whether β -catenin signaling was frequently activated in primary melanomas. We determined the subcellular localization of β -catenin in 65 malignant melanoma specimens. In addition, sequencing of β -catenin exon 3 was carried out. Although nuclear and/or cytoplasmic localization occurs in nearly one third of melanomas, only one case was found to have a β -catenin mutation.

Materials and Methods

Specimen Acquisition and Patient Population

All cases of metastatic malignant melanoma accessioned to the cytology or surgical pathology service at Yale-New Haven Hospital from 1994 through 1996 were identified. Tumor samples were obtained through the Critical Technologies Program at the Yale University School of Medicine in accordance with Yale University Human Investigation Committee protocol #8219. Of the 81 metastatic melanoma cases from 1994 to 1996, 65 were evaluable for both the immunostaining and DNA analyses. These 65 specimens represent 18 cytology and 47 surgical specimens. For 12 patients, both surgical and cytology specimens were obtained and studied; however, the surgical and cytology specimens were from independent sites.

Immunostaining Procedures

Standard histological sections were cut from paraffin blocks and prepared for immunostaining using a pressure cooker antigen retrieval method.¹³ In brief, each section was baked at 60°C overnight, then deparaffinized, and treated for antigen retrieval by immersion in 6.5 mmol/L sodium citrate (pH 6.0) for 5 minutes in a conventional pressure cooker (KMart Inc.). Sections were then blocked with 3% bovine serum albumin in Tris-buffered saline (TBS) (150 mmol/L NaCl, 20 mmol/L Tris, pH 8). An anti- β -catenin monoclonal antibody (Transduction Labs, Lexington, KY) was diluted to 2 to 7 μ g/ml and incubated in a humidity chamber overnight, followed by seven TBS washes including 0.01% Triton X-100 in the 6th wash. For increased sensitivity and better subcellular localization of β -catenin, Cy3-conjugated secondary antibodies were used instead of conventional enzymatic

reaction-based chromogens. A Cy3-conjugated goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Labs, West Grove, PA) was diluted 1:500 in TBS with 3% bovine serum albumin and incubated with the sections for 1 hour before washing as above and coverslipping. Cytological specimens were prepared for immunohistochemical analysis using the Cytoc (Boxborough, MA) 2000 Thin Prep processor. Slides were rinsed for 5 minutes in tap water followed by 5 minutes in TBS. Slides were then blocked for 20 minutes with diluted normal serum and washed once in TBS before overlay with the β -catenin monoclonal antibody diluted 1:250 in TBS. After 30 minutes of incubation, slides were subjected to 3 washes with TBS for 5 minutes each. Then 200 μ l of Cy3-Goat anti-mouse antibody (Jackson ImmunoResearch Labs), diluted 1:500 in TBS with 3% bovine serum albumin, was incubated for 30 minutes. Slides were then washed and coverslipped.

DNA Purification

For the cytological specimens, cells in 1 to 20 ml were sedimented to remove the PreservCytTM (Cytoc) and then resuspended in digestion buffer containing 10 mmol/L Tris, pH 8, 100 mmol/L NaCl, 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate, and proteinase K to 100 mg/ml. Each specimen was incubated overnight at 37°C before extraction in an equal volume of phenol/chloroform. DNA was extracted once, precipitated with cold ethanol, washed with 70% ethanol, and resuspended in Tris/EDTA buffer. For the formalin-fixed and paraffin-embedded specimens, DNA was purified from five 20- μ mol/L thick sections using standard proteinase K and phenol/chloroform extraction methods.

Polymerase Chain Reaction (PCR) Amplification and Sequencing of β -Catenin

PCR was carried out with *Taq* polymerase on the genomic DNA samples from the cytology and surgical resection specimens using a β -catenin exon 2 forward primer (5'-CGTGGACAATGGCT-ACTCAA-3'), a β -catenin exon 4 reverse primer (5'-TGCATACTGTCCAT-CAATA-3'), and the following conditions: hot start at 95°C, 95°C for 45 seconds, 52°C for 45 seconds, and 72°C for 1 minute. The 700-bp product was purified from agarose gels using the GeneClean Kit (Bio101, Vista, CA). Sequencing of both strands was carried out using Thermo-Sequenase and ³³P-labeled ddNTPs (Amersham Life Science Inc., Arlington Heights, IL) with internal primers (5'-TGGGT-CATATCACATTCTTTT-3' and 5'-CTCT-TACCAGCTACTTGTCTTGA-3').

Results and Discussion

Previous studies have shown that activation of the *wnt* pathway results in an increase in the free cytoplasmic pool of β -catenin and its translocation to the nucleus, presumably via the binding of β -catenin to Tcf/LEF family

members.¹⁴ Mutations in the *APC* tumor suppressor gene or the N terminus of β -catenin itself have also been shown to result in increased levels of β -catenin, its localization in the nucleus, and Tcf/LEF transcriptional activation.^{6,15} Hence, we undertook studies to assess the subcellular localization and abundance of β -catenin in 65 melanoma specimens. As an internal control (for the surgical specimens), the localization of β -catenin at the membrane of endothelial cells in capillaries was observed. Membrane localization of β -catenin was seen in a fraction of the neoplastic cells in nearly all specimens. This result was expected because melanocytes have previously been shown to express N-cadherin and other types of cadherin on their cell surfaces,¹⁶ and, as with E-cadherin, β -catenin is bound to the cytoplasmic domain of N-cadherin. Although nearly all specimens showed strong membrane staining, some cases also showed increased cytoplasmic staining and nuclear staining for β -catenin. In this study, the primary goal was to score the neoplastic cells in the melanoma specimens for clear evidence of nuclear and/or cytoplasmic β -catenin staining compared with the membranous pattern seen in normal melanocytes.

We detected nuclear and/or cytoplasmic β -catenin staining in 10 of 18 cytology specimens and 8 of 47 surgical specimens. In the 10 cytology specimens with nuclear β -catenin staining, a high percentage of the cells showed strong staining of the nucleus (Figure 1). In contrast, in the eight surgical specimens with nuclear and/or cytoplasmic β -catenin staining, only 1 to 3% of the nuclei in a given field showed nuclear and/or cytoplasmic staining, whereas the remainder of the neoplastic cells displayed membranous staining for β -catenin. (Figure 2). Table 1 summarizes the staining patterns found. For 12 of the 47 surgical specimens, a cytology specimen from the same patient had also been obtained but from an independent metastatic lesion. Of these, 10 cases showed the same pattern on cytology and surgical specimens, including three cases in which both specimens showed nuclear localization and seven in which both showed membrane staining. In contrast to these 10 cases in which similar staining patterns were seen, in 2 of the 12 cases nuclear and/or cytoplasmic β -catenin staining was seen in the patient's cytology specimen, but only membranous staining was seen in the surgical specimen. The differences in the percentage of cells with nuclear β -catenin between cytology and surgical specimens and the two cases with discordant results on the cytology and surgical specimens are quite curious. The findings may reflect tumor cell heterogeneity among different metastatic deposits within a single patient. An alternative and perhaps more likely explanation is that the consistent increase in the fraction of cells with β -catenin nuclear staining in cytology specimens may be specifically related to specimen preparation. For instance, the mild alcohol-based fixation methods used for the cytology specimens may more readily conserve nuclear β -catenin staining than the formalin fixation used for the surgical specimens.

To assess the prevalence of β -catenin mutations in primary melanomas and to determine whether there was a strong correlation between nuclear β -catenin staining

Figure 1. An example of nuclear and/or cytoplasmic localization of β -catenin in a cytology specimen. **A:** A Thin Prep specimen stained with the Papanicolaou stain shows a cluster of melanoma cells aspirated from a metastatic lesion from a malignant melanoma. **B:** A phase image of another slide of the same cytology specimen. **C:** The same field viewed with indirect immunofluorescence demonstrates staining of the nuclei with the β -catenin monoclonal antibody and the Cy3-conjugated secondary antibody. Scale bar, 20 μ m.

and β -catenin mutations, we carried out direct sequencing studies of PCR products generated from genomic DNA preparations of the specimens. The cytology specimens used for DNA isolation generally contained greater than 75% neoplastic cells, and the surgical specimens were selected such that the neoplastic cells represented greater than 75% of the total area of the section. Of the 50 specimens for which sufficient DNA was recovered for PCR, only one tumor specimen was found to have a β -catenin mutation. The mutation, a T to C transition at the first position of codon 45, resulted in a change from serine to proline, and it was confirmed in independent

Figure 2. β -catenin localization in surgical specimens shows membrane and scattered nuclear and/or cytoplasmic staining. A, C, E, and G show the hematoxylin and eosin stains of serial sections of the tissues. In B, D, F, and H, an adjacent section from each case has been stained with β -catenin antibody and visualized with the Cy3 fluorophore. H&E-stained slides cannot be studied using immunofluorescence; hence, adjacent sections were studied. Low and high power views are shown of two different cases, one that demonstrated only membrane staining (A-D) and another that demonstrated nuclear staining (E-H). Original magnification, $\times 200$ (A, B, E, and F); $\times 1000$ (C, D, G, and H). Scale bar, 100 μ m (A); 20 μ m (C).

PCR and sequencing reactions. The codon 45 mutation was found in a cytology specimen that showed nuclear staining for β -catenin (Figure 1). This serine to proline mutation has been seen in other studies including two cases of hepatocellular carcinoma,¹⁰ one colonic carcinoma,¹⁷ and (in our group) anaplastic carcinoma of the thyroid (Garcia-Rostan G, Tallini G, Herrero A, D'Aquila TG, Carcangiu ML, Rimm DL, unpublished manuscript). In the only other published study of melanoma speci-

Table 1. β -Catenin Expression and Localization in Metastatic Malignant Melanoma

Tissue type	Nuclear/ cytoplasmic	Membrane	Total
Cytology	10 (55%)	8	18
Surgical	8 (17%)	39	47
Total	18 (28%)	47	65

The distribution of localization of β -catenin expression in the cytology and surgical specimens. Note that nuclear and/or cytoplasmic localization was found in nearly 1/3 of the specimens but with higher frequency in cytology specimens.

mens (cell lines), this serine codon was mutated to tyrosine.⁶

In the study of β -catenin mutations in melanoma cell lines, Rubinfeld et al⁶ found 6 of 27 (22%) of the cell lines had mutations affecting exon 3. The frequency of clonal mutations in β -catenin in primary metastatic melanomas appears to be substantially lower with only 1 of 50 (2%) tumors in our study harboring a mutation. The disparate results raise the possibility that β -catenin mutations in many melanoma cell lines may not have been present in primary tumor tissue, but may have arisen during *in vitro* culture. Another possibility is that melanomas with β -catenin mutations may be more readily adapted to *in vitro* culture than melanomas lacking β -catenin mutations. Yet a third possibility is that β -catenin mutations may be present as a subclonal genetic defect in some melanomas, and those neoplastic cells with β -catenin mutations may have more robust growth properties *in vitro* than those cells lacking β -catenin mutations and thus may be more readily grown in culture. Consistent with this proposal, in prostate cancer, β -catenin mutations were seen in only selected regions of primary tumors.¹¹ In addition, in our immunohistochemical studies of melanomas, we found that β -catenin nuclear and/or cytoplasmic localization was a focal and a subclonal alteration in the majority of tumor specimens with nuclear β -catenin staining. Finally, it is also possible that in some cases a mutation was present in only a small minority of cells, and thus it escaped detection in this study.

Overall, nuclear localization of β -catenin was seen in 28% of the 65 metastatic melanoma specimens that we analyzed. In light of the low frequency of β -catenin mutations in our study, the immunohistochemical findings suggest that there may be other mechanisms through which β -catenin may be activated. Indeed, in the majority of colorectal cancers, mutations in the APC gene are responsible for β -catenin activation. Rubinfeld et al⁶ previously found evidence of β -catenin activation as a result of APC inactivation in 2 of the 27 melanoma lines. Hence, APC inactivation may underlie nuclear localization of β -catenin in some of the melanomas that we have studied. Similarly, mutations in GSK3 β , axin, or other elements in the *wnt* pathway may contribute to β -catenin nuclear localization in some tumors. Finally, it is important to emphasize that nuclear localization of β -catenin, particularly the focal and heterogeneous staining patterns that we and others have observed in primary tumors, might even reflect transient and physiological activation

of the *wnt* pathway in some cases. As such, at this time, it would be premature to conclude that nuclear and/or cytoplasmic localization of β -catenin in cancer cells provides definitive evidence of constitutive deregulation of the *wnt* pathway. Nevertheless, our studies establish that β -catenin is mutated in some primary melanomas and suggest that additional studies of the role of *wnt* pathway alterations in melanoma should provide further insights to its pathogenesis.

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