CYTOPLASMIC AND/OR NUCLEAR ACCUMULATION OF THE \(\beta\)-CATENIN PROTEIN IS A FREQUENT EVENT IN HUMAN OSTEOSARCOMA

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The molecular events that precede the development of osteosarcoma, the most common primary malignant tumor of bone, are unclear, and concurrent molecular and genetic alterations associated with its pathogenesis have yet to be identified. Recent studies suggest that activation of \(\beta\)-catenin signaling may play an important role in human tumorigenesis. To investigate the potential role of \(\beta\)-catenin deregulation in human osteosarcoma, we analyzed a panel of 47 osteosarcoma samples for \(\beta\)-catenin accumulation using immunohistochemistry. Potential activating mutations were investigated by sequencing exon 3 of the \(\beta\)-catenin gene in genomic DNA isolated from tumor samples. Our findings revealed cytoplasmic and/or nuclear accumulation of \(\beta\)-catenin in 33 of 47 samples (70.2%); however, mutation analysis failed to detect any genetic alterations within exon 3, suggesting that other regulatory mechanisms may play an important role in activating \(\beta\)-catenin signaling in osteosarcoma. In our survival analysis, \(\beta\)-catenin deregulation conferred a hazard ratio of 1.05, indicating that \(\beta\)-catenin accumulation does not appear to be of prognostic value for osteosarcoma patients. When analyzed against other clinicopathologic parameters, \(\beta\)-catenin accumulation correlated only with younger age at presentation (24.4 vs. 39.8 years). Nevertheless, our results demonstrate that the deregulation of \(\beta\)-catenin signaling is a common occurrence in osteosarcoma that is implicated in the pathogenesis of osteosarcoma.

Key words: Wnt signal; \(\beta\)-catenin; osteosarcoma; tumorigenesis; bone tumor

Osteosarcoma is the most common primary malignant tumor of bone, encompassing a class of osteoid-producing neoplasms that range in clinical behavior and responsiveness to therapeutic regimens.\(^1\) Best known of these lesions, the classic high-grade osteosarcoma, primarily afflicts individuals in the second decade of life and is distinguished by its locally aggressive character and early metastatic potential. Despite advances in chemotherapy, 5-year survival has remained around 60%.\(^2\)

The molecular events that precede the development of osteosarcoma are poorly understood. Given its relatively early age at presentation and predilection for early metastasis, osteosarcoma is likely the result of underlying early somatic and/or germline mutations. Alterations in tumor-suppressor genes such as retinoblastoma (\(Rb1\)) and \(p53\) have been identified in osteosarcoma and hypothesized to predispose individuals to this malignancy at an especially early age.\(^3,4,5\) Although several putative chromosomal regions have been suggested to harbor potential tumor-suppressor genes, the molecular nature and identity of these genes have not been elucidated.

\(\beta\)-Catenin is a cellular protein with multiple functions. As an important component of the adherens junction complex, it helps to anchor E-cadherin to the intracellular actin cytoskeleton through interactions with \(\alpha\)-catenin.\(^6,7\) As an important Wnt signal transducer, \(\beta\)-catenin plays an important role in many developmental processes. In normal cells, \(\beta\)-catenin protein is maintained at a very low level and thereby restricted to the cellular membrane. Wnt ligands initiate their signaling pathway by binding to the frizzled receptors, leading to phosphorylation of the diseveled protein.\(^9\) Through its association with Axin and the adenomatous polyposis coli (APC) tumor-suppressor, phosphorylated disheveled protein then prevents glycosyn synthase kinase 3\(\beta\) (GSK3\(\beta\)) from phosphorylating \(\beta\)-catenin.\(^10,11\) Unphosphorylated \(\beta\)-catenin is stabilized by escaping recognition by \(\beta\)-TrCP, a component of an E3 ubiquitin ligase.\(^7,8\) Stabilized \(\beta\)-catenin accumulates in the cytoplasm and eventually translocates to the nucleus, where it engages transcription factors LEF and Tcf-4 to activate expression of downstream target genes, such as c-Myc\(^12\) and cyclin D1.\(^13\)

The involvement of \(\beta\)-catenin in tumorigenesis was first established in colorectal cancer, where it was found to form a complex with the APC tumor-suppressor.\(^14,15\) The importance of \(\beta\)-catenin in regulating cell proliferation has been further highlighted by the discovery of oncogenic mutations of the \(\beta\)-catenin gene in colon cancers containing the wild-type APC gene.\(^16–18\) Mutant \(\beta\)-catenin protein becomes more stable because it is capable of bypassing APC-targeted degradation. Oncogenic forms of \(\beta\)-catenin induce tumor formation in transgenic animals, whereas mutations in the \(\beta\)-catenin gene have been frequently uncovered in tumors induced by either carcinogens or activated oncogenes.\(^19,20\) Furthermore, cytoplasmic and/or nuclear accumulation of the \(\beta\)-catenin protein has been extensively documented in the vast majority of human tumors, though \(\beta\)-catenin mutations have been uncovered at a low frequency in several forms of human tumor.\(^21,22\) Thus, these collective genetic data suggest that deregulation of \(\beta\)-catenin signaling may be involved in the development of a broad range of human malignancies. To increase our understanding of the molecular mechanisms underlying the development of osteosarcoma, we investigated the potential involvement of \(\beta\)-catenin signaling in human osteosarcoma. Immunohistochemical analysis of 47 osteosarcoma samples revealed significant cytoplasmic and/or nuclear accumulation of the \(\beta\)-catenin protein in 33 cases (70.2%). Interestingly, mutations of \(\beta\)-catenin exon 3 were not detected in any of the analyzed samples, indicating that while elevation of cytoplasmic and nuclear \(\beta\)-catenin protein is prevalent, mutations in the \(\beta\)-catenin gene are rare in osteosarcoma. Nevertheless, our results strongly imply that deregulation of \(\beta\)-catenin signaling is associated with the pathogenesis of osteosarcoma.

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MATERIAL AND METHODS

Osteosarcoma tumor samples

The use and selection of human tumor specimens followed guidelines approved by the Institutional Review Board of the University of Chicago. Forty patients diagnosed with osteosarcoma and treated at the University of Chicago Hospitals were selected, and 4 μm sections were prepared. Immunohistochemical staining was performed using an anti-β-catenin antibody (see Material and Methods). Immunostaining results were graded according to the location and intensity of the signal. Slides were examined at ×40 magnification under a light microscope. Negative specimens had no nuclear/cytoplasmic staining, though many had isolated membranous staining (top row). Positive samples showed significant staining within the nucleus (middle row) and/or cytoplasm (bottom row). Control staining was performed in the same conditions except that a mouse IgG was used. After immunostaining, each section was counterstained with light green to reveal cellular structures. HE staining was also performed to reveal the histology of the selected samples. See text for details. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Immunohistochemical staining with β-catenin

Paraaffin-embedded sections were deparaffinized using xylene at room temperature and then rehydrated in a graduated fashion. For antigen retrieval, deparaffinized samples were immersed in a 0.1 M citrate buffer (pH 6.0) and microwaved for 10 min. After fixation, slides were incubated with a mouse anti-β-catenin antibody (against the C terminus of the protein; Transduction Laboratories, Lexington, KY) at a dilution of 1:200 for 1 hr at room temperature. Super Sensitive Multilink and Super Sensitive Label (both from BioGenex, San Ramon, CA) were then applied to each slide for 30 min. To visualize the β-catenin protein, a diamobenzidine substrate (Pierce, Rockford, IL) was added for approximately 5 min, followed by counterstaining with light green (Fisher, Pittsburgh, PA) and mounting. A negative control exposed to mouse IgG antibody accompanied each specimen, and at least 1 positive control (i.e., a known positive section from human prostate cancer) was run with each series of specimens to ensure the validity of positive and negative findings. Stained samples were evaluated independently by 3 investigators. Samples were considered positively stained for β-catenin if significant staining was detected in the nucleus and/or cytoplasm. Conversely, staining restricted to the cytoplasmic membrane or no staining at all was considered negative.

Isolation of genomic DNA and DNA sequencing

Based on the immunostaining results of the β-catenin protein, a total of 23 cases were selected for genomic DNA isolation and subsequent sequencing analysis. Two 4 μm sections of each sample were prestained with hematoxylin and eosin (HE). Upon inspection of the original HE section, the specimens selected for sequence analysis consisted entirely of homogenous sheets of pleomorphic spindle cells, without any identifiable background stromal cells. Microdissection, therefore, was not used. The tumor region was carefully collected by scraping with a scalpel blade and then deparaffinized by placing in 100 μl of 0.5% Tween-20 and heating at 90°C for 10 min. Samples were allowed to cool down to 50°C, and proteinase K (Life Technologies, Gaithersburg, MD) was added to each sample at a final concentration of 200 mg/ml. Digestion was carried out at 50°C overnight. The following day, 100 μl of 5% Chelex-100 (Bio-Rad, Hercules, CA) were added to each sample, followed by heating at 99°C for 10 min. Samples were then centrifuged and the supernatants collected. Standard phenol:chloroform:isoamyl alcohol extraction was performed for each sample, followed by ethanol precipitation. PCR amplification of exon 3 of β-catenin was performed using the following primer pair: 5'-TATTTCATGGGTCTATATCACAG-3' and 5'-CTGTTCGCCACTCATACAGGAC-3'. A reaction volume of 25 μl was used, and touchdown PCR amplifications were performed on a PCR Express thermocycler (Hybaid, Franklin, MA) as follows: 94°C for 2 min, 1 cycle; 92°C for 20 sec, 67°C–56°C (10°C decrease/cycle) for 30 sec and 70°C for 30 sec, 12 cycles; 92°C for 20 sec, 55°C for 30 sec and 70°C for 30 sec, 30 cycles; 70°C for 5 min, 1 cycle. Amplification products were purified from 0.5% agarose gels. The sequencing primer was 5'-CTGATTGTGAGGAGGTTG-GCATG-3'. Sequence reactions were performed using the ThermoSequenase kit (USB, Cleveland, OH) and resolved on 6% DNA sequencing gels. DNA sequence was radiographically visualized.

Statistical analysis

Fisher’s exact test was performed on comparisons of noncontinuous variables (gender, stage, tumor location, grade and histologic subtype); Student’s t-test was performed to compare the means for age. Survival analysis and Kaplan-Meier curves were generated on a subset of 30 biopsy specimens from patients for whom outcome data were available. All patients were treated at the University of Chicago with preoperative chemotherapy and wide resection. Mean follow-up was 60.03 months (range 1–166 months). Statistical analyses were conducted using Stata® Statistics/Data Analysis software (Stata Corporation, College Station, TX).

RESULTS

Immunohistochemical evaluation of β-catenin in 47 samples of human osteosarcoma

Forty-seven osteosarcoma samples, representing a cross-section of histologic subtypes of osteosarcoma as well as both primary and metastatic lesions, were derived from 40 patients. Immunohistochemical staining was carried out using a well-characterized β-catenin antibody. Staining results were graded independently by 3 investigators according to the location and intensity of the signal. Samples where significant signal was detected in the nucleus were considered positive.
and/or cytoplasm but no staining was observed in the negative control (i.e., mouse IgG added) were designated positive. Negative staining was defined as no detectable signal or staining of the cytoplasmic membrane only. A representative case from each category is illustrated in Figure 1. HE staining was also used as a histologic reference to ensure that tumor regions were properly analyzed.

As summarized in Table 1, 33 cases of 47 osteosarcoma samples (or 70.2%) exhibited significant cytoplasmic and/or nuclear accumulation of the β-catenin protein. The immunohistochemical staining results were then tabulated and compared to the following clinical variables: age, gender, Enneking stage, disease stage, histologic subtype, histologic grade and tumor location. Statistical analyses were performed to assess whether β-catenin accumulation was associated with specific histologic subtypes of osteosarcoma, disease stage (e.g., primary lesions, local recurrence or distal metastases) or age at presentation of disease. There was no clear association between β-catenin accumulation and any clinicopathologic variable (p > 0.20, Fisher’s exact test), with the exception of patient age at presentation. Specifically, patients with elevated β-catenin in tumors were, on average, younger (mean age 26.38 vs. 39.78, p = 0.02).

In a few selected cases, we analyzed tumor samples from the same patient at different stages of treatment. Interestingly, 2 cases consisted of prechemotherapy biopsies compared to postchemotherapy resections. One case demonstrated detectable β-catenin accumulation before chemotherapy as opposed to afterward, and the other demonstrated the reverse. Overall, no distinct pattern existed among the comparisons within the same patient.

**Mutational analysis of exon 3 of the β-catenin gene**

To investigate the cause of β-catenin deregulation, we performed a mutational analysis in 23 of the 33 samples that exhibited marked accumulation of β-catenin in the cytoplasm and/or nucleus. Exon 3 of β-catenin was selected for analysis because it harbors the majority of β-catenin mutations identified in other human malignancies. Genomic DNA was isolated from paraffin-embedded sections and amplified using oligonucleotides that flanked the region of exon 3 containing each of the GSK3β phosphorylation sites of β-catenin where mutations have been documented in other malignancies. In all analyzed samples, we failed to detect any apparent genetic alterations within this region. Figure 2 illustrates the wild-type genomic DNA sequences of the hot spots in 5 cases with markedly elevated β-catenin staining. These results suggest that the β-catenin protein may be frequently elevated, but mutations of the β-catenin gene are rare in osteosarcoma.

**Statistical correlation analysis of β-catenin accumulation with clinical outcome in 30 patients**

We performed a survival analysis on a subset of 30 patients for whom follow-up data were available and found a hazard ratio of 1.05 (SD = 0.563). Furthermore, the Kaplan-Meier curves that were generated were almost identical between the 2 cohorts (data not shown). Although the sample size was relatively small, these results suggest that the prognostic value of β-catenin accumulation may be limited in osteosarcoma.

**DISCUSSION**

The molecular events leading up to the development of osteosarcoma remain an area of continuing investigation. Mutations in the Rb1 and p53 tumor-suppressor genes, e.g., have been widely documented in osteosarcoma, and with most sarcomas in general. While Rb1 loss of heterozygosity may be a poor prognostic sign, p53 has not emerged as a meaningful prognostic marker and neither has been effectively used as a therapeutic marker.

**TABLE 1 – SUMMARY OF CLINICOPATHOLOGIC DATA AND β-CATENIN IMMUNOSTAINING RESULTS ON SELECTED OSTEOSARCOMA SPECIMENS**

<table>
<thead>
<tr>
<th>Total</th>
<th>β-Catenin+</th>
<th>β-Catenin-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>47</td>
<td>33 (70.2%)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>31.37</td>
<td>26.38</td>
</tr>
<tr>
<td>Histology of primary tumor</td>
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<td></td>
</tr>
<tr>
<td>Osteoblastic</td>
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<td>17</td>
</tr>
<tr>
<td>Chondroblastic</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Fibroblastic</td>
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<td>1</td>
</tr>
<tr>
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<td>1</td>
</tr>
<tr>
<td>Paget’s sarcoma</td>
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<td>1</td>
</tr>
<tr>
<td>Small cell</td>
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<td>1</td>
</tr>
<tr>
<td>Soft tissue</td>
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<tr>
<td>Grade of primary tumor</td>
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</tr>
<tr>
<td>High</td>
<td>37</td>
<td>31</td>
</tr>
<tr>
<td>Low</td>
<td>2</td>
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<tr>
<td>Location of primary tumor</td>
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<td>Pelvis</td>
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<tr>
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</tr>
<tr>
<td>Soft tissue</td>
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</tr>
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<tr>
<td>Prechemotherapy primary tumor</td>
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</tr>
<tr>
<td>Postchemotherapy primary tumor</td>
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<td>Local recurrence</td>
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<td>Metastasis (lung)</td>
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<td>5</td>
</tr>
<tr>
<td>Metastasis (chest wall)</td>
<td>1</td>
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</tr>
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</table>
target in osteosarcoma. The INK4 products p14ARF and p16 were underexpressed in over 50% of cases of osteosarcoma.34 These data support a central role for both p53 and Rb1 in the development of osteosarcoma; however, mutational analysis of INK4 revealed no sequence alteration, suggesting that the observed downregulation of p14ARF and p16 was not central to the tumorigenesis process. Furthermore, cytogenetic analyses of osteosarcoma reveal a broad variety of chromosomal abnormalities, including allelic loss of 3q, 6p, 13q, 15q, 17p and 18q, each of which occurs in 50–75% of cases.32 While Rb1 and p53 largely account for the allelic loss reported on 13q and 17p, respectively, further attempts to identify tumor-suppressor genes that map to other regions of chromosomal loss have failed to identify additional genes critical to the development of osteosarcoma. In this respect, osteosarcoma has largely eluded our attempts to generate molecular markers for clinical surveillance.

The contribution of β-catenin deregulation in the pathogenesis of sarcoma remains to be elucidated. In a broad survey of several types of sarcoma, including a small group of osteosarcomas, Iwao et al.33 reported that 4 of 6 tumors exhibited significant elevation of β-catenin. Their results are comparable to our data, suggesting that β-catenin deregulation occurs in the majority of osteosarcomas. Furthermore, our findings are consistent with similar studies in other human tumors, in which β-catenin accumulation was a frequent event but β-catenin mutations were infrequent.34–38 It is conceivable that β-catenin accumulation can also be caused by loss-of-function mutations of the negative regulators of β-catenin signaling (e.g., APC, Axin, and GSKβ). However, thus far, mutations of the APC tumor-suppressor gene have not been reported in noncolon cancer, and genetic alterations of Axin have been reported only in a small fraction of hepatocellular carcinomas, colon adenocarcinomas and sporadic medulloblastomas.39–41 Thus, the lack of mutations in β-catenin and its negative regulators strongly suggests that deregulation of β-catenin signaling may be caused by other mechanisms.

Our data, therefore, indicate that β-catenin deregulation is common in osteosarcoma, indeed more common than loss of heterozygosity for either Rb1 or p53. However, our data do not support the use of β-catenin accumulation as a clinical prognostic marker. Although the sample used for survival analysis was relatively small, clinical outcome was nearly identical between patients with tumors exhibiting β-catenin accumulation and those with β-catenin-negative tumors. This similarity was evident in both the estimated hazard ratio as well as the Kaplan-Meier curves. This lack of prognostic value should not diminish the importance of β-catenin deregulation in human osteosarcoma. For instance, although activation of β-catenin signaling is the primary cause for most human colorectal cancers, its activation has not been shown to correlate with the clinical prognosis of colon cancer patients.42 Alternatively, other tumors have demonstrated a correlation between elevated cytoplasm/nuclear β-catenin and poor prognosis or disease progression.43–46 When we compared β-catenin accumulation to a variety of clinicopathologic data, no identifiable pattern emerged, with the important exception of age at presentation.

In summary, our results demonstrate that deregulation of β-catenin signaling is a common occurrence in osteosarcoma and appears to correlate with younger age at initial presentation. The lack of association with other clinicopathologic parameters may reflect the possible early involvement of β-catenin signaling in osteosarcoma. Taken together, our findings suggest that deregulation of β-catenin signaling may represent an important new mechanism underlying the development of osteosarcoma.

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