Tissue Microarray Analysis of β-Catenin in Colorectal Cancer Shows Nuclear Phospho-β-catenin Is Associated with a Better Prognosis

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ABSTRACT

**Purpose:** β-Catenin is involved in homotypic cell-cell adhesion and the wnt signaling pathway. Deregulation of β-catenin levels, caused in part by mutations of the adenomatous polyposis coli gene, is thought to play a role in the development of colorectal and other cancers. To further elucidate their roles, the expression pattern of β-catenin and phosphospecific β-catenin was correlated with clinical outcomes in a series of patients with colorectal cancer.

**Experimental Design:** Immunohistochemical analysis of a tissue microarray with 650 colorectal cancer specimens was performed to study the expression and subcellular localization of β-catenin and phosphospecific β-catenin. These results were correlated with other clinicopathological factors and with overall survival.

**Results:** The majority of cancers retained some degree of β-catenin membranous staining, whereas cytoplasmic or nuclear expression was seen in 42.5% and 20.4% of specimens, respectively. Phospho-β-catenin showed nuclear staining in 9.5% of specimens, and there was no apparent membranous or cytoplasmic staining. There was no significant association between β-catenin or phospho-β-catenin and grade or stage. However, there was a positive correlation between β-catenin and phospho-β-catenin (P = 0.039), with phospho-β-catenin representing a subset of nuclear β-catenin. Patients with nuclear expression of β-catenin did not have an altered survival compared with those that did not (P = 0.5611). Nuclear expression of phospho-β-catenin, however, was associated with an improved survival (P = 0.0006). In multivariate analysis, only stage and phospho-β-catenin were independently predictive of overall survival (P < 0.001 and P = 0.0034, respectively).

**Conclusions:** These findings support a role for β-catenin overexpression in colorectal tumorigenesis and provide initial evidence that phospho-β-catenin may be a marker for improved overall survival independent of stage and grade.

INTRODUCTION

β-Catenin is a member of the cadherin-catenin complex that mediates homotypic cell-cell adhesion (1). It also plays a role in the wnt signaling pathway, which is important in embryogenesis (2). In the absence of wnt stimulation, this pathway is regulated at least in part by a multiprotein complex consisting of axin, GSK3β, and the tumor suppressor protein APC (3–5).

Formation of this complex facilitates the phosphorylation of critical residues by GSK3β in the NH2 terminus of β-catenin, targeting it for degradation by the ubiquitin/proteasome pathway (6, 7). Wnt signaling or a disruption of the regulatory complex leads to stabilization of cytosolic β-catenin, translocation to the nucleus, and T-cell factor/lymphoid enhancer factor family-mediated transcriptional activation, resulting in the transcription of target genes such as cyclin D1 (8) and matrialsylin (9).

Whereas the APC gene is mutated in approximately 80–100% of sporadic and hereditary colorectal cancers (10), this has not been established in other malignancies. The discovery that APC binds to β-catenin and facilitates its degradation provided a link between these pathways and offered insight into the possible roles of APC. Several lines of evidence point to APC-associated β-catenin deregulation as an important step in the development of colorectal tumors and possibly other tumors as well. Colon cancer cells containing mutated APC have increased levels of β-catenin that can be down-regulated by exogenous wt APC (11), a high percentage of colon cancers cells with wt APC have β-catenin mutations (12), β-catenin-mediated transcription is constitutively active in APC-deficient colon cancer cells (13), and point mutations in the putative GSK3β phosphorylation sites of β-catenin that impede its degradation have been found in a variety of cancers (14, 15).

Previous immunohistochemical studies with β-catenin have shown distinct subcellular expression patterns in colorectal carcinomas, adenomas, and benign epithelium. Whereas benign tissue almost universally expressed β-catenin on the plasma membrane only, adenomas and carcinomas expressed β-catenin.
to varying degrees in the cytoplasm and in the nucleus as well (16–18). This finding is not surprising, given the intracellular pathway described above. In addition, correlations between alterations of β-catenin expression in colorectal cancer and outcome variables have not been consistent (19–21).

To assist in the high throughput immunohistochemical analysis and to minimize variability of experimental conditions among a large number of specimens, we used the TMA technology (22). Using this technique, a much larger cohort of patient samples can be evaluated in a more standardized and rapid fashion.

We used antibodies to β-catenin and to phospho-β-catenin (specific for phosphorylated serine 33, serine 37, and threonine 41 residues in the GSK3β phosphorylation sites of β-catenin) to immunohistochemically evaluate the expression levels and subcellular localization patterns in a TMA containing a cohort of 650 colorectal cancer specimens. These data were correlated with standard clinicopathological parameters and with overall survival. To date, this is the largest cohort of colorectal cancer patients analyzed for β-catenin expression and the first study to investigate expression using phosphospecific β-catenin antibodies.

### MATERIALS AND METHODS

#### Cell Lines

MDCK cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin.

#### Site-directed Mutagenesis

Mutants S33A and K49A were made with the MORPH site-specific plasmid DNA mutagenesis kit (Eppendorf 5 Prime, Boulder, CO), and mutants S37A and T41A were produced using the Quik-change PCR-mutagenesis kit (Stratagene, La Jolla, CA). Template for mutagenesis reactions in both kits was wt full-length β-catenin in pcDNA3 with a FLAG tag directly upstream of the β-catenin start codon. All target codons were changed to alanine. Mutants were confirmed by automated DNA sequencing of both strands.

#### Transient Transfections

Large-scale isolation of the plasmid was done using a Qiagen Maxiprep kit (Qiagen, Valencia, CA). MDCK cells were transfected with LipofectAMINE 2000 (Life Technologies, Inc., Rockville, MD). Four-chamber slides were transfected with 1 μg DNA:3 μl LipofectAMINE in OptiDMEM. Eight to 24 h after transfection, growth media were added for 48 h.

#### Immunofluorescence

Transiently transfected MDCK cells were fixed in 4% paraformaldehyde/TBS, permeabilized in 0.2% Triton, and blocked in 3% BSA/TBS. Primary antibodies were phospho-β-catenin S33-S37-T41 (1:500; Cell Signaling Technologies, Beverly, MA) and FLAG (1:1500; Sigma Chemical Co., Saint Louis, MO). All primary antibodies were diluted in 3% BSA/TBS. Secondary antibodies were GAR-cy3 (phospho-β-catenin) and GAM-Alexa 488 (FLAG).

#### TMA

The construction of TMAs has been described previously and reviewed recently (23). Briefly, formalin-fixed, paraffin-embedded tissue blocks containing colorectal cancer were retrieved from the archives of the Yale University Department of Pathology. Areas of invasive carcinoma were identified on corresponding H&E-stained slides, and the tissue blocks were cored and transferred to a recipient “master” block using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). Each core is 0.6-mm wide spaced 0.7–0.8 mm apart. After cutting of the recipient block and transfer with an adhesive tape to coated slides for subsequent UV cross-linkage (Intronmedics, Inc., Hackensack, NJ), the slides were dipped in a layer of paraffin to prevent oxidation (24). For this study, a cohort of 650 cases of colorectal cancers was divided between two slides, each with 10 normal colon controls. A second core was arrayed for each specimen, representing 2-fold redundancy for each case. A multitumor TMA was also obtained from the National Cancer Institute (Tissue Array Research Program) containing 42 ovarian cancer, 89 breast cancer, 91 colorectal cancer, 25 brain cancer, 45 lymphoma, 97 prostate cancer, 90 lung cancer, 24 melanoma, and 63 normal tissue specimens. This array was also stained with the phospho-β-catenin antibody using the method described below.

#### Immunohistochemistry of TMA

TMA slides were deparaffinized with a 30-min incubation at 60°C and xylene rinses and then rehydrated in a series of alcohol and water rinses. Endogenous peroxidase activity was blocked with a 30-min incubation in a 2.5% hydrogen peroxide/methanol buffer (phospho-β-catenin) or a 5-min incubation in 0.03% hydrogen peroxide/sodium azide buffer (β-catenin). Antigen retrieval was performed by boiling in citrate buffer in a pressure cooker (25). To reduce background nonspecific staining, slides were incubated with a 0.3% BSA/TBS solution for 1 h. Primary antibody was then applied at appropriate dilutions for 30 min at room temperature (β-catenin) or overnight at 4°C (phospho-β-catenin). Primary antibodies were monoclonal anti-β-catenin (1:2500; Transduction Laboratories, Lexington, KY) and polyclonal anti-phospho-β-catenin (1:200; Cell Signaling Technologies). Phospho-β-catenin antibody was directed at β-catenin phosphorylated specifically at serine 33, serine 37, and threonine 41 residues in exon 3. Detection of bound antibody was accomplished with the DAKO Envision TM + System (DAKO, Carpinteria, CA). After incubation with primary antibody, slides were rinsed once in TBS (β-catenin) or with a series of 2-min washes in TBS, TBS/0.01% Triton, and TBS (phospho-β-catenin). Horseradish peroxidase-labeled polymer conjugated to goat antimouse (or rabbit) secondary antibody was incubated for 30 min (β-catenin) or 1 h (phospho-β-catenin) followed by rinses identical to those performed after incubation with the primary antibody. Sections were developed with 3,3’-diaminobenzidine for 5 min, counterstained with hematoxylin, and mounted with ImmunoMount (Shandon, Pittsburgh, PA). Slides were also stained in the absence of primary antibodies to evaluate nonspecific secondary antibody reactions.

#### Evaluation of Immunostaining

Areas of most intense and/or predominant staining pattern were scored. The membranous, cytoplasmic, and nuclear staining was determined separately for each specimen. Only nuclear staining was seen with phospho-β-catenin, and thus this was the only variable that was graded. The staining intensity was graded as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. For specimens that were interpretable, a score of NA was given. In each case, the staining was scored as an average throughout the spot. Conventional immunohistochemistry quantitation often includes parameters for both intensity and percentage area within the tumor. The histo-spots in TMAs are only 600 μm in diameter and tend to be homogenous. Thus, no area
variable was included in assessment of the scores. Scoring of phospho-β-catenin was performed by two independent observers, and the average score was obtained for cases of disagreement. There was a high level of correlation between the two scorers, and thus β-catenin was scored by a single observer but with 2-fold redundancy of cases. Discrepant scores from the same tumor or from the two observers were averaged to arrive at a single score.

Statistical Analysis. The χ² test was used to measure relationships between the expression of β-catenin or phospho-β-catenin and standard clinicopathological parameters. Multivariate analysis using the Cox proportional hazards model was carried out to assess the independent prognostic significance of variables on overall survival. Survival curves were calculated using the Kaplan-Meier method, and the differences were estimated using the log-rank (Mantel-Cox) test. All cases were collected from specimens resected between 1970 and 1982. Follow-up ranged from 13 days to 31 years, with median follow-up 4.4 years and a median survival of 3.4 years. However, because of a significant number of patient deaths >10 years after the date of diagnosis and the unlikelihood that these events would be related to their initial oncological diagnosis, surviving patients were censored at 10 years. All of the analyses were performed with the Statview 5.0.1 package (SAS Institute Inc., Cary, NC).

RESULTS

Immunostaining of Colorectal Cancer TMA with β-Catenin. Normal colonic epithelium showed membranous staining only with β-catenin (data not shown). In general, two distinct staining patterns were seen in the malignant cases: (a) one that was similar to benign tissue showing predominantly membranous with no cytoplasmic or nuclear staining (Fig. 1A), and (b) one that was predominantly nuclear, often with concomitant cytoplasmic and reduced membranous staining (Fig. 1B). Most specimens showed minimal intratumoral heterogeneity within the histo-spots of the array.

Of 650 specimens, 633 (97%) had adequate survival data, and 91 (14%) were not interpretable secondary to loss of tissue on the TMA, no cancer on the histo-spot, or minimal staining on either of the redundant slides. Of the remaining 543 (84%) evaluable specimens, the majority retained some degree of membranous staining with 93% having a score ≥ 1 and 38% having a score ≥ 2 (Fig. 2A). Elevated cytoplasmic levels were found in 91% (score ≥ 1) and 42% (score ≥ 2) of specimens
Nuclear accumulation of β-catenin was seen in 46% (score ≥ 1) and 21% (score ≥ 2) of specimens (Fig. 2C). Nuclear accumulation correlated positively with elevated cytoplasmic ($P < 0.0001$) and reduced membranous ($P < 0.0001$) expression.

Validation of Phospho-β-catenin Antibody. Residues 33, 37, and 41 of β-catenin are putative targets for GSK3β phosphorylation that target the protein for degradation, and point mutations of these residues have been found in a mutually exclusive fashion with APC in colorectal cancer. The phospho-33/37/41 β-catenin antibody was generated using a β-catenin phosphopeptide phosphorylated at these three residues. To determine the specificity of this antibody, transient transfections of MDCK cells with FLAG-tagged wt β-catenin and with β-catenin with point mutations at residues 33, 37, 41, and 49 were performed. The wt transfectants showed a bright signal throughout the cytoplasm and nucleus and minimal membranous staining when stained with antibody to FLAG, representing the subcellular distribution on transient overexpression of β-catenin (Fig. 3A). When stained with phospho-β-catenin, however, there was punctate nuclear staining that correlated with FLAG localization (Fig. 3B). Although phospho-β-catenin should be degraded by APC-mediated pathways, the massive overexpression of β-catenin seen in these transfection experiments results in punctate nuclear expression. This is probably due to overexpression exceeding the capacity of homeostatic degradation mechanisms. There was minimal cytoplasmic staining. This pattern was repeated when mutant 49 cells were stained with FLAG and phospho-β-catenin (Fig. 3, I and J). In contrast, when cells transfected with either mutant 33, 37, or 41 were stained with FLAG, the staining pattern was again predominantly nuclear and cytoplasmic, but when these cells were stained with phospho-β-catenin, the previously seen punctate nuclear localization was abolished (Fig. 3, C–H). Thus, phospho-β-catenin specifically recognizes only β-catenin phosphorylated at residues 33, 37, and 41, and this recognition was seen only in the nucleus.

Immunostaining of Colorectal TMA with Phospho-β-catenin. Normal colon tissue and the majority of colorectal cancer specimens showed no staining with phospho-β-catenin (Fig. 1C). This may be a reflection of the rapid degradation of phosphorylated β-catenin that is predicted by the model described above. The remaining colorectal cancer specimens showed, as in the MDCK studies, nuclear staining without evident membranous or cytoplasmic staining (Fig. 1D).

The number of uninterpretable histo-spots was 39 (6%). In the remaining 594 (91%) evaluable specimens with adequate survival data, 26% demonstrated nuclear staining with a score ≥ 1, and 9.5% demonstrated nuclear staining with a score ≥ 2 (Fig. 2D). Immunostaining of the multitumor TMA with phospho-β-catenin showed a variable degree of nuclear staining among the different tumor histologies; staining was notably highest in colon cancer and essentially absent in lymphoma, prostate cancer, and benign controls (Fig. 4).

Survival Analyses. As expected, advanced tumor stage was highly correlated with a poorer survival ($P < 0.0001$), and there was a trend toward worse outcome for poorly differentiated tumors (data not shown). To determine whether β-catenin expression level is correlated with a worse outcome, we chose to score our TMAs on a 4-point graded system (0, 1, 2, or 3). Kaplan-Meier survival curves were then generated using ≥1, ≥2, or ≥3 to represent a “positive” score. Using this method, we did not find a significant difference in terms of overall survival for any of these scoring systems for membranous, cytoplasmic, or nuclear staining with β-catenin (data not shown). Fig. 5A shows a representative Kaplan-Meier survival curve for nuclear staining using ≥2 as a positive score. When a similar method was used for the phospho-β-catenin nuclear staining specimens, however, we found no significant difference in overall survival when using ≥1 as the positive cutoff but found a highly significant improvement in overall survival for patients with a nuclear score of ≥2 ($P = 0.0006$; Fig. 5B).
All photographs are nuclear staining with phospho-

- catenin was abolished in mutants S33A/H9252.

Although the correlation between nuclear phospho-

- catenin positivity as well (Table 2).

DISCUSSION

Since the discovery of the interaction between the β-cate-

nin/wnt signaling pathway and APC, it has been clear that β-catenin plays an important role in colorectal tumorigenesis, yet the details of this relationship and its contribution to overall clinical outcome remain to be elucidated. Using immunohisto-

chemistry on a large number of colorectal cancer specimens, we have demonstrated that nuclear localization of β-catenin was seen in a subset of patients but that this altered immunostaining pattern did not predict worse overall survival as seen in one previous study (20). Phospho-β-catenin showed exclusively nuclear staining in an even smaller subset of patients. Surprisingly, they had a significantly improved overall survival.

Other immunohistochemical studies of β-catenin in colorectal cancer have shown wide variability in terms of proportion with nuclear, cytoplasmic, or membranous staining (16–18). Several studies have looked at β-catenin expression and localization and their correlations with clinical outcome in colorectal cancer. One study looked at 60 patients and showed 32% focal and 18% widespread nuclear localization, but only the latter was independently predictive of a shorter survival (20). The second study showed 68% cytoplasmic and 66% nuclear accumulation in 96 patients, but only cytoplasmic localization correlated with a significantly worse metastasis-free survival (21). Finally, a third study showed that in rectal cancer, nuclear expression was seen in 20% of the cases and did not correlate with disease-free survival (19). Many reasons could explain these differing results with each other and with ours, including small sample size, intrinsic tumor heterogeneity, different immunohistochemical staining and visualization methods with varying degrees of sensitivity, and lack of standardization of what constitutes a “positive” or “negative” result. In addition, although it is reasonable to focus on patients with nuclear localization as a separate group, given what we know about the transcriptional role of β-catenin in the nucleus, it is not clear whether other variables such as cytoplasmic staining or loss of membranous localization may be more appropriate when looking at clinical outcome. We have used TMA technology to allow us to easily study a much larger number of samples in a high-throughput format. Although not yet validated for colon cancer, 2-fold redundancy has been shown to be highly representative of standard breast cancer sections (26). For prostate (27) and fibrous (28) tumors, 3-fold or greater redundancy has been recommended. Unpublished data in our laboratory suggest that 2-fold redundancy will be appropriate for colon cancer.4

The role of phosphorylated β-catenin is even more unclear. Although much is known about the phosphorylation of β-catenin, localization studies using the phospho-33/37/41 antibody have not yet been published. The punctate nuclear localization of this antibody is somewhat surprising because the wnt signal-

Correlations with Clinicopathological Variables and Multivariate Analysis. There was no significant association between β-catenin or phospho-β-catenin nuclear expression patterns and known prognostic factors stage and grade (Table 1). Although the correlation between nuclear phospho-β-catenin and nuclear β-catenin localization was not perfect, there was a statistically significant positive correlation, with a fraction of the latter showing phospho-β-catenin positivity as well (Table 2). Multivariate analysis with the Cox proportional hazards model using the variables stage, grade, β-catenin nuclear staining, and phospho-β-catenin nuclear staining showed that only stage and phospho-β-catenin were statistically significant independent predictors of overall survival ($P < 0.0001$ and $P = 0.0034$, respectively; Table 3).

4 R. L. Camp, unpublished data.
ing dogma suggests that phosphorylated β-catenin is degraded via an APC/axin/ubiquitin-mediated pathway. Nonetheless, we have found nuclear localization in both cultured cells and numerous tumor tissues. There are a number of potential explanations for this observation. In cultured cells, the most likely observation is that nuclear localization is related to massive overexpression. During transient or stable overexpression, protein levels of transfected proteins may exceed normal levels by $10^6$–100-fold or more. When this occurs, it is likely that the normal degradative, homeostatic mechanisms are overwhelmed, and the total β-catenin pool is enlarged. This likely results in nuclear translocation, independent of the phosphorylation status. In some ways, this may be similar to events that occur in tumors. That is, there may be mutations in the β-catenin degradation pathways (at either the APC/axin/GSK3 node or the F-Box proteins further down the degradative pathway) that result in spurious high-level expression of β-catenin. This could also result in phosphorylation state-independent translocation of β-catenin to the nucleus.

Although a number of studies have looked at the prognostic value of β-catenin expression, this study is the first such study to examine phospho-β-catenin antibodies. When studying phospho-β-catenin, there was close correlation between the percentage positive in the multitumor TMA and in our own 650-case cohort array. Additionally, phospho-β-catenin was again seen only in the nucleus in all of the tumor histologies studied. It is notable that although exon 3 mutations of β-catenin have been detected with variable frequency among different tumors (29, 30), the distribution does not mimic the distribution of phospho-β-catenin seen in our multitumor TMA. For example, β-catenin mutations have not been detected in breast cancer (31), contrasting with the 4.5% nuclear phospho-β-catenin seen with immunostaining in our study. Alternatively, we did not find any staining of prostate cancer with phospho-β-catenin, although mutations in prostate cancer have been seen, including residues 33 and 41 (32).

Although APC is mutated in a majority of colorectal cancers and presumably contributes to β-catenin overexpression, our study shows that in fact only a minority actually have nuclear localization of β-catenin and phospho-β-catenin. Other regulatory mechanisms for β-catenin levels and translocation into the nucleus may explain this discrepancy. Phospho-β-catenin at the putative GSK3β phosphorylation sites would be expected to be rapidly degraded via ubiquitin-mediated pathways. However, phospho-β-catenin in our study was detected in the nucleus only in a small percentage of patients, possibly representing a fraction of the phosphorylated form that has escaped degradation, perhaps due to alterations in the degradation machinery, and translocated into the nucleus. Once in the nucleus, the two forms of β-catenin may have different functions. It is possible that the phosphorylated form may be unable to serve as a transcriptional activating agent. A study looking at the immunohistochemical correlation between β-catenin and matrilysin (a downstream target protein of the β-catenin/T-cell factor complex) expression in colorectal cancer has shown that there was complete positive correlation, but the sample size was only 16 (9). In an analogous fashion, Qiao et al. (33) have shown that in pancreatic cancer, reduced membranous β-catenin
expression and accumulation of β-catenin in the cytoplasm correlated with cyclin D1 overexpression and that cytoplasmic overexpression of β-catenin was correlated with worse outcome. The effects of β-catenin versus phospho-β-catenin-positive cancers on the expression levels of downstream target genes such as matrilysin and cyclin D1 are an important question that we will address in future studies. In conclusion, immunostaining of a large number of colorectal cancers on a TMA showed that nuclear β-catenin and phospho-β-catenin positivity was designated as a score of 2 or greater.

Table 1 Association between nuclear β-catenin or phospho-β-catenin and histological grade and clinical stage

<table>
<thead>
<tr>
<th>Histological grade (n = 430)</th>
<th>Nuclear β-catenin</th>
<th>Nuclear phospho-β-catenin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Low&lt;sup&gt;c&lt;/sup&gt;</td>
<td>86</td>
<td>294</td>
</tr>
<tr>
<td>High</td>
<td>7</td>
<td>43</td>
</tr>
<tr>
<td>Stage (n = 515)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low&lt;sup&gt;d&lt;/sup&gt;</td>
<td>62</td>
<td>201</td>
</tr>
<tr>
<td>High</td>
<td>42</td>
<td>210</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nuclear β-catenin and phospho-β-catenin positivity was designated as a score of 2 or greater.
<sup>b</sup> Ps were calculated by the χ² test.
<sup>c</sup> Low histological grade was well or moderately differentiated, and high histological grade was poorly differentiated.
<sup>d</sup> Low stage was clinical stage I or II, and high stage was stage III or IV.

Table 2 Association between nuclear β-catenin and phospho-β-catenin

<table>
<thead>
<tr>
<th>Nuclear phospho-β-catenin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Negative</th>
<th>Positive</th>
<th>Totals</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear β-catenin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>401</td>
<td>37</td>
<td>438</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>97</td>
<td>17</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>498</td>
<td>54</td>
<td>552</td>
<td>0.039</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nuclear β-catenin and phospho-β-catenin positivity was designated as a score of 2 or greater.
<sup>b</sup> Ps were calculated by the χ² test.

Table 3 Multivariate analysis of prognostic factors with overall survival

Multivariate analysis was performed using a Cox proportional hazards model.

<table>
<thead>
<tr>
<th>Variable</th>
<th>OS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>P</th>
<th>Hazard ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4763</td>
<td>1.14 (0.79–1.64)</td>
<td></td>
</tr>
<tr>
<td>Stage&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
<td>1.67 (1.31–2.12)</td>
<td></td>
</tr>
<tr>
<td>Nuclear β-catenin (neg)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.8781</td>
<td>1.02 (0.73–1.31)</td>
<td></td>
</tr>
<tr>
<td>Nuclear phospho-β-catenin (neg)</td>
<td>0.0034</td>
<td>2.18 (1.30–3.68)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> OS, overall survival; CI, confidence interval; β-cat, β-catenin; neg, negative.
<sup>b</sup> High grade was poorly differentiated.
<sup>c</sup> High stage was clinical stage III or IV.
<sup>d</sup> Nuclear β-catenin and phospho-β-catenin negativity was designated as a score of 0 or 1.

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