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ORIGINAL ARTICLE

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β-Catenin expression pattern in primary oesophageal squamous cell carcinoma. Relationship with clinicopathologic features and clinical outcome

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Abstract β-Catenin has an essential role in intercellular adhesion and signal transduction. β-catenin functions as a transcriptional activator downstream in the Wnt signalling pathway. Cytoplasmic stabilisation of β-catenin, mainly due to inactivating mutations of the adenomatous polyposis coli (APC) tumour suppressor gene or activating mutations in exon 3 of the β-catenin gene, can activate this important pathway in the development of several carcinomas. To determine whether this pathway for malignant transformation is important in oesophageal cancer, we analysed 39 primary oesophageal squamous cell carcinomas (OSCC). Immunohistochemical expression of β-catenin was studied in formalin-fixed, paraffin-embedded tissue samples. Results were correlated with clinicopathological parameters and immunohistochemical expression of the proteins p53, E-cadherin, bcl-2 and Ki-67. All examined OSCC had β-catenin expression localised in the cellular membrane, frequently with a heterogeneous pattern. Seven (18%) cases also showed immunoexpression in the cytoplasm and nuclei of the tumour cells. These seven tumours were localised in the upper (three) or in the middle third (four) of the oesophagus. Only one patient had p53 expression and all had bcl-2 expression. The consensus sequence for glycogen synthase kinase (GSK) 3β phosphorylation in exon 3 of the β-catenin gene was studied using polymerase chain reaction and direct sequencing in the seven cases with nuclear β-catenin expression. No genetic alteration was found. These results suggest that β-catenin expression may characterise a subset of OSCC.

Keywords Oesophageal squamous carcinoma · β-catenin · p53 · bcl-2 · Ki-67 · E-cadherin

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Introduction

β-catenin, the vertebrate homologue of armadillo protein in *Drosophila*, is a multifunctional protein involved in two apparently independent processes: cell–cell adhesion and signal transduction [33, 34, 36].

Recently, it has been demonstrated that cytoplasmic β-catenin participates in the transduction of *wingless*-Wnt signals and activates transcription by forming complexes with DNA-binding proteins [2, 24]. Intracellular concentrations of β-catenin are mainly regulated by degradation, which is probably initiated by interaction with the adenomatous polyposis coli protein (APC) [12] and phosporylation on serine or threonine residues of codons 33, 37, 41 and 45 through the action of glycogen synthase kinase (GSK)-3β [31].

The APC tumour suppressor gene has frequently been found to be mutated in human colorectal adenomas and colon carcinomas but rarely in other malignancies, such as oesophageal carcinomas [35]. The mutant APC proteins found in colon carcinomas result in β-catenin stabilisation and a significant increase of this protein within the cell which may then activate β-catenin/Tcf signalling [20]. In the same manner, dominant activating β-catenin mutations that render the protein insensitive to APC/GSK-3β-mediated degradation could lead to a dysregulation of the signalling function of β-catenin and thus to carcinogenesis [26]. Oncogenic activation of β-catenin by amino acid substitutions or interstitial deletions has been reported in colorectal [13, 15, 40] and melanoma [37] cell lines, sporadic colorectal cancers [18], desmoid tumours [22], ovarian carcinomas [32],

hepatocellular carcinomas [6, 19, 23, 29], prostate carcinomas [43], endometrial carcinomas [7], medulloblastomas [45], pylomatricomas [5] and anaplastic thyroid carcinomas [10].

Oesophageal squamous cell carcinoma (OSCC) is an aggressive disease with a poor prognosis, and the genetic mechanism of its carcinogenesis is unsolved. The progression of this tumour is associated with multiple genetic alterations, including loss of heterozygosity in chromosomes 3p, 5q, 9p, 9q, 13q, 17p, 17q and 18q, and amplification of epidermal growth factor receptor (EGFR), human EGFR (HER)-2, c-myc, and cyclin D1 [25]. The most frequent genetic alteration in OSCC is a point mutation in the p53 gene (40–60%) which occurs at a relatively early stage of tumour development [3]. The temporal sequence of these events, however, is far from being understood. In this paper, the expression pattern of β-catenin and the mutations in exon 3 of the β-catenin gene (CTNNB1) was investigated and correlated with the clinicopathological factors of OSCC.

Material and methods

Samples

This study was conducted on 39 primary epidermoid oesophageal carcinomas diagnosed at the Department of Pathology of La Paz Hospital (Madrid, Spain). Histological, immunohistochemical and DNA studies were performed on formalin-fixed, paraffin-embedded tissue samples obtained from endoscopic biopsies.

Immunohistochemistry

Immunostaining for the proteins β-catenin, p53, E-cadherin, bcl-2 and Ki-67 was performed using the avidin–biotin peroxidase method, as previously reported [32]. A heat-induced antigen retrieval step was performed for all deparaffinized sections of the antigens that were then immersed in 0.01 M sodium citrate buffer, pH 6.0, and incubated in a pressure cooker for 3 min. The following mouse antihuman monoclonal antibodies were used: β-catenin monoclonal antibody (C19220, Transduction Laboratories, Lexington, Ky., USA), E-cadherin monoclonal antibody (clon 36, Transduction Laboratories), p-53 monoclonal antibody (DO-7, Novocastra Laboratories), bcl-2 monoclonal antibody (Clon 124, Dako Laboratories, Denmark) and Ki-67 monoclonal antibody (MM-1, Novocastra Laboratories), at the respective dilutions of 1:500, 1:100, 1:100, 1:50 and 1:200, for 1 h at room temperature. In negative controls, the primary antibody was omitted or replaced by an irrelevant antibody.

Evaluation of immunohistochemical staining

Two patterns of β-catenin expression were considered. Expression was membranous if β-catenin was localised in the cell membrane only and nuclear when β-catenin was expressed in the nucleus, irrespective of the percentage of stained nuclei or simultaneous expression of β-catenin in membrane and cytoplasm (Fig. 1a, b).

A semiquantitative estimation of membranous β-catenin and E-cadherin expression was done using a composite score obtained by adding the values of the immunoreaction intensity and relative abundance of the β-catenin or E-cadherin immunoreactive cells, as previously reported for cadherins [8]. Briefly, the intensity was graded from 0 (equivalent to background staining of the acellular stroma) to +3 (intense stain equivalent to normal surface epithelium). The abundance of β-catenin or E-cadherin positive cells was graded from zero to four (zero, less than 5% of positive cells; one, 5–25%; two, 26–50%; three, 51–75%; four, 76–100%). β-catenin or E-cadherin expression was considered preserved when the composite score was six or seven. Cases with scores between zero and five were considered to be tumours with reduced β-catenin or E-cadherin expression.

The CAS 200 analyser (cell analysis system, Lombard Ill.) was used to quantitate p53 and Ki-67 expression. Only nuclear staining in the tumour tissue was scored. When there was less than 15% positive nuclei area, it was considered negative staining, while greater than 15% positive nuclei area was considered positive staining. The same method was used to quantitate bcl-2 expression. Bcl-2 expression was considered positive when more than 25% of the cells stained positive for bcl-2.

DNA analysis

Polymerase chain reaction (PCR) and sequence analysis were performed on the seven tumours with nuclear β-catenin expression. Normal tissue samples from each of the seven patients were also subjected to PCR and sequencing. DNA was extracted from 10 μm of five paraffin sections by meanse of proteinase K digestion without further purification.

The crude extract (5–10 μl) was amplified using PCR as previously reported [32]. The primers used were 240 F (5'-ATG GAA CCA GAC AGA AAA GC) and 439F (5'-GCT ACT TGT TCT GAG TGA AG). These were used to amplify a 200-bp fragment of exon 3 of the *CTNNB1* gene, encompassing the sequence for GSK-3B phosphorylation by means of PCR, as previously reported [32].

PCR products were directly sequenced in a 310 automated sequencer (Applied Biosystems, Foster City, Calif.) using the ABI PRISM rhodamine kit (Applied Biosystems). The PCR primers were used as the primers for sequencing the two strands of the amplified products. Cases with abnormal DNA sequences were subjected to DNA extraction from additional tissue sections, PCR and sequencing to confirm the presence of true mutations.

Statistical methods

The χ^2 and Fisher's exact tests were used to analyse the statistical significance of the relationship between β-catenin expression pattern and the clinicopathological and immunohistochemical variables. The overall survival (OS), defined as the time from diagnosis to death or latest contact (withdrawal), was used as a measure of prognosis. Univariate survival curves were estimated using the Kaplan-Meier method and compared using the log rank test. Multivariate analysis was done using Cox's proportional hazards regression model. Analysis was carried out using SPSS software.

Results

Clinicopathological features

Thirty-nine patients with OSCC were studied. All of them were men with ages ranging from 43 years to 69 years (mean, 59 years). Thirty-six patients (90%) were smokers and 25 (62%) had a history of alcohol consumption. All patients had a good performance status (0, 1 or 2, ECOG scale) at diagnosis. In 6, 24, and 9 cases, the tumour was localised in the upper third, the middle third and the lower third of the oesophagus, respectively. Four OSCC were well differentiated, 16 were moderately differentiated and 19 poorly differentiated. There were

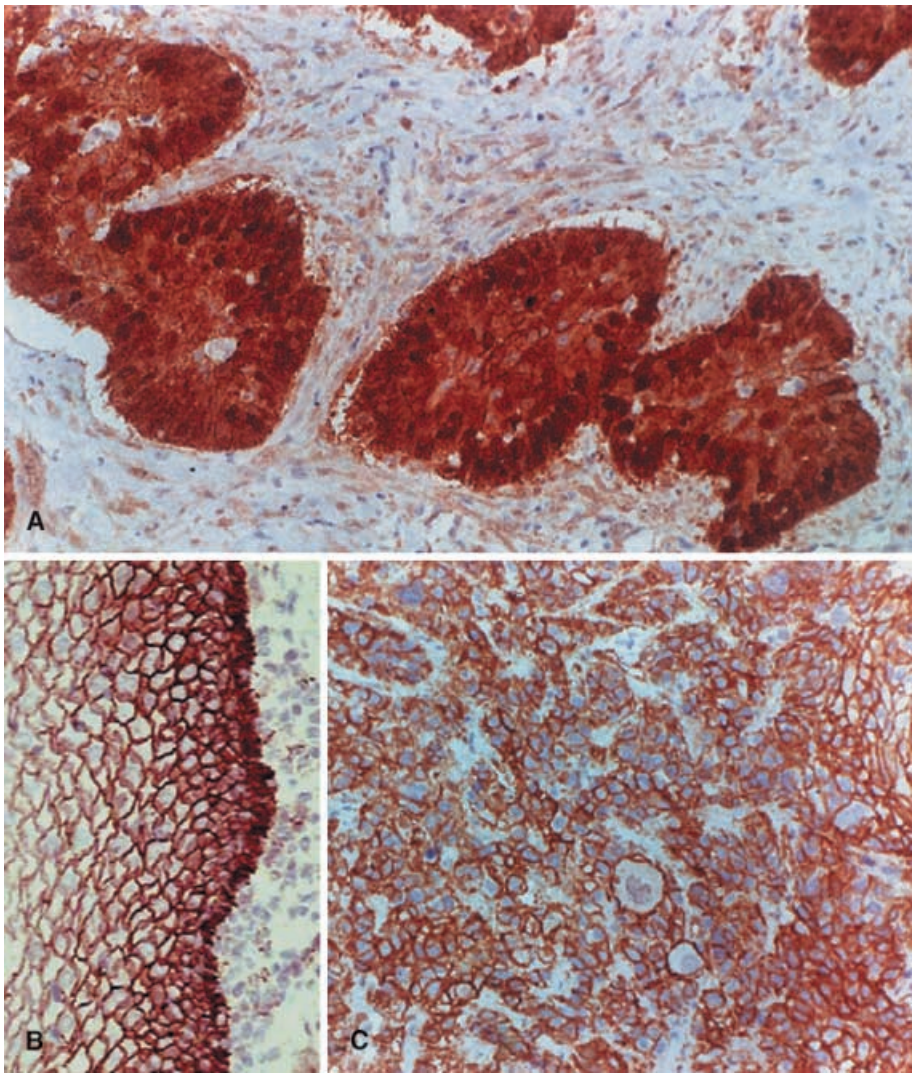

Fig. 1 **A** β-catenin nuclear expression pattern in an oesophageal squamous carcinoma. **B** Conserved homogenous β-catenin staining localised at the cellular membrane in normal oesophageal mucosa. **C** β-catenin membranous expression pattern in an oesophageal squamous carcinoma

19 (50%) stage II, 10 (25%) stage III and 10 (25%) stage IV tumours (AJCC/UICC classification). All cases were initially treated with chemotherapy (cisplatin and 5-fluorouracil). Chemotherapy response was evaluated according to the revised version of the UICC/WHO criteria. Fifteen patients underwent curative surgery and 13 patients received additional radiotherapy.

Immunohistochemichal analysis showed conserved homogenous β-catenin staining localised at the cellular membrane in all normal oesophageal mucosa at a similar level. In contrast, only 12 (31%) OSCC cases had conserved membrane expression (scores 6–7), frequently with a heterogenous pattern (areas of intense and of weak immunoreactivity were admixed in the same tumour). In addition, seven (18%) cases also showed immunoexpression in the cytoplasm and nuclei of tumour cells. In these cases, nuclear immunostaining was present in 25–100% of neoplastic cell nuclei. All cases were localised in the upper third (three) or in the middle third (four) but none in the lower third of the oesophagus. We found a statistically significant correlation between the expression patterns for β-catenin, p53 and bcl-2. Only one case with nuclear β-catenin expression was positive for p53, but all cases with β-catenin nuclear expression showed bcl-2 expression (Table 1). No relationship was observed between β-catenin expression pattern (nuclear versus membrane expression) and stage, differentiation grade, chemotherapy response, Ki-67 and E-cadherin expression. There were no significant differences between survival rate and the β-catenin expression pattern. However, the patients who had nuclear β-catenin expression presented longer median survivals than the others did (18 months vs12 months).

Reduced β-catenin membrane expression (scores 0–5) was observed in 20 (51%) cases, and this was concomitant with reduced E-cadherin expression (77%). However, when β-catenin expression was localised at the nucleus, no correlation was shown with E-cadherin. No correlation was observed between membrane β-catenin expression level (preserved vs reduced) and grade or stage of the patient's tumour, survival, and immunohistochemical expression of the proteins p53, bcl-2, Ki-67.

Table 1 Relationships between the β-catenin expression pattern and clinicopathological features in oesophageal squamous cell carcinomas

	Nuclear β-catenin expression		β-catenin membrane expression	
Localisation				
Upper third	3	(50%)	3	(50%)
Middle third	4	(17%)	20	(83%)
Lower third	0	(0%)	9	(100%[a])
Chemotherapy response				
Complete/partial responses	3/2	(22%)	5/13	(78%)
No responses	2	(13%)	13	(87%[a])
p53 Expression				
Positive	1	(5%)	21	(95%)
Negative	6	(35%)	11	(65%[b])
bcl-2 Expression				
Positive	7	(100%)	0	(0%)
Negative	0	(0%)	32	(100%[c])
E-cadherin expression				
Preserved	1	(11%)	8	(89%)
Reduced	6	(20%)	24	(80%[a])
Ki-67 Expression				
<15%	1	(25%)	3	(75%)
≥ 15%	6	(17%)	29	(83%[a])
Median survival (months)	18		12	([a])

[a] Not statistically significant using the Fisher's exact test
[b] Statistically significant $P<0.05$ using the Fisher's exact test
[c] Statistically significant $P<0.001$ using the Fisher's exact test

β-catenin gene mutations

Sequence analysis of the PCR amplification products showed no mutations of CTNNB1 gene exon 3. To further confirm sequencing, PCR products were digested with *Xmn*I and *Hinf* I restriction endonucleases, which detect mutations at codon 37 and at codon 32 and 33, respectively. All cases showed a normal restriction pattern.

Discussion

β-catenin is a multifunctional protein involved in two apparently independent processes: cell–cell adhesion and signal transduction. Disruption of these two β-catenin pathways could be important in tumour development and progression. Previously, several reports have detected alterations of β-catenin in different neoplasms [41] and have suggested that this protein plays a critical role in carcinogenesis. However, little is known about alterations of β-catenin in oesophageal cancer.

β-catenin plays an important role in the E-cadherin-mediated cell adhesion system because it binds to the cytoplasmic domain of cadherins and to α-catenin, which in turn links the cadherin–catenin complex to the actin cytoskeleton [39]. Moreover, β-catenin mediates the interactions of the cadherin–catenin complex with the c-erbB-2 gene product [16] and EGFR [11]. Disruption of β-catenin binding to cadherin could result in loss of cell–cell adhesion and tissue disorganisation. Because normal squamous oesophageal mucosa has strong membranous β-catenin staining in the boundaries, reduced expression in tumour cells could impair the cadherin–catenin adhesion system. We detected reduced β-catenin membrane expression in 51% of OSCC, but no survival advantage was demonstrated for patients whose tumours had preserved expression of this protein. This data are consistent with those of previous studies. Nakanishi et al. [27] observed reduced β-catenin expression in 73% of OSCC. Although these authors detected a positive correlation between β-catenin expression and loss of tumour differentiation, they were not able to assign it a significant prognostic value. They concluded that β-catenin abnormalities could be involved in early human oesophageal cancer development. Sanders et al. [38] analysed sequential changes in cadherin–catenin expression associated with the progression of OSCC and reported significant down-regulation of membranous cadherin and catenin expression in poorly differentiated OSCC. In both studies, only membranous β-catenin expression was reported, but recently Kimura et al. [17] observed β-catenin nuclear expression in 14% of studied OSCC cases. In addition, Krishnadath et al. [21] observed a correlation between reduced expression of β-catenin and poor prognosis in oesophageal adenocarcinomas. Another two studies [1, 44] simultaneously reported focal nuclear staining for β-catenin in oesophageal adenocarcinomas and suggested that β-catenin nuclear expression could mediate some nuclear function like transcriptional regulation, as had already been seen in other tumours.

We observed localisation of β-catenin expression in the cytoplasm and nuclei of a subset of tumours with specific clinical and immunohistological features. The seven patients with nuclear expression of β-catenin had a tumour localised in the upper or middle third of their oesophagus. Survival in these patients was longer than in those with membranous expression alone (18 months vs

12 months), although this was not significant in the statistical analysis. The overall response rate to chemotherapy was higher than in the rest (71% vs 58%). Moreover, the immunohistochemical analysis detected important differences. Most of the patients showed no expression of p53 protein (86%). In the group with exclusively membranous β-catenin expression, p53 was positive in 65% of the cases, as has been reported before in OSCC [3]. Only the seven patients with nuclear β-catenin presented bcl-2 expression. Bcl-2 expression has been observed in the early stages of oesophageal carcinomas, particularly in non-keratinizing lesions [30]. Furthermore, the seven cases that showed β-catenin nuclear expression presented a higher proliferative activity, as defined by the Ki-67% immunohistochemical method, than the complete group (71% vs 47%). Finally, despite these evident characteristics among patients with nuclear expression of β-catenin, no differences were detected in statistical analysis, although some trends were observed. These results can be explained by the fact that the number of patients was too small to find significant differences. Another reason for the lack of significance could be the presence of other variables which have been shown to be unfavourable prognostic factors with a statistical significance that might work as intermediate variables and mask the value of altered β-catenin expression as a predictive factor.

The nuclear and cytoplasmic accumulation of β-catenin, as observed in our seven cases, suggests activation of the APC/β-cat/Tcf pathway as previously reported in colon cancer [20]. β-Catenin mutations usually lead to stabilisation of this protein whose expression can be demonstrated immunohistochemically by the accumulation of β-catenin in the cytoplasm or nucleus. To date, mutations in exon 3 of the β-catenin gene have been described in several neoplasms, such as carcinomas of the colon [40], ovary [32, 9] endometrium [7], liver [6], prostate [43], desmoid tumours [22], medulloblastomas [45] and anaplastic thyroid carcinomas [10]. All of the identified mutations were single base missense mutations on serine/threonine residues (codons) [33, 37, 41, 45]. They altered the GSK-3β phosphorylation consensus motif, which participates in the degradation of β-catenin. Cytoplasmic β-catenin is rapidly turned over, under control of the APC protein and GSK-3β, by targeted phosphorylation of highly conserved serine and threonine residues and ubiquitination in the NH2 terminus.

Because we detected nuclear expression of β-catenin in OSCC tumours, the next step was to question whether this abnormal immunostaining pattern might be due to mutations in the β-catenin gene (CTNNB1) itself. However, we could not find β-catenin mutations in the CTNNB1 exon 3. A similar result was reported by Sanders et al. who studied 22 OSCC [38]. Nonetheless, samples obtained by means of endoscopic biopsy were very small; their DNA analysis was limited. Others have also described genetic alterations other than mutations in the sequence for GSK-3β phosphorylation, such as deletions involving the complete exon 3. Mutations in APC that result in truncated APC protein lead to increased cytoplasmic β-catenin. Alteration of β-catenin expression has been reported in colonic epithelial cells of familial APC patients [14] and in adenomatous polyps from patients without familial APC and has also been observed in sporadic aggressive fibromatosis associated with somatic APC mutations. Nevertheless, mutations of APC have not been reported in oesophageal carcinomas [28] despite a high frequency of loss of heterozygosity at the APC locus and on the long arm of chromosome 5.

Other molecules acting upstream from APC may hypothetically activate the APC/β-catenin/tcf pathway. The Frizzled (Fz) family has been detected acting as receptors for Wnt proteins [4]. Recently, Tanaka et al. [42] reported a novel member of the human Fz family that could be altered in OSCC and may down-regulate APC function and enhance β-catenin-mediated signals. This third path could explain β-catenin activation in the absence of APC or β-catenin gene mutations.

Altered β-catenin function has been reported in several adenocarcinomas as an important factor in their carcinogenic processes. However, the role of β-catenin in squamous carcinomas is not yet clear. This study has demonstrated abnormalities in the β-catenin expression pattern in OSCC, particularly in the group that presented nuclear expression who also presented distinctive clinical and pathological features. These results suggest that β-catenin could be involved in the carcinogenesis of a subset of OSCC, but additional studies are needed.

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