Implications of Differential Expression of β-Catenin in Oral Carcinoma

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Abstract. *Background: Alterations of β-catenin can promote cancer progression, but there has been little analysis of this protein in oral squamous cell carcinoma (OSCC). The objective of this study was to investigate the role of β-catenin in OSCC development. Materials and Methods: Expression of β-catenin and Ki-67 from 92 OSCC was immunohistochemically studied in tissues close to the tumor invasion point and, in 37 cases, in tissues distant from the invasion point. Results: Loss of membranous β-catenin expression was observed in 77.2% of "close" samples and 94.4% of "distant" samples. Nuclear β-catenin expression was detected in 35.9% of adjacent samples and 44.4% of "distant" samples. Nuclear β-catenin expression in ≥10% of cells was observed in 6.6% of "close" samples and 25% of "distant" samples. No relationship was found between β-catenin expression and cell proliferation. Conclusion: β-Catenin plays a major oncogenic role in OSCC, principally through increase in invasiveness due to loss of its membranous expression.*

β-Catenin, a member of the Armadillo family of proteins, has multiple functions according to its cellular localization. They derive from its interaction with other cellular proteins, on the membrane and both in the cytoplasm and nucleus (1, 2). β-Catenin forms a complex with the adhesion molecule E-cadherin, promoting cell–cell adhesion and contributing to the structural formation of the stratified squamous epithelium of the oral mucosa, thereby preventing cell dissociation required for cancer invasion and progression. Besides its membranous localization, there is a dynamic pool of cytoplasmic β-catenin that serves as connection between the extracellular microenvironment and nucleus through the plasma membrane (3). In its cytoplasmic localization, β-catenin acts as signal transcription factor to the nucleus in the canonical Wingless-type MMTV integration site family (WNT) pathway, activating the transcription of genes with different cell functions (4). Cytoplasmic β-catenin accumulation, secondary to the lack of degradation of this molecule, is essential for its function as a transcription factor (3). The activation of β-catenin degradation mechanisms in the cytoplasm mainly depends on the status of the canonical WNT signaling pathway. In the absence of WNT signaling, cytoplasmic β-catenin is constantly degraded by the action of a multiprotein complex formed by axin, adenomatous polyposis coli (APC) protein, and the kinases casein kinase 1 (CK1) and glycogen synthase 3 (GSK3) (3). Axin plays a key role in this complex by coordinating the sequential phosphorylation of β-catenin through independent domains for interacting with the other proteins in the complex, first on serine 45 for CK1, and then on threonine 41, serine 37, and serine 33 for GSK3 (4). These β-catenin phosphorylation events create a binding site for β-transducin repeat-containing protein E3 ubiquitin ligase, that induces β-catenin ubiquitination and its proteasomal degradation (5). The constant removal of β-catenin prevents it from reaching the nucleus and acting as transcription factor for its target genes. The translocation of β-catenin from the cytoplasm to the nucleus is the final step of the canonical WNT pathway and permits the transcription of β-catenin target genes, notably the cyclin D1 gene (6). The function of β-catenin as nuclear transcription factor requires its binding with the family of T-cell factor (TCF)/lymphoid enhancer binding factor transcriptions factors, which constitute the main β-catenin pair for gene regulation (7, 8). Over the past few years, it has been confirmed that structural or functional alterations of β-catenin can promote cancer progression at different localizations, either by increasing cell mobility and invasiveness secondary to the loss of cell adhesive functions or by promoting oncogene

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transcription linked to a dysregulation of the canonical WNT pathway. Immunohistochemical studies are of great interest in analyzing the oncogenic activity of multifunction proteins that, like β-catenin, develop physiological or oncogenic functions depending on their presence or absence in different cellular localizations. Although the study of oncogenesis linked to β-catenin began over a decade ago, there has been little analysis in cancer of the oral cavity, despite its high prevalence and the poor prognosis of 50% of patients (9).

The objectives of the present study were to: immunohistochemically analyze β-catenin expression in 92 OSCC samples from 71 patients, examine the role of alterations in this expression in malignant epithelia and the biopathological significance of its different cellular localizations, and to identify possible cell proliferation changes linked to β-catenin expression.

Materials and Methods

We studied 92 OSCC samples from 71 patients (Tables I and II) aged 45-87 years (mean=64 years) under treatment at the Jaen Hospital Complex (Spain); 51 (73.9%) were males. Patients' hospital records were reviewed and data were gathered on the clinicopathological characteristics of lesions. Tumor tissue samples were classified as "distant" when >1 cm from the tumor invasion point or "close" when ≤1 cm from this point (10). Close tumor tissue was available for all OSCCs, whereas distant tissue was only available in 37 cases. Clinicopathological data not found in the clinical records were recorded as missing. For the immunohistochemical study, one 4-μm section was cut from paraffin blocks of each case and the peroxidase–antiperoxidase technique was applied, using the avidin–biotin method. The study was performed automatically with Autostainer Link equipment (Dako, Carpinteria, CA, USA) and EmVision™ *FLEX* reagents (K8002; Dako) according to the manufacturer's instructions. In this system, de-waxing and rehydration is followed by recovery of the heat-induced epitope, and the reproducibility of the process is ensured by loading the entire slideholder, guaranteeing the identical heating of all sections in each cycle. We used monoclonal mouse anti-human beta-catenin clone β-catenin-1 (NHC-38; Dako), as primary antibody for β-catenin and the Mib-1 primary antibody (Dako) recommended by the manufacturer for this automatic system. Counterstaining was performed with the EmVision™ *FLEX* hematoxylin system (K8008; Dako), which gives a light blue nuclear stain, and the samples were then permanently mounted in DPX®. For negative controls, the primary antibody was replaced with phosphate-buffered saline; for positive controls, tissue was used from an OSCC known to intensively express β-catenin or Ki-67. The immunohistochemical result was considered positive when a brown color appeared in the membrane, cytoplasm, or cell nucleus for β-catenin and in the nucleus for Ki-67. Marker expression was assessed in four randomized high-power fields (×40). An optic micrometer was used to count the total cells and positive cells in each field, obtaining a mean percentage expression in every case. All measurements were made by a single experienced observer (MAGM). The impact of subjectivity on the color evaluation was further reduced by applying an automated immunohistochemical technique and using positive and negative controls. In one case, the tumor tissue corresponding to the distant tumor suffered deterioration during the immunohistochemical processing and could not be evaluated.

Table I. *Description of patients with oral cancer (71 patients with 92 tumors).*

Variable	n (%)
Gender	
Female	18 (26.1)
Male	51 (73.9)
Missing, n	2
Age (years) range	45-87
Mean (±SD)	64±11
Missing data, n	15
Tobacco	
Non-smoker	11 (27.5)
1-10 cigs/day	3 (7.5)
11-20 cigs/day	12 (30.0)
>20 cigs/day	7 (17.5)
Former smoker	1 (2.5)
Unknown quantity	6 (15.0)
Missing data (n)	(31)
Alcohol	
Non-drinker	11 (29.7)
Mild	1 (2.7)
Moderate	3 (8.1)
Severe	13 (35.1)
Unknown quantity	9 (24.3)
Missing data, n	(34)

cigs: Cigarettes.

Table II. *Number of tumors per patient and tumor site (n=92 tumors).*

	n (%)
Number of tumors	
1	56 (78.9)
2	11 (15.5)
3	2 (2.8)
4	2 (2.8)
Tumor site	
Floor of mouth	17 (18.4)
Tongue	48 (52.1)
Floor + tongue	9 (9.7)
Trigone	5 (5.4)
Buccal mucosa	5 (5.4)
Soft palate + buccal mucosa	5 (5.4)
Lower lip	2 (2.1)
Gingiva	5 (4.3)

Results

Table III shows β-catenin expression at the different cellular localizations for the tumor tissues. Loss of membranous β-catenin expression was observed in 77.2% (71/92) of close tumor tissue samples and 94.4% (34/36) of distant tumor tissue samples (Figure 1A and B). Cytoplasmic β-catenin expression in >25% of neoplastic cells was observed in 53.3% of close samples and 61.1% of distant samples

Table III. *Percentage of cells with β-catenin expression in close and distant tumor tissues.*

	Expression (positivity)					
Tumor zone	0%	1-25%	26-50%	51-75%	76-100%	Mean±SD
Close (n=92)						
Membrane	77.2	6.5	4.3	5.4	6.5	12.2±27.6
Cytoplasm	34.8	12.0	12.0	13.0	28.3	40.0±38.6
Nucleus	64.1	34.8	0	1.1	0	3.1±8.5
Distant (n=36)						
Membrane	94.4	2.8	2.8	0	0	1.5±6.3
Cytoplasm	27.8	11.1	11.1	27.8	22.2	44.9±37.1
Nucleus	55.6	41.7	2.8	0	0	5.2±8.1

Table IV. *Percentage of cells with Ki-67 expression in close and distant tumor tissues.*

	Expression					Mean±SD
Tumor zone	0%	1-25%	26-50%	51-75%	76-100%	
Close (n=92)	1.1	22.8	39.1	28.3	8.7	43.4±21.3
Distant (n=37)	0	13.5	40.5	32.4	13.5	48.8±21.9

(Figure 1C). Nuclear β-catenin expression was observed in 35.9% (33/92 cases) of distant samples and 44.4% (16/36) of close samples, although nuclear β-catenin in ≥10% of neoplastic cells was found in only 6.6% (6/92) of close samples and 25% (9/36) of distant samples (Figure 1D).

A complete lack of β-catenin expression in membrane, cytoplasm and nucleus was observed in 21.7% (20/92 cases) of close samples and in 25% (9/36 cases) of distant samples (Figure 1E). Only one case had lost membranous β-catenin expression in close tumor tissue and retained this expression in distant tumor tissue (0.36%; 1/36 cases); 11.1% (4/36 cases) of cases that did not express cytoplasmic β-catenin in close tumor tissue expressed it in distant tumor tissue, and 13.9% (5/36 cases) exhibited negative nuclear β-catenin expression in close tumor tissue and positive expression in distant tumor tissue. In some cases, loss of membranous β-catenin expression was observed with expression of nuclear β-catenin in 30.4% (28/92 cases) of close tumor tissue samples and 41.7% (15/36) of distant samples. Positive β-catenin expression on membrane, cytoplasm and nucleus was exhibited by 5.4% (5/92) of close samples and 2.8% (1/36) of distant samples. Cytoplasmic β-catenin expression without nuclear β-catenin expression was detected in 29.3% (27/92) of close samples and 27.8% (10/36) of distant samples. However, nuclear β-catenin expression without cytoplasmic expression was never observed, while cytoplasmic and nuclear expression of the marker appeared together in 35.9% (33/92) of close tumor tissue samples and in 44.4% (16/36) of distant samples.

Table IV presents the Ki-67 expression results. We observed no relationship between mean Ki-67 and β-catenin expression in close and distant tumors.

Discussion

The results of this study demonstrate that oncogenic activity linked to β-catenin is mainly related to invasiveness due to loss of its membranous expression. A high proportion of tumor samples close to and distant from the invasion point showed loss of β-catenin expression on the membrane (77.2% of close samples and 94.4% of distant samples), and the proportion with expression in <25% of OSCC cells was even higher. Complete loss of membranous β-catenin was previously reported in around 50% of oral carcinomas (11) and has been related to a worse histological grade (12) and greater lymph node involvement (13). According to the present results, this oncogenic mechanism, related to cell dissociation and increase in invasiveness due to β-catenin loss, operates from the earliest phases of OSCC invasion, facilitating the local expansion of malignant cell clones. In addition, our finding of completely negative membranous, cytoplasmic and nuclear β-catenin expression in 21.7% (20/92 cases) of close tumors and 25% (9/36 cases) of distant samples indicates that this may be the sole β-catenin-related oncogenic mechanism in a substantial proportion of cases. Only one case (0.36%; 1/36) exhibited loss of membranous β-catenin expression in close tumor and its recovery in distant tumor. From a molecular perspective, loss of β-catenin expression is related to loss of E-cadherin expression, frequently due to methylation of the *CDH1* gene, which encodes E-cadherin. Interestingly, *CDH1* methylation is not irreversible. Researchers isolated *in vitro* highly invasive OSCC clones (MSCC-Inv1 and MSCC-Inv2) with reduced or absent E-cadherin/β-catenin expression in comparison to parental cells and reported the reversible hypermethylation of *CDH1* gene (14). In other words, the hypermethylated gene can be demethylated and return to expressing E-cadherin and therefore β-catenin, restoring cellular adhesiveness. This mechanism appears to be important for restoring the growth advantage of tumor nests, linked to the establishment of metastatic colonies favored by cell–cell contact upon arrival of the cells to lymph nodes or distant organs. However, our results appear to indicate that re-expression of β-catenin, possibly linked to this reported molecular phenomenon, is not frequent in the local expansion of OSCC, in which priority may be given to mechanisms linked to a gain in invasiveness rather than to canonical activation of WNT.

Nuclear β-catenin expression in tumor cells, *sine qua no* condition for complete activation of canonical WNT pathway, was frequent in our OSCC series. Thus, both

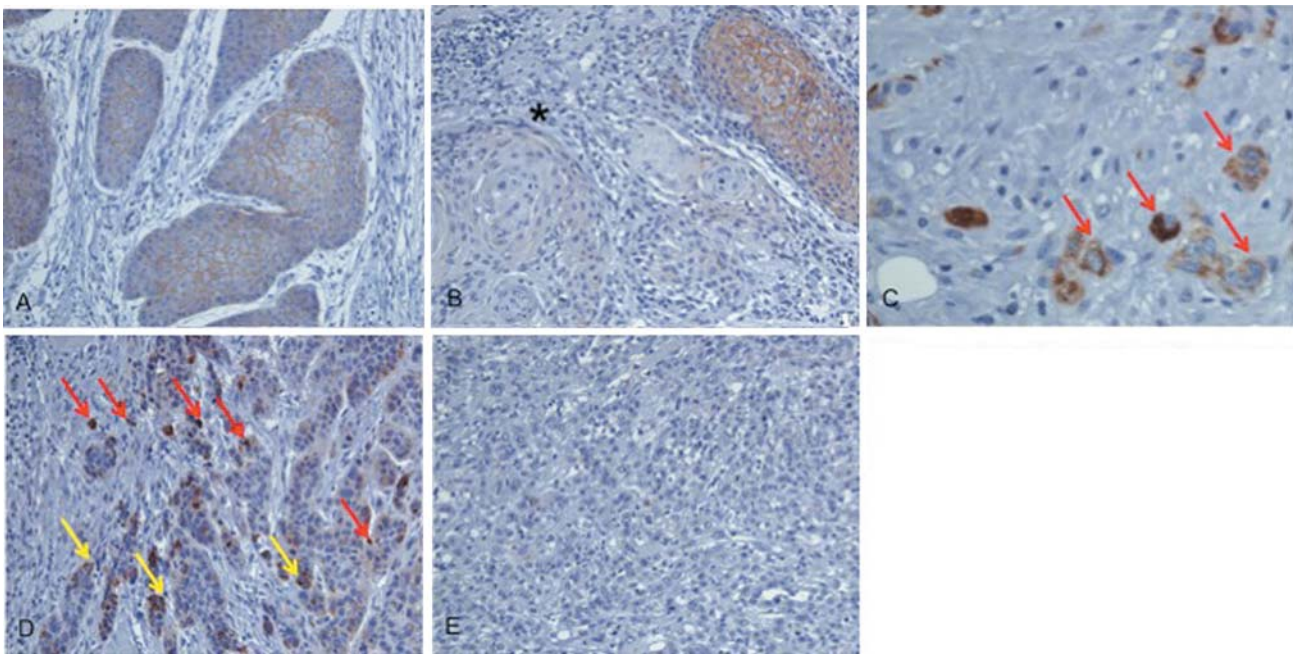

Figure 1. *A: Membranous β-catenin expression with absence of cytoplasmic and nuclear expression (×200). B: Loss of β-catenin expression in membrane (asterisk) with preservation of membranous expression in an adjacent area (×200). C: Cytoplasmic expression of β-catenin with absence of nuclear expression (arrows) (×400). D: Nuclear and cytoplasmic β-catenin expression (arrows); some cells show cytoplasmic expression without nuclear expression (yellow arrows) (×200). E: Complete absence of β-catenin expression in membrane, cytoplasm, and nucleus (×200).*

distant and close tumors exhibited nuclear β-catenin expression in 35.9% (33/92) and 44.4% (16/36) of cases, respectively, although the proportion of tumors that expressed nuclear β-catenin in ≥10% of neoplastic cells was considerably lower (6.6% of close samples and 25% of distant samples). Hence, the well-documented activation of canonical WNT in oral carcinogenesis may not affect the majority of tumor cells. In this regard, it was recently suggested that the canonical WNT pathway may be involved in maintaining the population of cancer stem cells (CSCs), a scarce population of malignant cells that maintains tumor growth, while nuclear β-catenin expression has been proposed as a marker of this cell population (6, 15). The low rate of malignant cells expressing nuclear β-catenin is consistent with the small number of CSCs in a tumor, although further research is required to establish its value as a CSC marker. Nuclear expression of the protein was associated with loss of its membranous expression in 30.4% (28/92) of close tumor samples and 41.7% (15/36) of distant tumor samples in our series, consistent with previous reports (16, 17). This observation in close tumor samples indicates that the role of β-catenin in oral carcinogenesis frequently involves all its oncogenic properties. However, our results suggest that the exclusive participation of the canonical WNT pathway in oral carcinogenesis is rare, with only 5.4% (5/92) of close tumors and 2.8% (1/36) of distant tumors exhibiting positive membranous, cytoplasmic, and nuclear β-catenin expression. As noted above, the expression of β-catenin in the cell nucleus and its function as an oncogene transcription factor require its aberrant accumulation in the cytoplasm due to the failure of physiological degradation mechanisms. This is corroborated by the absence of cases with nuclear β-catenin expression and negative cytoplasmic expression in the present study. Cytoplasmic accumulation of β-catenin, simulating activation of the canonical WNT pathway, can be produced under certain molecular conditions, including the mutation of *β-catenin* gene or of genes encoding the proteins that degrade it in the cytoplasm (18). In general, however, β-catenin mutations are rare in human carcinogenesis (19, 20). Thus, no β-catenin gene mutations were found in most studies of oral or head and neck carcinomas (18, 21-24) and, when observed, they did not translate into a gain-of-function (21). Mutations of the *APC* gene that encodes the APC protein, necessary for the cytoplasmic degradation of β-catenin, are rare in OSCC (25) or are not accompanied by amino-acid substitution (18). Similar observations have been made for mutations in the *axin-1* gene encoding the axin protein, essential in the β-catenin cytoplasmic degradation complex. Most authors found no mutations of this gene in OSCC cell lines or in

clinical samples, with only one study reporting three polymorphisms of the axin-1 gene, two of which did not result in amino-acid substitution (26). Another possible mechanism for inactivating β-catenin degradation is loss of heterozygosity in the *APC* gene. This phenomenon has been reported in 25-75% of OSCC samples, especially in exons 11 and 15 (23, 25, 27). However, this inactivating mechanism has not been studied in other genes that participate in the canonical WNT pathway. The rareness of mutations in β-catenin, *APC*, and axin-1 genes suggests that the aberrant activation of the canonical WNT pathway may be attributable to epigenetic changes. Thus, frequent epigenetic inactivation of secreted frizzled-related proteins genes that encode WNT inhibitors has been observed in OSCC (28), although another study detected no CpG island methylation in β-catenin, axin-1, or GSK3β genes in oral cancer (21).

The action of nuclear β-catenin expression as a transcription factor gives rise to the activation of oncogenes involved in increased cell proliferation. The expression of *CD1* oncogene, one of the targets of β-catenin, strongly depends on the β-catenin/TCF complex and has a direct effect on cell proliferation in some types of carcinomas, *e.g.* hepatocellular carcinoma (29). In the present study, however, no association was observed between nuclear β-catenin expression and the Ki-67-assessed increase in proliferative activity of tumor cells. There are two possible explanations for this apparently contradictory result. Firstly, the low frequency of nuclear β-catenin expression suggests that other oncogenic mechanisms may be involved and play a greater role in increasing tumor cell proliferation in comparison to β-catenin. In addition, we recently reported that Ki-67 expression does not faithfully represent the fraction of tumor cells undergoing oncogenic events, given that some proliferative cells in a tumor may represent a fraction capable of behaving as transitory amplifying cells of oral epithelium destined for terminal differentiation, as in normal epithelium (30).

In conclusion, β-catenin plays a major oncogenic role in OSCC, largely through the increase in invasiveness that results from the loss of its expression, while activation of target oncogenes of the canonical WNT pathway appears to be less relevant. It is important to consider immunohistochemical parameters whose evaluation could serve to avoid misinterpretations of the oncogenic functions of this molecule. Most studies of oral carcinomas and pre-malignant epithelia attribute the same oncogenic capacity to cytoplasmic and nuclear β-catenin expression, and many do not differentiate between them in their statistical analyses. In our view, solely nuclear β-catenin expression should be considered as a marker of canonical WNT pathway activation, while loss of its membranous expression may reflect an increase in invasiveness. Expression in the cytoplasm would be an intermediate step in the physiopathology of this multifunctional molecule, where it can be degraded by the ubiquitin proteasome system or stored for translocation to the nucleus.

Conflicts of Interest

The Authors declare no conflict of interest.

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