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# The relationship between beta-catenin and apoptosis: A cytological and immunocytochemical examination

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ABSTRACT

Disruption of the adhesive role of beta-catenin by caspases has been reported; however, the relationship between the Wnt/beta-catenin signaling pathway and apoptosis remains unclear. Therefore, we aimed to evaluate squamous epithelial cells in cervicovaginal smears by using cytological and immunocytochemical methods to observe changes in the presence and localization of beta-catenin during apoptosis, death receptor-, and mitochondria-mediated apoptosis. We investigated 224 cervicovaginal smears using the Papanicolaou method. Anti-beta-catenin and anti-cleaved caspase 3, 8, and 9 antibodies were used for immunocytochemical staining. Apoptotic cells were negative for beta-catenin. This showed that the Wnt/beta-catenin signaling pathway was inactive in apoptotic cells. However, beta-catenin showed intense positivity in the membrane, cytoplasm, and nucleus of non-apoptotic epithelial cells around these apoptotic cells. Therefore, the Wnt/beta-catenin signaling pathway was active in non-apoptotic epithelial cells, and this activity in non-apoptotic cells may have been induced by apoptotic cells. A highly significant association between the presence of death receptor-mediated apoptosis and the activity of the Wnt/beta-catenin signaling pathway was also found ($P < 0.001$). In conclusion, the Wnt/beta-catenin signaling pathway was found to be inactive in apoptotic cells, but apoptotic cells may induce the Wnt/beta-catenin signaling pathway in non-apoptotic cells to compensate for a decrease in epithelial cells because of apoptosis in order to maintain epithelial tissue integrity.



## 1. Introduction

Beta-catenin (β-catenin) was first identified as an essential molecule in mediating cell–cell adhesion. During this cell–cell adhesion, β-catenin binds to the cytoplasmic tail of E-cadherin and forms a cadherin–catenin complex, which acts as a molecular bridge between actin filaments and E-cadherin (Ozawa et al., 1989; Aberle et al., 1996). β-catenin is also a target protein of the Wnt/β-catenin signaling pathway (Willert and Nusse, 1998). The Wnt/β-catenin signaling pathway regulates embryonic development and homeostasis of adult tissues by controlling cell proliferation, differentiation, survival, and biological processes such as adipogenesis and angiogenesis (Clevers, 2006; Christodoulides et al., 2009; Dejana, 2010).

Many studies have assessed the relationship between apoptosis and β-catenin. Brancolini et al. (1997) demonstrated that β-catenin was cleaved by activated caspases during apoptosis in fibroblast and epithelial cell cultures. Brancolini et al. (1998) and Steinhusen et al. (2000) manifested the dismantling of the cadherin–catenin complex and decrease in β-catenin-mediated transactivation after cleavage by these proteolytic caspase enzymes. The relationship between the Wnt/β-catenin signaling pathway and apoptosis has been widely investigated through several reports; however, this relationship is not fully understood. In a previous study using *Drosophila melanogaster* embryos, it was shown that the Wg (Wnt) signaling pathway induces apoptosis in retinal neurons (Ahmed et al., 1998). In contrast, other reports showed an inhibition of apoptosis by the Wnt/β-catenin signaling pathway in fibroblasts, epithelial cells, and colon carcinoma cell lines. These authors suggested that the inhibition of apoptosis by the Wnt/β-catenin signaling pathway was through its oncogenic activity (Chen et al., 2001; Ueda et al., 2002; You et al., 2002).

In previous studies, the presence of β-catenin was examined using immunocytochemistry of cervicovaginal smears and endometrial thin layer specimens (Politi et al., 2008; Norimatsu et al., 2008a; Norimatsu et al., 2008b). Apoptosis was detected in cervical biopsy samples using immunohistochemistry (Zanotti et al., 2003). In other studies using fine-needle aspiration of breast and ovarian follicular fluid, immunocytochemistry was performed to detect apoptosis. In an immunofluorescence-based study of tra-

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cheal aspirates, immunocytochemistry was also used to detect apoptosis (Zoog et al., 2010; Glamočlija et al., 2005; Cheah et al., 2005). However, there are no previous studies elucidating the relationship between β-catenin and apoptosis in cervicovaginal smears using immunocytochemistry. In the present study, we have investigated whether there is a relationship between β-catenin and apoptosis in cervicovaginal smears by using cytological and immunocytochemical techniques. Furthermore, we have also determined the presence and localization of β-catenin in apoptosis and the activity of the Wnt/β-catenin signaling pathway in apoptotic and non-apoptotic cells.

## 2. Materials and methods

### 2.1. Case sellection

In our study, 224 patients with various gynecological complaints were examined at the Gynecology and Obstetrics Clinics in Ankara, Turkey (March–October 2014). Pregnant women were not included in the study. The mean age of the patients was 41.3 ± 12.3 years (20–76 years). This study was approved by the local ethics committee (LUT 2012-12/170). Cervicovaginal fluid was obtained from women by using a cytobrush, and evaluated using cytological and immunocytochemical methods.

### 2.2. PAP smear microscopy

Cervicovaginal fluid was smeared on a slide in one direction and fixed with 96% ethanol without air drying. These smears were stained using the routine Papanicolaou (PAP) method and examined by light microscopy in detail to view infections and atypical cellular changes.

### 2.3. Immunocytochemistry

After fixation with 96% ethanol, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, followed by an avidin-biotin peroxidase complex technique using VECTASTAIN Elite Universal ABC detection kit (Vector Laboratories, USA). The following antibodies were used in the immunocytochemical methods: to examine the presence and localization of β-catenin, rabbit monoclonal anti-β-catenin antibody (1:100 dilution, Cell Signaling Technology, USA) was used, and to detect apoptosis, anti-cleaved caspase 3 (CC3) rabbit monoclonal antibody (1:400 dilution, Cell Signaling Technology, USA), anti-cleaved caspase 8 (CC8) rabbit monoclonal antibody (1:100 dilution, Cell Signaling Technology, USA), and anti-cleaved caspase 9 (CC9) rabbit polyclonal antibody (1:100 dilution, Thermo Scientific, Pierce antibodies, USA) were used. 3,3′-Diaminobenzidine was used as a chromogen (Vector Laboratories, USA). The samples were counterstained with Harris' hematoxylin (MERCK, Germany), followed by dehydration and mounting. All steps were performed at room temperature and in a humidity chamber. For the negative control, the same technique of immunocytochemistry staining was applied in the absence of primary antibodies (Fig. 1a–d). As positive controls, normal colon tissue was used for anti-cleaved caspase antibodies, and colon carcinoma tissue for β-catenin (Fig. 2a–d).

Ten random fields were counted in each slide under a light microscope at 40× magnification without knowledge of the cases. Results were presented as (−) negative, (+) weak, (++) moderate, (+++) strong, and (++++) very strong. For semi-quantitative analyses, H-scores were calculated as the sum of the percentages (%) of positively stained cells multiplied by the intensity of staining for all antibodies. The scoring method was performed according to previously published work (Detre et al., 1995).

The Wnt/β-catenin signaling activity was evaluated based on the following scores: weak (+), moderate (++), strong (+++), and very strong (++++) positivity of membranous staining indicated inactive signaling. In addition to membranous staining, weak (+) moderate (++), strong (+++), and very strong (++++) positivity of cytoplasmic and nuclear staining showed activated signaling.

To detect apoptosis, cases in which cells were stained with CC3 were accepted as being apoptotic (+). When CC3 and CC8, or CC3, CC8, and CC9 were positive together, these cases were defined as death receptor-mediated apoptosis (+), whereas CC3 and CC9 positive cases were defined as mitochondria-mediated apoptosis (+).

### 2.4. Statistical analysis

The Statistical Package for the Social Sciences (version 22.0) was used for statistical analysis. Chi-square and Fisher's exact tests were used to compare the relationship between the activity of the Wnt/β-catenin signaling pathway and the presence of apoptosis, death receptor- and mitochondria-mediated apoptosis. To compare the mean of H-scores, Mann-Whitney *U* test was used because H-scores were not normally distributed in all groups. *P* value less than 0.05 was considered statistically significant.

## 3. Results

Cervicovaginal smears from 224 cases were cytologically examined by the PAP method to observe infections and atypical cellular changes. Subsequently, β-catenin, CC3, CC8, and CC9 were detected using the immunocytochemical method.

During the examination of PAP-stained smears, infections and atypical cellular changes were observed in 80 of 224 cases (35.7%). Cytological findings are given in Table 1. Smears that were positive for bacterial vaginosis, *Trichomonas vaginalis*, cytolytic vaginosis, fungal infections, inflammation, and atypical changes, including atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion, and high-grade squamous intraepithelial lesion, were excluded from the study in order to compare β-catenin and apoptosis without these influences (included cases: 144/224, 64.3%).

In the immunocytochemical examination, the membranous (Fig. 3a–d), cytoplasmic and nuclear positivity for β-catenin were evaluated (Fig. 4a–d). Epithelial cells were only positive for membranous staining in 128 of 144 (88.9%) cases, which were considered to be inactive in view of the Wnt/β-catenin signaling pathway. β-catenin was localized in the cytoplasm and/or nucleus in 26 of 144 cases (18.1%). The Wnt/β-catenin signaling pathway was considered to be positive in these cases. As seen in Fig. 5, the mean membranous, cytoplasmic, and nuclear H-scores of β-catenin increased substantially because of the activity of the Wnt/β-catenin signaling pathway ($P < 0.001$).

In apoptotic cells, the membrane, cytoplasm, and nucleus were not stained for β-catenin (Fig. 6a). Therefore, the Wnt/β-catenin signaling pathway was inactive in apoptotic cells. The most striking finding was the observation of positive cytoplasmic and/or nuclear staining in non-apoptotic epithelial cells around the apoptotic cells (Fig. 6b).

Statistical analyses were performed to detect correlation between apoptosis (Fig. 7a–c) and the activity of the Wnt/β-catenin signaling pathway (Table 2). In cases with signaling activity ($n = 26$), apoptosis was positive in 18 of them (18/26, 69.2%). The activity of the Wnt/β-catenin signaling pathway was significantly associated with the presence of apoptosis ($P < 0.01$). Furthermore, death receptor-mediated apoptosis was positive in 17 of 26 cases (65.4%). There was a significant relationship between the activity of the Wnt/β-catenin signaling pathway and death receptor-mediated