Detection of the WNT/Beta-Catenin Signaling Activity by Using Immunocytochemical Technique in Cervical Smears

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OBJECTIVES: The Wnt/β-catenin signaling pathway controls cell fate and homeostasis of embryonic and adult tissues. The aim of the study is to evaluate the activity of Wnt/β-catenin signaling in cervical smears by using immunocytochemistry.

STUDY DESIGN: Cervical smears taken from 200 patients were examined in terms of immunostaining of β-catenin. The membranous staining with moderate (++) or strong (+++) cytoplasmic and/or nuclear staining was scored positive for signaling activity.

RESULTS: Positive Wnt/β-catenin signaling was found in 21 of 200 cases (10.5%). The nucleus was not found to be positive in any of the cells. However, some nuclear membranes were positive. In addition, when signaling was active, the membranous expression of β-catenin was observed to increase by a statistically significant amount epithelial cells ($p<0.05$).

CONCLUSION: Our data indicates that immunocytochemical techniques can be used to detect the activity of the Wnt/β-catenin signaling pathway in routine cervical smears.

Key Words: Wnt signaling, Beta-catenin, Cervical smear, Immunocytochemistry

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Introduction

Wnt/β-catenin signaling regulates cell proliferation, differentiation, apoptosis, adipogenesis, and also homeostasis of tissues and organs.[1,2] However, the aberrant activation of the signaling has been determined in various cancers including colorectal, cervical, uterine, and ovarian carcinomas, and formation of several serious diseases including osteoporosis and tetra-amelia.[3,4]

In the past, immunohistochemical techniques have commonly been used to detect the activity of Wnt/β-catenin signaling in gynecological specimens and tissue samples.[5,6] In a few previous studies, Norimatsu et al reported the expression of β-catenin in normal endometrium, benign endometrial lesions and endometrial carcinoma by using thin layer specimens.[7,8] Moreover, Politi et al reported the presence of β-catenin in the cell membrane by using conventional cervical smears.[9] Since there are few studies about Wnt/β-catenin signaling in cervical smears, we conducted the present study in order to contribute new insight into this issue.

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Material and Method

Case selection

In this study, 200 patients aged 18-73 years with varied gynecological complaints were seen at the Gynecology and Obstetrics Clinics of Hacettepe University in Ankara, Turkey (October 2009-February 2010). Pregnant women were not included in the study. This study was approved by the local ethics committee and applied according to the principles of The Declaration of Helsinki.

Immunocytochemistry

Cervical samples, including cells from the ectocervix and the endocervical canal, were taken with a cytobrush from each patient before a pelvic examination, and two slides were prepared for immunocytochemical technique. All samples were fixed with 96% ethanol for 30 minutes without drying in the air. These fixed samples were stained with an immunocytochemical method.

The Envision HRP/DAB plus kit (DAKO, Denmark) was used to detect β-catenin expression. After fixation, endogenous peroxidase activity was blocked with 0.3% hydrogen

peroxide for 5 minutes, and then the samples were washed twice with distilled water. The samples were blocked with 2% normal bovine serum (Merck, Germany) for 30 minutes, incubated for 30 minutes with a monoclonal mouse anti-β-catenin antibody (Clone Catenin beta 1, DAKO, USA) at a dilution ratio of 1:100. After washing the samples with phosphate buffer saline (pH: 7.4), a secondary antibody was added; it was then incubated an additional 30 minutes and washed three times with phosphate buffer saline. Next, Diaminobenzidine (DAB) was used as a chromogen. The samples were counterstained with Harris' hematoxyline (Merck, Germany), then dehydrated and mounted. All steps were performed at room temperature. For the negative control, the same technique for immunocytochemistry staining was applied without the primary anti-β-catenin antibody (Figure 1a).

Scoring

Immunostaining with the anti-β-catenin antibody was performed to evaluate the activity of Wnt/β-catenin signaling in epithelial cells. During the procedure, we examined parts of the cell where DAB turned certain β-catenin locations brown. In our study,[1] membranous staining with weak (+) cytoplasmic staining was scored negative for signaling activity, and[2] membranous staining with moderate (++) or strong (+++) cytoplasmic and/or nuclear staining was scored positive for signaling activity.

Membrane staining was also scored as weak (+), moderate (++) and strong (+++) separately from cytoplasm and the nucleus. The percentages of positive β-catenin staining of membranes were estimated by counting and scoring a hundred cells of each squamous epithelial cell type.

Statistical analysis

Immunocytochemical data were analyzed using the Independent t-test with the Statistical Package for the Social Sciences 11.5. Software Program (SPSS Inc., USA). P values less than 0.05 were accepted as significant.

Results

Evaluation of membranous staining

We evaluated the presence of β-catenin in cell membrane (Figure 1b, 1c). We observed that membranous staining is much stronger when epithelial cells adhere to another epithelial cell membrane (Figure 1d). The effects of Wnt/β-catenin signaling on the presence of β-catenin in the membrane were analyzed statistically by using an Independent t-test. There was a statistically significant difference between the percentages of positively scored membranous cases with both activated and inactivated signaling ($p<0.05$). Membranous expression of β-catenin was found to increase with increasing activity of Wnt/β-catenin signaling. All percentages were given in table 1.

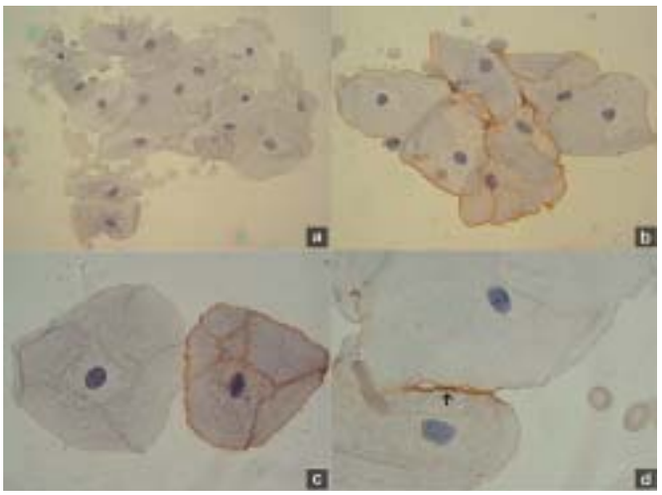

Figure 1:(a) Negative control without primer antibody (Immuno. x400), (b) Different degree of membranous positivity in an epithelial cell group (Immuno., x400), (c) A negative and a (+++) positive membrane for two intermediate cells (Immuno., x1000), (d) Strong membranous positivity of β-catenin (arrow head) where epithelial cells bind each other (Immuno., x1000).

Evaluation of cytoplasmic staining

The presence of cytoplasmic and/or nuclear β-catenin is an important hallmark of the activated signaling mechanism. Weak (+) cytoplasmic positivity was not important for signaling due to low levels of free beta catenin was found in the cytoplasm. Twenty one of 200 cases (10.5%) showed moderate

Table 1: Correlation of membranous and cytoplasmic immunoreactivity for beta catenin

Types of cells	All Cases (%)				Active Signaling (%)				Inactive Signaling (%)				*p value
	Total	+	++	+++	**Total**	+	++	+++	**Total**	+	++	+++	
Superficial cells	9.1	6.7	1.9	0.5	28.8	13.4	10.1	5.3	7.3	6.1	1.1	0.1	*0.000
Intermediate cells	30.7	24.7	5.8	0.2	39.8	22	17	0.8	29.9	25	4.7	0.2	*0.048
Parabasal cells	36.4	17.4	13.7	5.3	51.3	14.8	24.9	11.6	30.6	18.5	9.4	2.7	*0.036

**: Independent t test, p<0.05.*

(++) and/or strong (+++) cytoplasmic staining (Figure 2a, 2b). These cases were scored positive for signaling activity.

In many cases, cytoplasmic staining was most prominent in the parabasal cells, and the number of cells with positive cytoplasmic staining decreased from parabasal to superficial cells. As seen in Figure 2c, we observed that cytoplasmic positivity of β-catenin was predominantly found around the nucleus in some epithelial cells.

Evaluation of nuclear staining

None of the squamous epithelial cells showed positive nuclear staining, but interestingly, nuclear membranes were scored as positive in such cases (Figure 2d).

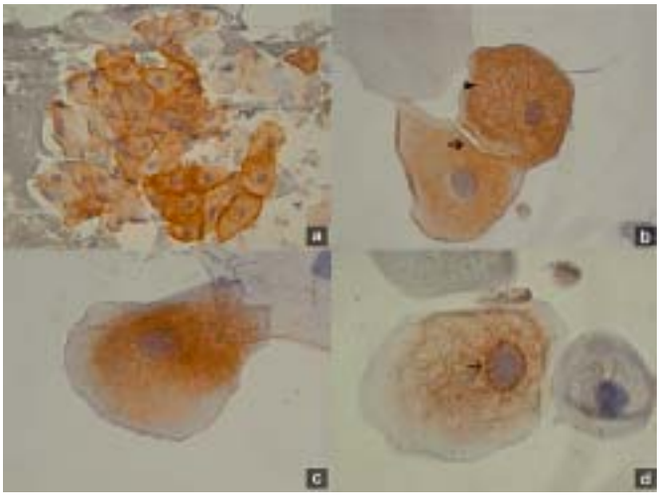

Figure 2: (a) Positive cytoplasmic staining in epithelial cell group (Immuno., 200x), (b) Moderate (arrow) and strong (arrow head) positive cytoplasm in intermediate cells (Immuno., 1000x), (c) Cytoplasmic positivity of β-catenin condensated in the perinuclear area of an intermediate cell (Immuno., 1000x), (d) β-catenin was positive (arrow) on the nuclear membrane, (Immunocyto., 1000x).

Discussion

Membranous positivity of β-catenin in superficial cells was less than in other types of cells, such as intermediate and parabasal cells. In addition, membrane staining was much stronger in cases where epithelial cells interact with another cell membrane (Figure 1d). β-catenin is associated with the cytosolic tail of E-cadherin and functions in mediating zonula adherence in addition to its role as a signal molecule in Wnt/β-catenin signaling.[10] Junctional complexes decrease during maturation of stratified epithelial tissue. According to Cowin et al, desmosomal proteins intensity reduce toward superficial layers.[11] Blaskewicz et al reported that expression of tight, desmosomal and adherence junction proteins decrease from basal to superficial layers.[12] In an earlier immunohistochemical study with cervical biopsies, β-catenin expression on the cell membrane is also more prominent in basal and parabasal cells.[13] Our results were consistent with those of previous studies.

In the absence of Wnt/β-catenin signaling, β-catenin is found only low level in cytoplasm, as a result of quick phosphorylation, ubiquitination and then degradation. In the presence of signaling activity, β-catenin does not degraded and accumulates in the cytoplasm. This accumulation shows that Wnt binds its receptors on the cell membrane and an extracellular signal pass through the cytoplasm, and then nucleus. Positive β-catenin staining of the cytoplasm and/or nucleus is used as a marker for activity of Wnt/β-catenin signaling.[14,15] Thus, only moderate (++) and strong (+++) cytoplasmic positivity which shows accumulation of β-catenin in the cytosol was positive for signaling activity, weak cytoplasmic positivity was not.

In our study, 21 of 200 cases (10.5%) showed moderate and/or strong cytoplasmic β-catenin staining but negative for nucleus (Figure 2a). There are two models of how β-catenin may function in Wnt/β-catenin signaling. Some authors suggest that when Wnt proteins induce signaling, β-catenin translocates to the nucleus and binds to transcription factors in the nucleus that activate transcription of target genes.[16,17] Other authors proposed that nuclear translocation of β-catenin is not required for Wnt/β-catenin signaling.[18,19] In the light of these data, although cytoplasmic β-catenin did not correlate with nuclear positivity in our study, cytoplasmic accumulation was shown to be related to active Wnt/β-catenin signaling.

We observed that a high level of β-catenin was present in the cytoplasm around the nucleus. According to Cox et al, nuclear β-catenin can be observed only in fully activated Wnt/β-catenin signaling.[20] Our results might indicate that after β-catenin reaches a certain level in the cytoplasm, it then accumulates in the nucleus.

In the presence of active Wnt/β-catenin signaling, we found increased amounts of positivity in the membranes of superficial, intermediate and parabasal cells compared with an inactive state of signaling ($p<0.05$). To our knowledge; this is the first immunocytochemical study to examine the relationship between signaling and adhesion in cervical smears. Heuberger and Birchmeier reported that signaling caused a decrease in β-catenin-dependent cell adhesion.[21] Contrary to these findings, Cox et al suggested that expression of β-catenin increased until all E-cadherin binding sites were saturated.[22] Similarly, Howard et al explained that E-cadherin recruited cytoplasmic β-catenin into the cell membrane with a high affinity. In light of these data, our finding suggests that when signaling is active, cells may direct free cytoplasmic β-catenin to the membrane. Only after all binding sites of E-cadherin are saturated, cytoplasmic pool of β-catenin may increase in the cytoplasm.

β-catenin was detected in the nuclear membrane of some cells in our study (Figure 2d). These cells were found only in active signaling cases. We suggest that β-catenin may bind to the nuclear membrane before entering the nucleus. To our knowledge, this is the first light microscopic observation of β-catenin in the nuclear membrane by using immunostaining. Translocation of β-catenin into the nucleus has not yet been fully understood. There are different opinions about how β-catenin migrates to the nucleus. In accordance with our findings, Fagotto et al reported that β-catenin migrates into the nucleus by direct binding of the nuclear pore complex, which controls bidirectional exchange of biomolecules between the cytosol and the nucleus.[23] Alternatively, cytoplasmic proteins such as Importin-β or Lymphocyte enhancer factor-1 (LEF-1) may bind β-catenin in order to take it into the nucleus.[24,25]

In many of our cases, cytoplasmic staining was most prominent in the parabasal cells. Parabasal cells have mitotic activity and form germinative layers of squamous epithelial tissue in addition to basal cells.[26] It was indicated that Wnt/β-catenin signaling controls cell proliferation via the regulation of genes such as c-MYC and CYCLIN-D1 in mitotic cells.1 Thus, signaling in parabasal cells was more active than in intermediate and superficial cells in our study, and this could be related to mitotic activity in parabasal cells.

In conclusion, we evaluated the roles of β-catenin in cell-cell adhesion and Wnt/β-catenin signaling. Membranous staining was scored to determine the adhesive role of β-catenin. As shown in our data, membranous staining for β-catenin increased with an increase in Wnt/β-catenin signaling. As a signal molecule, β-catenin accumulated in the cytoplasm and positivity was most prominent in parabasal cells. Due to these results, cervical smears may be used to indicate the activity of the Wnt/β-catenin signaling pathway.

WNT/Beta - Katenin Sinyal Aktivitesinin İmmünositokimyasal Teknik Kullanılarak Servikal Simirlerde Saptanması

AMAÇ: Wnt/β-katenin sinyal yolu hem hücre kaderini hem de embriyonik ve erişkin doku homeostazisini kontrol eder. Çalışmamızın amacı servikal simirlerde Wnt/β-katenin sinyal yolu aktivitesinin immünositokimya kullanılarak değerlendirilmesidir.

GEREÇ VE YÖNTEM: 200 hastadan alınan servikal simirler β-katenin açısından incelendi. Membranöz boyanmayla birlikte orta (++) veya kuvvetli (+++) sitoplazmik ve/veya çekirdek boyanması sinyal yolu aktivitesi açısından pozitif olarak kabul edildi.

BULGULAR: 200 olgunun 21'inde (%10.5) Wnt/β-katenin sinyali pozitif olarak bulundu. Hiçbir hücrede çekirdek pozitifliğine rastlanmadı ancak bazı çekirdek zarları pozitifti. Ayrıca, sinyal yolu aktif durumdayken epitel hücrelerde β-katenin'in zardaki ifadesinin istatistiksel olarak anlamlı bir miktarda arttığı gözlendi. (p<0,05).

SONUÇLAR: Çalışmamız rutin servikal simirlerde Wnt/β-katenin sinyal yolu aktivitesinin immünositokimyasal teknikler kullanılarak saptanabileceğini gösterdi.

Key Words: Wnt sinyali, Beta katenin, Servikal simir, İmmünositokimya

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