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ORIGINAL ARTICLE

β-catenin gene mutation in invasive ductal breast cancer

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Summary

Purpose: *Aberrant accumulation of β-catenin plays an important role in a variety of human neoplasms. In this study we analyzed the somatic mutations of the β-catenin gene and the immunohistochemical localization of β-catenin and cyclin D1 in invasive ductal breast cancer.*

Materials and methods: *We investigated 65 human invasive ductal breast cancer samples for somatic mutations in the exons 3, 4, 5 and 6 of β-catenin gene (N-terminal region) by the combined use of polymerase chain reaction (PCR), single-strand conformation polymorphism (SSCP) and sequencing. Sample tissues were also analyzed using β-catenin and cyclin D1 immunocytochemistry staining.*

Results: *No β-catenin mutation was detected in any of the tumor samples. Accumulation of aberrant β-catenin protein in cellular compartments in the same breast cancer samples was confirmed with a related experiment by immunocytochemical methods.*

Conclusion: *Our results suggest that genetic defects in β-catenin is not common in invasive ductal breast cancers, whereas mutations in other components of the Wnt signaling pathway should be considered.*

Key words: breast cancer, β-catenin, cyclin D1, localization, mutation

Introduction

Breast cancer ranks first among the different cancers in Turkish women. According to the data obtained from the Turkish Ministry of Health, breast cancer constitutes 24.1% of all cancers in women [1]. Several genes and chromosomal changes seem to be involved in the pathogenesis of breast carcinogenesis. However, the molecular mechanism that contributes to tumor progression remains unknown. In order to investigate one of the genetic alterations that could play a role in tumor progression in breast cancer, we decided to analyze the status of the β-catenin gene.

β-catenin protein is a key regulator of the cadherin-mediated cell-cell adhesion system by linking the cytoplasmic domain of cadherins to α-catenin, which anchors the adhesion complex to cytoskeleton [2,3]. It is also involved in the Wingless/Wnt signaling cascade, a transcription-activating pathway effective on cell proliferation, cell polarity and migration [4-6]. In this pathway, β-catenin forms a heterodimeric complex with the Tcf/Lef family of DNA binding proteins and regulates transcription of target genes e.g., cyclin D1, c-MYC and MMP7 [7]. Modulation of the level of the β-catenin protein is crucial in the regulation of Tcf/β-catenin controlled genes which in turn is regulated through the ubiquitin/proteasome-mediated degradation. Activating mutations in exon 3 of β-catenin gene at phosphorylation sites for ubiquitination and degradation of β-catenin appear to be crucial steps in the progression of a variety of cancers [8-10]. It has been demonstrated that the APC gene product regulates the cytoplasmic level of β-catenin by direct binding and promoting its NH2-terminal phosphorylation by GSK-3β. GSK-3β phosphorylates multiple serine and threonine residues within the NH2-terminal of β-catenin. Phosphorylated β-catenin is targeted for degradation by the proteasome system. Mutations in any of these

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genes can result in accumulation of unphosphorylated free β-catenin and activation of TCF/LEF transcription factors. Interestingly, cyclin D1 and c-MYC have recently been identified as target genes for TCF/LEF transcriptional activation [11,12].

At present, β-catenin gene exon 3 mutations and abnormal accumulation of β-catenin protein in tumor cell nuclei have been reported for a variety of human malignancies, such as colorectal carcinoma, hepatoblastoma, hepatocellular carcinoma, desmoid tumor, Wilms' tumor, melanoma, ovarian carcinoma and prostate cancer [13-15]. Furthermore single-nucleotide changes causing missense mutations in β-catenin gene were also detected in several carcinoma cell cultures. Such changes include codon 183 (exon 4) of HCA 46, codon 245 (exon 5) of Caco 2, and codon 287 (exon 6) of Colo 201/205 cell lines [16].

In this study, we attempted to investigate the β-catenin gene for possible mutations by examining β-catenin exon 3, 4, 5, and 6 regions in tissues samples from invasive breast cancer.

Materials and methods

Tumors samples and DNA extraction

We analyzed samples of breast cancer tissues from 65 patients who had undergone surgical operation at the Department of Surgery, Izmir Education and Research hospital. After resection of the tumors, a piece of the material was snap frozen and stored at –80° C. Formalin-fixed and paraffin-embedded samples of all tumors were examined at the Pathology Department of the same hospital. Genomic DNA was extracted from frozen tissues with NucleoSpin® Tissue (Macherey-Nagel, Duren, Germany) DNA isolation kit.

Mutation analysis

Tumor DNAs were evaluated for mutations in the GSK-3β phosphorylation consensus motif (exon 3), codon 183 (exon 4), codon 245 (exon 5), and codon 287 (exon 6) of the β-catenin gene by PCR-SSCP. Primers for each of the regions indicated were designed using the Fast PCR® primer design program (Institute of Biotechnology University of Helsinki, Finland) (Table 1).

PCR was carried out in 30 μl of reaction mixture containing 20-100 ng genomic DNA, 20 pmol of each primer, 250 μM each dNTP (MBI Fermantas, Ontario, Canada), 2 mM $MgCl_2$, 10X High Fidelity PCR Enzyme Mix Buffer (MBI Fermantas), and 2.5 units of High Fidelity PCR Enzyme Mix (MBI Fermantas). The mixture was heated for 10 min at 94° C for initial DNA denaturation, followed by 35 cycles of denaturation (at 94° C for 1 min), annealing (at 55° C for 2 min), and extension (at 72° C for 3 min) on the PTC-100 Thermal Cycler (MJ Research, Minnesota, USA). The PCR products were applied to electrophoreses in a 3% Nusieve: Seakem LE agarose gel (FMC BioProducts, Philadelphia, USA) (2:1) and visualized with ethidium bromide. For SSCP analysis, 30-40 ng of PCR products were denatured by adding 2.5–5 volumes of stop solution (95% formamide, 5.0 M NaOH, 0.1% bromphenol blue, and 0.1% xylene cyanol) and heated at 95° C for 5 min. After quick chill on ice, samples were loaded onto a 12.5% non-denaturant polyacrylamide gel and run for 3 h in SE 600 vertical gel apparatus (Hoefer Scientific, San Francisco, USA) at 400 V and 18° C. Gel temperature was regulated with a cooling circulating bath. After electrophoresis, the gels were stained for 10-20 min with SYBR-Green II (Amresco, Ohio, USA) and diluted 1:10,000 in TE Buffer (pH 7.4). Images were captured on an Eagle Eye® II Imaging System (Stratagene, Jola, California, USA) using 254 nm or 313 nm UV transillumination and a SG-3 filter.

Immunocytochemical staining

Immunocytochemical analysis was performed with antibodies against β-catenin (Santa Cruz, California, USA; Cat No: sc-7963), and cyclin D1 (Santa

Table 1. β-catenin gene exon primers

Exon 3 (227bp)	Sense	5'-GCTGATTTGATGGAGTTGGA-3'
	Anti-sense	5'-GCTACTTGTTCTTGAGTGAA-3'
Exon 4 (253bp)	Sense	5'-GACAGTATGCAATGACTCGAGC-3'
	Anti-sense	5'-TTGCTTACCTGGTCCTCGTC-3'
Exon 5 (254bp)	Sense	5'-GAATTCCTGTATTACAGGTGGTGG-3'
	Anti-sense	5'-AAGCATTTTCACCAGGGCAG -3'
Exon 6 (217bp)	Sense	5'-TCTTCCCAGTTCACCAGTGG -3'
	Anti-sense	5'-TCTTACCTTGCTTTCTTGGTTGC -3'

Cruz, California, USA; Cat No: sc-8396). The histologic sections of formalin-fixed and paraffin-embedded material were deparaffinized in xylene and dehydrated in ethanol. After dehydration, the endogenous peroxidase was blocked by methanol containing 0.3% hydrogen peroxide for 30 min. The sections were incubated with the primary antibody at 48° C overnight, followed by staining with UltraVision Large Volume Detection System Anti-Polyvalent, HRP (Lab Vision, Fremont, California, USA). The sections were finally reacted in a 3,3' diaminobenzidine peroxytrichloride substrate solution, then counterstained with hematoxylin. Dilutions of primary antibodies were 1:200 for anti-β-catenin and 1:250 for anti-cyclin D1.

Results

Mutational analysis of β-catenin gene and immunocytochemistry of β-catenin protein

PCR-SSCP was used to detect mutations in exon 3, 4, 5 and 6 of the β-catenin gene. No mutation was detected in 65 breast carcinoma samples (Figure 1). In addition, immunocytochemistry of β-catenin protein was carried out to investigate its localization. In normal epithelial cells β-catenin staining was localized on the cell membrane, whereas cytoplasmic and nuclear staining was absent. In the tumor cases, the immunostaining patterns of β-catenin could be divided into two groups: in 63 (96.9%) cases there was membranous but not cytoplasmic or nuclear staining, exactly as in normal epithelial cells, whereas 2 (3.1%) cases showed cytoplasmic and nuclear, as well as membranous staining for β-catenin (Figure 2). Furthermore, immunohistochemistry of cyclin D1 showed positive staining in 61 (93.8%) cases.

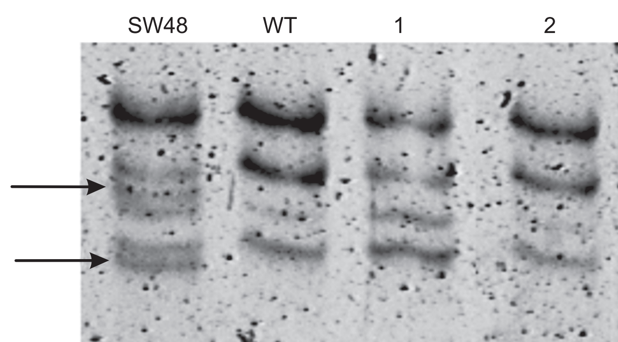

Figure 1. PCR-SSCP shows aberrant bands in SW48 positive control cell line (arrows), wild type (WT) and sample no. 1 and no. 2.

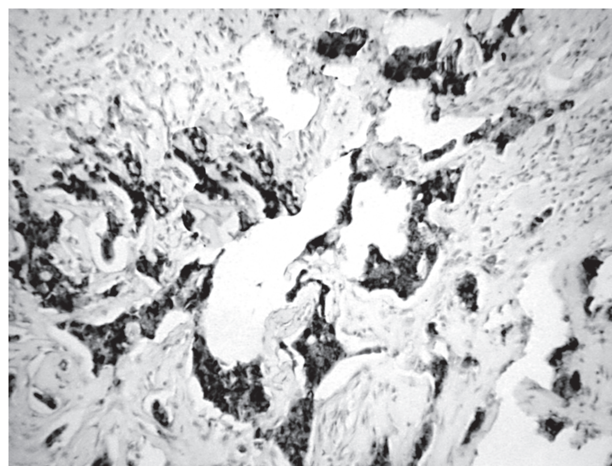

Figure 2. Sample no 23 showing positive cytoplasmic, nuclear and membranous staining for β-catenin.

Discussion

Our study demonstrated that β-catenin was a poor prognostic marker in human breast cancer. How β-catenin activity is upregulated in breast cancer is not clear at this moment. It is possible that activated Wnt pathway may contribute to this upregulation [17-20]. Further studies are required to elucidate the detailed mechanisms.

In the past, β-catenin pathway has been studied mainly in colon carcinoma. Almost 100% of colon cancers have either mutated β-catenin or deleted APC, which is supposed to activate the β-catenin pathway. However, several studies have shown cyclin D1 as the β-catenin target in colon carcinoma [20-24]. Nevertheless, it is worth mentioning that cyclin D1 overexpression has been found in only 30% of colon cancers which might not be consistent with the almost 100% deregulation of the β-catenin pathway, suggesting that overexpression of cyclin D1 in colon cancer may be more complicated than pure upregulation by β-catenin [25,26]. In this study, we showed that cyclin D1 was one of the targets for β-catenin in breast cancer. More importantly, we demonstrated the significant role of activated β-catenin in breast cancer both by molecular studies in cell culture and breast tumor samples. Consisted with these findings, our study provides strong evidence supporting the biological significance and clinical relevance of this pathway in human breast cancer. In contrast to colon carcinoma, the strong correlation between β-catenin activity and cyclin D1 expression was found in both breast cancer cell lines and breast patient tissue samples. Thus, the data presented in this study may open a new direction in the research of breast cancer, involving both cancer development

and progression and provide an opportunity for development of potential therapy by blocking β-catenin pathway in breast cancer cells.

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