β-catenin-independent noncanonical Wnt pathway might be induced in gastric cancers

Gastrik kanserlerde β-catenin'den bağımsız nankanonikal Wnt yolunun indüklenmesi mümkün olabilir

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Background/aims: *Abnormal Wnt signaling is often observed in human cancers. Wnt5a is a representative Wnt ligand that can activate both β-catenin-dependent canonical andβ-catenin-independent noncanonical Wnt pathways. However, the role of Wnt5a in carcinogenesis is controversial. This study was designed to understand whether Wnt5a in the Wnt pathway and its key downstream molecules such as MMP-7 and β-catenin are involved in gastric cancers.* ***Methods:*** *We analyzed the expressions of Wnt5a, MMP-7 and β-catenin genes in 40 primary gastric normal and tumor biopsies by RT-PCR and the subcellular localization of β-catenin by immunohistochemistry.* ***Results:*** *Our results showed a specific combination of genes expressed significantly in the gastric tumor tissues: 65% of the tumor samples containing non-nuclear β-catenin were Wnt5a-positive, 42.5% were MMP-7-positive, and 35% of the samples involved both. Interestingly, normal samples did not show any relevant coexpression of Wnt5a and MMP-7 in the β-catenin-containing samples.* ***Conclusions:*** *These results suggest that the noncanonical Wnt pathway might be critically important in gastric carcinogenesis.*

Key words: Gastric cancer, Wnt signaling, noncanonical pathway, β-catenin, RT-PCR

Amaç: *İnsan kanserlerinde Wnt5a sinyal bozuklukları sıkça gözlemlenmektedir. Wnt5a β-katenin'e bağımlı kanonikal ve β-katenin'den bağımsız nankanonikal Wnt yolunu aktive eden tipik bir Wnt ligantıdır. Ancak, Wnt5a'nın kanser oluşumundaki rolü çelişkilidir. Bu çalışma Wnt sinyal yolunda yer alan Wnt5a'nın ve onun aşağısındaki anahtar moleküllerden MMP-7'nin ve β-katenin'in gastrik kanselerdeki durumunu anlamak üzere tasarlanmıştır.* ***Yöntem:*** *Bunu anlamak için, 40 primer gastrik normal ve tümörlü biyopside Wnt5a, MMP-7 ve β-katenin genlerinin ekspresyonları RT-PZR ile, β-katenin'in hücre içi lokalizasyonu ise immunohistokimya ile analiz edildi.* ***Bulgular:*** *Sonuçlarımız gastrik tümör dokularında spesifik gen kombinasyonlarının önemli derecede ifade edildiğini gösteriyor. Nükleer olmayan β-katenin içeren %65 tümör dokusu aynı zamanda Wnt5a pozitiftir, bu dokulardan %42.5'i MMP-7 pozitiftir ve %35'i ise her iki gen ekspresyonunu da ihtiva etmektedir. β-katenin ekspresyonu saptanan normal dokularda ise Wnt5a ve MMP-7 genlerinin birlikte ekspresyonları gözlemlenmemiştir.* ***Sonuç:*** *Bu bulgular gastrik kanser oluşumunda nankanonikal Wnt sinyal yolunun son derece önemli olabileceğini ileri sürmektedir.*

Anahtar kelimeler: Gastrik kanser, Wnt sinyali, nankanonikal yol, β-katenin, RT-PZR

INTRODUCTION

The Wnt pathway was originally described in Drosophila as the Wingless pathway and is highly conserved among flies, frogs and mammals. This pathway is involved in cellular processes such as development, tissue homeostasis and cancer (1-5). In the "canonical" Wnt signaling pathway, Wnt binding to Frizzled receptors induces β-catenin protein stabilization and entry to nucleus to induce transcription of target genes including c-Myc, cyclin D1, matrix metalloproteinase (MMP)-7, gastrin, and ITF-2 (6-11). In addition to the canonical pathway, evidence for noncanonical Wnt signaling pathways independent of β-catenin has accumulated.

Wnt5a is involved in activation of both canonical and noncanonical Wnt signaling. However, it has specifically been classified as a noncanonical Wnt

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family member (12). This protein is a representative ligand involved in cell migration and invasion through activation of the β-catenin-independent pathway. The functions of Wnt5a in tumorigenesis have been documented. Some reports indicate that Wnt5a acts as a tumor suppressor because it has an ability to inhibit the β-catenin pathway. For instance, Wnt5a has been shown to induce down-regulation of β-catenin through Siah2 (11) or to inhibit the transcriptional activity of Tcf/Lef (13-15). Antisense Wnt5a mimics Wnt-1-mediated C57MG cell transformation (16). Wnt5a negatively regulates B-cell proliferation, and Wnt5a heterozygous mice develop B-cell lymphoma (17). Furthermore, Wnt5a inhibits proliferation, migration and invasiveness in thyroid tumor and colorectal cancer cell lines (18,19). In contrast to these observations, it has also been suggested that Wnt5a has oncogenic properties based on the findings that the Wnt5a mRNA level is up-regulated in lung cancers, prostate cancers and breast cancers (20). There is a correlation between Wnt5a expression and increased cell motility and invasiveness in melanoma, breast cancer and gastric carcinoma cells with tumor-associated macrophages (21-23). Thus, the roles of Wnt5a in human cancers are controversial and still unclear. Although abnormal activation of the β-catenin-dependent pathway is often observed in human cancers, the relationship between the β-catenin-independent pathway and tumorigenesis remains unclear (23, 24).

Gastric cancer is the fourth most common malignancy worldwide. However, the relationship between gastric carcinogenesis and β-catenin-independent Wnt signaling is not yet fully understood (25, 26). This study was designed to determine the involvement of Wnt5a and its key downstream effectors in gastric cancers. In order to establish this, we first explored the expression patterns of Wnt5a in primary gastric tumors. It is well known that nuclear β-catenin is the characteristic of an active canonical Wnt pathway. Moreover, studies directed to MMP analysis in tumor invasion and metastases have attracted the attention of scientists, since these proteins have the capability to degrade all the components of connective tissue, which is an acquired characteristic for a tumor cell. The transcription of MMP-7, one of the MMPs involved in the majority of connective tissue destruction during invasion and metastasis of a tumor, is activated by nuclear β-catenin (26). Therefore, we secondly investigated the expression of MMP-7 in the same patients by real-time polymerase chain reaction (RT-PCR). Finally, we determined the expression of β-catenin in gastric tumor biopsy samples by RT-PCR and detected its localization in these patients by immunohistochemistry using paraffin-embedded tissues in order to understand whether the canonical Wnt pathway is activated or whether the noncanonical Wnt pathway is involved in these patients.

MATERIALS AND METHODS

Tissue Preparation

Normal and gastric tumor tissue biopsy samples were taken from the gastrectomy specimens of 40 gastric carcinoma patients. The material was collected with the permission of the Ethical Committee of Istanbul University, Cerrahpasa Medical Faculty (#2405/296). These samples were immediately immersed in liquid nitrogen and then stored at -80°C. Paraffin- embedded blocks from tumor biopsies were prepared and the clinicopathological features were determined.

Reagents and Kits

Kits used in this study were as follows: Total RNA extraction kit (NucleoSpin RNA II, Macherey-Nagel), cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, Fermentas), PCR kit, dNTP and molecular weight marker (Fermentas), primers (Iontek), immunohistochemical detection of, β-catenin kit (DakoCytomation LSAB2 System-HRP Kit), and mouse monoclonal β-catenin antibody (1:1000 dilution, Abcam) and hematoxylin (Sigma).

RT-PCR

Levels of the mRNA transcripts encoding Wnt5a, MMP-7, β-catenin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (a housekeeping gene used to understand quality of the samples) genes were determined by RT-PCR. Samples were homogenized to break them up. RNA was extracted from biopsy samples according to the manufacturer's instructions. RNA concentration and purity of each extract were determined by A_{260}/A_{280} absorptions using UV-1202 Shimadzu spectrophotometer. RNA extracts were stored at -80°C for long-term use. cDNA was synthesized using oligo$(dT)_{18}$ primer according to the manual of RevertAid™ First Strand cDNA Synthesis Kit. Primers for amplification of Wnt5a, MMP-7, β-catenin, and GAPDH and the PCR conditions are shown in Table 1. To determine the optimum number of

cycles required for the amplification of these genes, an aliquot of first strand cDNA generated from normal and gastric tumor tissue biopsy samples was amplified with the respective primers using an increasing number of PCR cycles (20-35). Initial denaturation was performed at 94°C for 4 min. The subsequent cycling programs consisted of denaturation at 95°C for 30 sec, annealing temperature (Table 1) 30 sec, and extension at 72°C for 30 sec, followed by a final extension at 72°C for 5 min. The amplified products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed using Gel Imaging System (BioRad, USA). PCR reactions in which the first strand cDNA was omitted served as negative controls. To avoid technical error, each PCR experiment was repeated three times.

Immunohistochemistry

The avidin-biotin peroxidase complex was used for immunohistochemical staining. Sections were cut in 4μm thicknesses, incubated at 37°C overnight, deparaffinized in xylene, rehydrated with alcohol, and heated in a citrate buffer solution (0.01 mol/L sodium citrate, pH6.0) at 95°C for 15 min. Following this, DakoCytomation LSAB2 System-Horseradish Peroxidase (HRP) Kit protocol was followed. Sections were blocked with hydrogen peroxide solutions for 10 min (peroxidase blocking), and then incubated with the primary antibody against β-catenin at room temperature for 2 h. The sections were treated for 30 min at room temperature first with biotinylated goat anti-rabbit and goat anti-mouse IgG and then with streptavidin-HRP solution. Between incubations, sections were washed with 1X phosphate buffered saline (PBS) (pH7.4). 3-3'- diamino benzidine tetrahydrochloride (DAB) was used as HRP substrate. Sections were incubated with substrate-chromogen solution for 5 min and counterstaining was performed with Mayer's hematoxylin to visualize the nucleus.

Table 1. Primers for RT-PCR

Gene symbol	Primer sequence	Product size (bp)
Wnt5a	5'-GGGAGGTTGGCTTGAACATA-3'	141
	5'-GAATGGCACGCAATTACCTT-3'	
MMP-7	5'-TCCCGCGTCATAGAAATAATG-3'	451
	5'-AGGAATGTCCCATACCCAAAG-3'	
β-Catenin	5'-CAGAAGCTATTGAAGCTGAGG-3'	326
	5'-TTCCATCATGGGGTCCATAC-3'	
GAPDH	5'-GACCTGCCGTCTAGAAAAAC-3'	126
	5'-TTGAAGTCAGAGGAGACCAC-3'	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

RT-PCR Analysis of mRNA Expressions

RT-PCR expression analysis of Wnt5a, MMP-7 and β-catenin genes of normal and tumor tissue samples is demonstrated in Figure 1. Our results showed that Wnt5a, MMP-7 and β-catenin genes were expressed in 20 normal (50%) and 30 tumor (75%); 0 normal and 19 tumor (47.5%); and 19 normal (47.5%) and 25 tumor (62.5%) samples, respectively (Figure 2-a). β-catenin and Wnt5a coexpression was demonstrated in 14 (35%) normal samples while no other coexpression was observed in any normal samples. Unlike the normal samples, coexpressions of β-catenin-Wnt5a, β-catenin-MMP-7, β-catenin-Wnt5a-MMP-7, and Wnt5a-MMP-7 were observed in 19 (47.5%), 15 (37.5%), 10 (25%), and 15 (37.5%) tumor samples, respectively (Figure 2-b). The obtained data in this study illustrated a specific combination of genes expressed significantly in the gastric tumor tissues: 65% of the tumor samples containing non-nuclear, β-catenin were Wnt5a-positive, 42.5% were MMP-7-positive, and 35% of the samples involved both. Interestingly, the normal tissues from the patients did not show any relevant coexpression of Wnt5a and MMP-7 in the β-catenin containing samples.

Immunohistochemistry

Subcellular localization of β-catenin was examined in tumor samples by immunohistochemical staining as demonstrated in Figure 3. Our results illustrated that β-catenin was mainly located in the membrane and cytoplasm of the tumor samples. Among 30 Wnt5a-positive tumor samples only 4 (10%), among 19 MMP-7-positive samples only 2 (5%), and among 15 Wnt5a- and MMP-7-positive samples only 1 (2.5%) had nuclear β-catenin (Figure 2-c). As shown in Table 2-a only 6 (15%) of the patients that we examined had nuclear β-catenin localization, of which 4 (10%) were gastric adenocarcinomas. In contrast to adenocarcinomas, signet-ring adenocarcinomas had no nuclear β-catenin but only cytoplasmic and membranous β-catenin staining (Table 2-a). In addition, as shown in Table 2-b, cytoplasmic β-catenin was detected in only 7 patients (17.5%), while cytoplasmic and membranous was demonstrated in 27 (67.5%), cytoplasmic and nuclear in 4 (10%), and cytoplasmic, membranous and nuclear in 2 (5%) of them.

Clinicopathological Features of Patients

As shown in Table 3, the tumor tissues that were obtained from the patients were histologically of

two kinds: 55% were adenocarcinomas and 22.5% were signet-ring adenocarcinomas. Five percent of the tumors had T1, 47.5% had T2, and 35% showed T3 level of invasion depth. Furthermore, among 15 tumor samples in which coexpression of MMP-7 and Wnt5a was observed, 9 (22.5%) had T3 level of invasion depth, while 6 (15%) had T2 level; there was no T1 level of invasion among these samples (Table 4).

DISCUSSION

Gastric cancer is one of the leading causes of death worldwide. The molecular mechanism leading to gastric tumors remains to be identified. In many

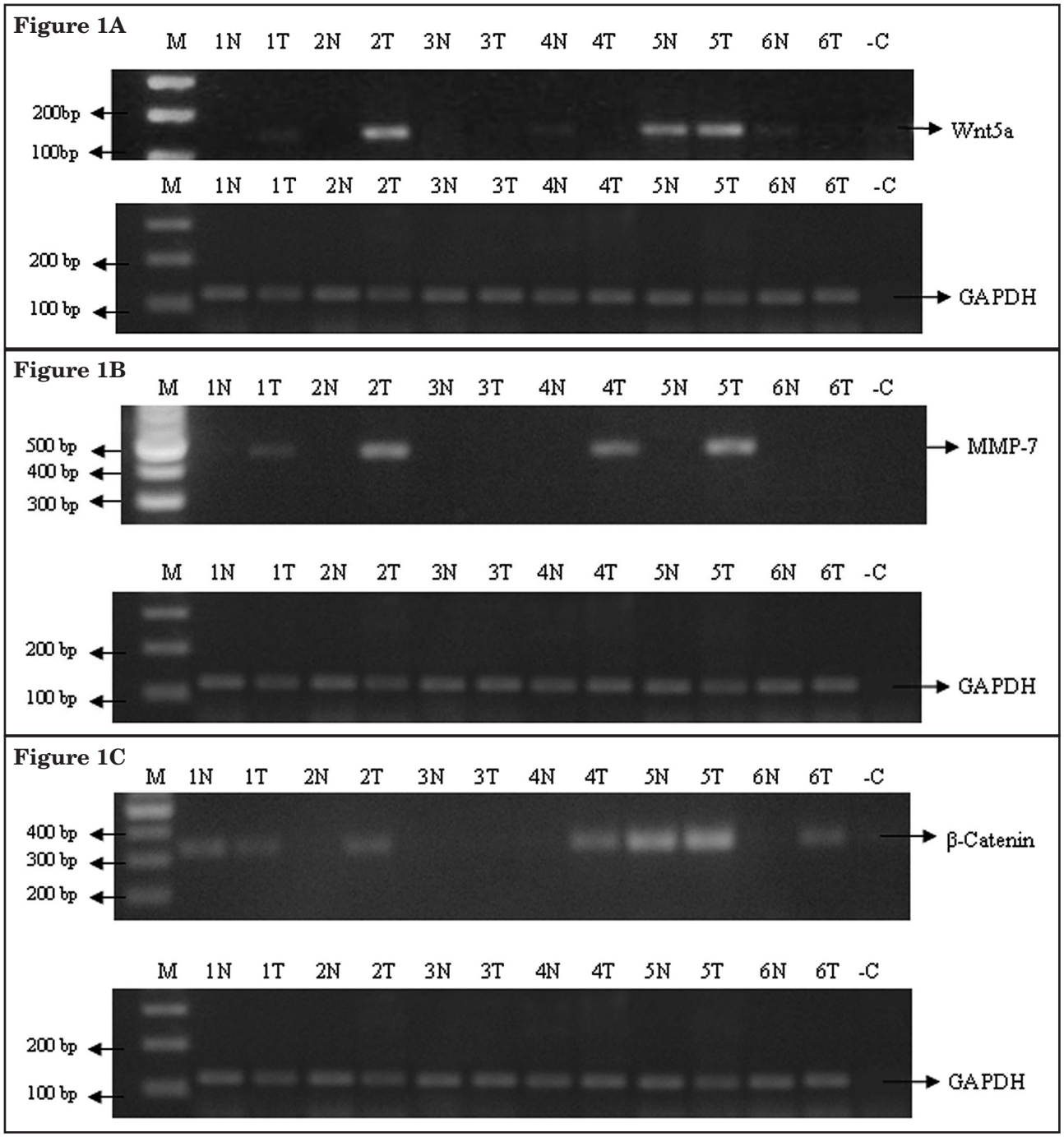

Figure 1. Representative RT-PCR analysis of mRNA expressions for Wnt5a, MMP-7 and β-catenin genes for six gastric cancer patients. N and T: matched samples from normal and tumor tissues, respectively, M: Molecular weight marker (100 bp), C: negative control. RT-PCR results of Wnt5a gene for patients **(A)**, RT-PCR results of MMP-7 gene for patients **(B)**, and RT-PCR results of β-catenin gene for patients **(C).**

cancer types, Wnt5a, β-catenin, and MMP-7 genes are highly upregulated as documented (27, 28). In our study, we determined the expression patterns of these genes in normal and tumor gastric biopsies. As expected, normal and tumor biopsy samples showed Wnt5a and β-catenin expression while only tumor samples had MMP-7 expression, as MMP-7 is documented to be expressed during tissue invasion of the tumor (Figure 1) (26). For all three genes that we analyzed, more tumor samples demonstrated the gene expressions compared to normal samples (Figure 2-a). In parallel, the number of normal and tumor samples differed in coexpression patterns of the genes, with almost no normal samples showing coexpression (except β-catenin and Wnt5a coexpression), revealing that the set of genes with oncogenic potential that are expressed by the tumor tissue increases, giving the multistep genetic disorder nature of tumorigenesis (Figure 2-b).

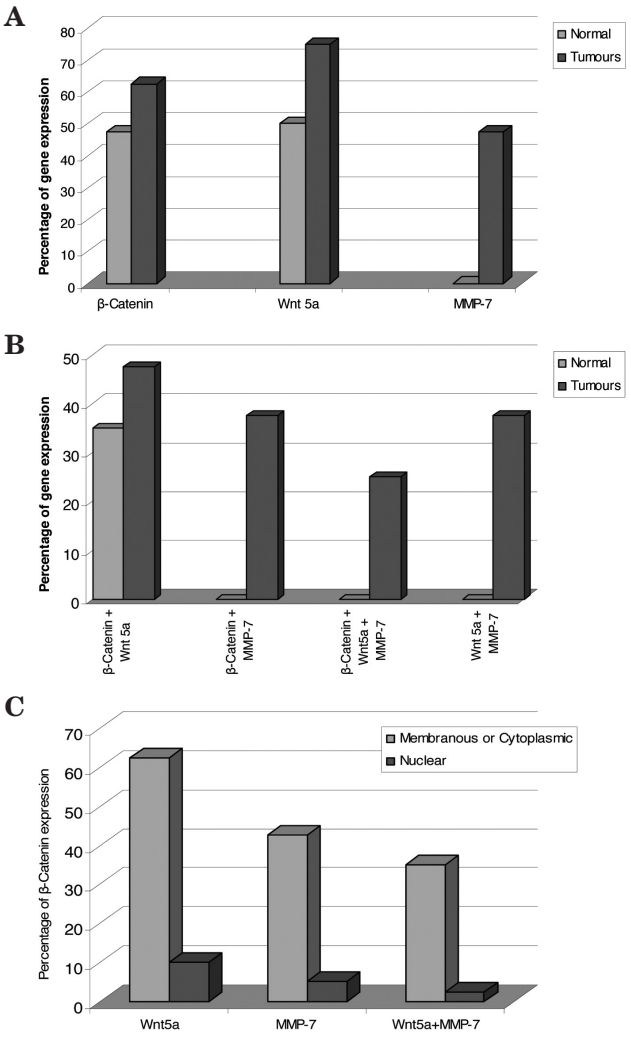

Figure 2. Comparison of β-catenin, Wnt5a, and MMP-7 expressions in normal and tumor tissues **(A)**, Comparison of β-catenin, Wnt5a, and MMP-7 coexpression in normal and tumor tissues **(B),** and Comparison of β-catenin localization with Wnt5a and MMP-7 coexpression in tumor tissues **(C).**

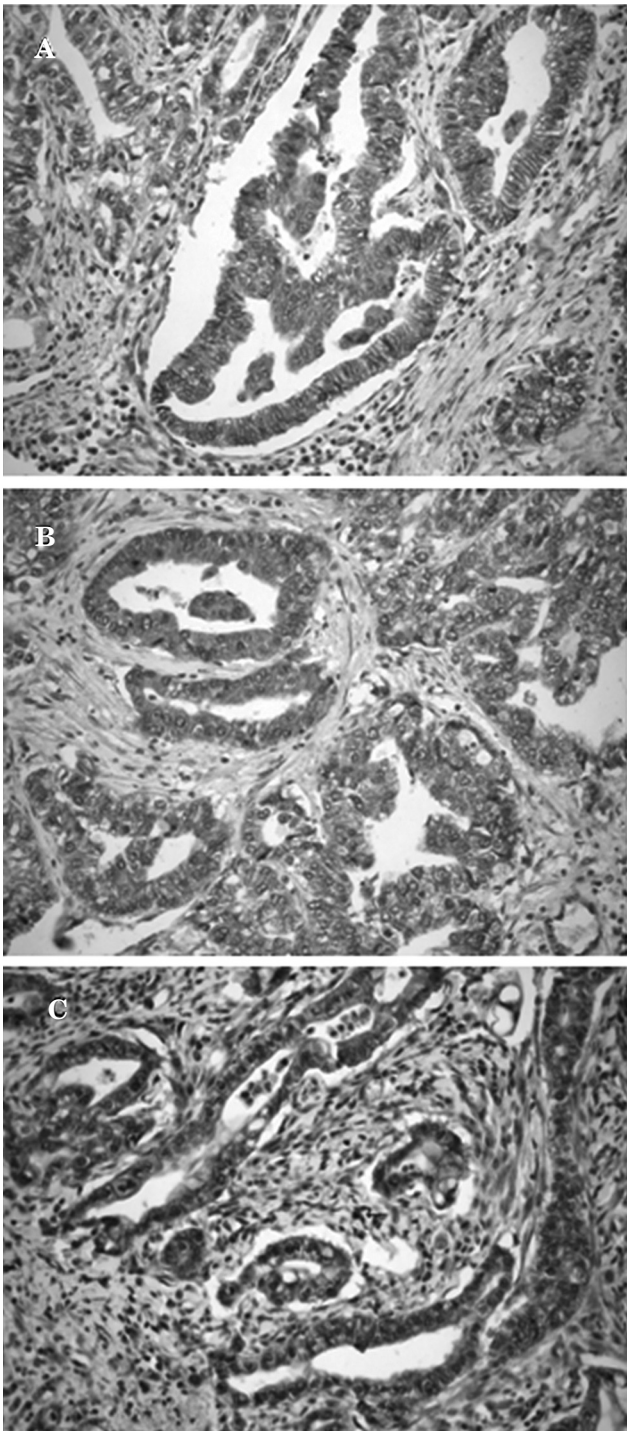

Figure 3. Immunohistochemical analysis of gastric cancer tissue samples with monoclonal antibody against β-catenin protein. Sides were detected at x200 magnifications. Membranous localization of β-catenin **(A)**, Cytoplasmic localization of β-catenin **(B)**, and Nuclear localization of β-catenin **(C).**

Table 2-a. Immunohistochemical expression of β-catenin in tumor samples

	Adenocarcinoma n (%)	Signet- ring n (%)	Others n (%)	Total n (%)
Cytoplasmic	18 (45)	8 (20)	17.5)	33 (82.5)
Membranous	18 (45)	6 (15)	4 (10)	28 (70)
Nuclear	4 (10)	—	2 (5)	6 (15)

Table 2-b. Subcelluler localization of β-catenin in tumor samples

	C n (%)	M n (%)	N n (%)	C+M n (%)	C+N n (%)	M+N n (%)	C+M+N n (%)
β- Catenin	7 (17.5)	---	---	27(67.5)	4 (10)	---	2 (5)

C: Cytoplasmic, M: Membranous, N: Nuclear

Table 3. Clinicopathological features of the 40 patients included in this study

Variables	Patient characteristics (n), (%)
Total number	40 (100)
Histology	
Adeno	22 (55)
Signet-ring	9 (22.5)
Others	9 (22.5)
Invasion deep	
T1	2 (5)
T2	19 (47.5)
T3	14 (35)
Unknown	5 (12.5)

Table 4. The relationship between invasion deep and MMP-7 and Wnt5a expression

Invasion deep	MMP-7 n (%)	Wnt5a n(%)	MMP-7 and Wnt5a n (%)
T1	2 (5)	---	---
T2	8 (20)	14 (35)	6 (15)
T3	9 (22.5)	16 (40)	9 (22.5)
Total	19 (47.5)	30 (75)	15 (37.5)

It is well documented that nuclear, β-catenin is the key molecule in the activation of the canonical Wnt pathway. In this study, we analyzed the localization of β-catenin with immunohistochemistry (Figure 3). Our results showed that 6 out of 40 (15%) patients had nuclear localization of, β-catenin, suggesting activation of the canonical Wnt pathway. Among these 6 patient biopsies with nuclear β-catenin, 2 of them showed no Wnt5a expression, suggesting only canonical Wnt pathway activation in 5% of the patients. On the other hand, 4 patients had Wnt5a expression as well as nuclear β-catenin, implying the activation of both canonical and noncanonical Wnt pathways in 10% of the patients (Table 2-a and Figure 2-c). Moreover, 26 out of 40 patients had Wnt5a expression but cytoplasmic or membranous β-catenin, revealing the activation of only the noncanonical pathway in 65% of these patients (Figure 2-c). Based on the data we obtained, the number of patients with only noncanonical Wnt pathway activation was much higher than of those with only canonical Wnt pathway activation. This suggests that in gastric carcinogenesis, instead of nuclear β-catenin, Wnt5a expression might be a key molecule, the signals of which are transduced to the noncanonical Wnt signaling cascades for the control of tissue polarity, cell adhesion and cell movement (28).

Moreover, according to the histopathological features, the tumor samples were distributed as 55% adenocarcinomas and 22.5% signet-ring adenocarcinomas. We found that signet- ring adenocarcinoma and adenocarcinoma types had both cytoplasmic and membranous β-catenin localization as shown in Table 2. While adenocarcinomas had nuclear β-catenin localization, signet-ring adenocarcinoma types had no nuclear β-catenin, which supports the literature (29). This study illustrated that the active canonical Wnt pathway is observed in adenocarcinoma types, while cytoplasmic and membranous β-catenin localization and Wnt5a expression suggesting noncanonical pathway signal transduction are observed in both adenocarcinoma and signet-ring adenocarcinoma types.

In parallel, MMP-7 is involved in cell movement by destruction of connective tissue during invasion and metastasis of the tumor. As seen in Figure 2-a, MMP-7 was expressed in 19 out of 40 tumor tissues (47.5%). Two of these MMP-7-positive tumor tissues had nuclear β-catenin while the rest had cytoplasmic or membranous β-catenin localization, suggesting the fact that in most of the cases, MMP-7 is transcriptionally regulated by means other than β-catenin in the nucleus in gastric cancers. The fact that MMP-7 in nonnuclear β-catenin- containing samples is coexpressed together with Wnt5a in 35% of the tumor but in none of the normal cases may imply that the noncanonical Wnt pathway in the presence of MMP-7 might be critically important in some cases of gastric carcinomas.

According to clinicopathological features of the patients, 47.5% of the gastric tumors that we analyzed had T2 level of invasion, while 35% had T3 level of invasion (Table 3). As is seen in Table 4, 9 pa-

tients (22.5%) that coexpressed Wnt5a and MMP-7 had a T3 tumor invasion level, which demonstrates that coexpression of these two genes might disrupt cell adhesion and cell movement abilities of tumor cells, as these two genes are involved in invasive characteristics of tumor tissues. Additionally, among the tumor samples that coexpressed Wnt5a and MMP-7, only one sample had nuclear β-catenin localization with a T3 tumor invasion level, suggesting severity of the tumor as a result of coexpression of all the genes that we considered.

In conclusion, most of our patients had Wnt5a expression with nonnuclear β-catenin. Our findings suggest mostly a potential involvement of the noncanonical Wnt pathway and rarely of the canonical Wnt pathway in gastric carcinogenesis. This work might help in understanding the importance of studying the noncanonical Wnt pathway in gastric cancers, which is yet not clear.

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