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Canonical and noncanonical Wnt pathway: A comparison among normal ovary, benign ovarian tumor and ovarian cancer

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Abstract. The Wnt family is involved in tumorigenesis of several tissues. In ovarian cancer, the role played by Wnts and its pathways is not clearly defined. In order to analyze the canonical and noncanonical Wnt pathway in normal ovary, benign ovarian tumor and ovarian cancer, we evaluated the immunohistochemical expression of Wnt1, Frizzled-1 (FZD1), Wnt5a, Frizzled-5 (FZD5) and ß-catenin. Ovarian specimens were obtained from surgeries performed between 1993 and 2004. The patients were divided in three groups: group A, epithelial ovarian cancer (n=38); group B, benign epithelial neoplasia (n=28); and group C, normal ovaries (n=26). Immunoreactivity for Wnt1, FZD1, Wnt5a, FZD5 and ß-catenin was scored for each group. The proportion of Wnt1 positive women in group A (29.4%) was significantly higher than in group B (4.3%) and C (9.1%) (p=0.020). The proportion of FZD1 positive patients in group C (54.5%) was significantly lower than in group A (97.1%) and B (90.0%) (p<0.001). The proportion of Wnt5a positive women was significantly higher for group A (80.0%) compared to group B (25.0%) and C (27.3%) (p<0.001). The proportion of ß-catenin positive patients in group C (95.8%) was significantly higher than group B (52.4%) (p=0.004). Comparison of the survival curves in group A according to Wnt5a expression showed a significant difference between positive and negative patients, whereas the Wnt5a positive women showed worse results (p=0.050). Our findings suggest that the pathways related to Wnt5a have an important role in ovarian malignant neoplasia. Furthermore, Wnt5a was found to be a predictor of poor prognosis for ovarian cancer.

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Introduction

In recent years, several studies have shown the importance of Wnt family in tumorigenesis (1-5). Wnts are an evolutionarily highly conserved family of genes/proteins that act through four signaling pathways (6).

The canonical pathway: In the absence of Wnt signaling, a multiprotein complex that includes adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and Axin ensure the degradation of ß-catenin, thereby limiting the free intracytoplasmic pool of ß-catenin. The presence of Wnt signal through the Frizzled (FZD) receptor and low density lipoprotein receptor-related Protein 5 and 6 (LRP5/6) receptor complex inactivates GSK3 and causes its dissociation from Axin preventing the phosphorylation of ß-catenin. The intracytoplasmic pool of ß-catenin thus increases and it translocates to the nucleus where it complexes with members of the LEF/TCF family of transcription factors to mediate transcriptional induction of target genes such as c-myc, cyclin D, VEGF and others.

The noncanonical pathway: In noncanonical or planar cell polarity (PCP) signaling, Wnt signaling is transduced through Frizzled independent of LPR5/6. This pathway mediates cytoskeletal changes through activation of the small GTPases Rho and Rac (7).

Certain Wnts can activate both the canonical and the noncanonical pathway, such as Wnt3a; others appears to be specific to the noncanonical pathway, such as Wnt5a (6,7).

The $WntCa^{2+}$ pathway: Wnt signaling via Frizzled mediates activation of heterotrimeric G-proteins, which engage Dsh, phospholipase C calcium-calmodulin kinase 2 (CamK2) and protein kinase C (PKC). This pathway modulates cell adhesion and motility.

The protein kinase A pathway: Chen *et al* demonstrated that adenyl cyclase signaling via protein kinase A (PKA) and its target transcription factor cAMP responsive element-binding protein (CREB) are required for Wnt-directed myogenic gene expression. They have shown that Wnt proteins can also stimulate CREB-mediated transcription (6,8).

In 1990, six members of the Wnt family were identified, including Wnt5a. It encodes a protein involved in cell-cell signaling during embryonic development (9-13). In 1997,

Olson *et al* suggested that Wnt5a inactivation would lead to tumorigenesis (14). Thereby, some authors affirm that the noncanonical pathway would assume an antagonistic effect on the canonical pathway through different mechanisms and the Wnt5a would be a tumor suppressor (15-18). It can antagonize the canonical pathway at TCF/LEF level, in the nucleus (17). Furthermore, Wnt5a was able to increase the expression of the APC and Siah2 proteins which would lead to ß-catenin degradation independent of CAMK2 (18).

However, many authors found Wnt5a amplified in several malignancies such as stomach, esophagus, prostate, melanoma, colon/rectum and breast. Ricken *et al* considered the possibility that the Wnt5a could be involved in ovarian carcinogenesis (19).

Patients and methods

Ovarian specimens were obtained from surgeries performed between 1993 and 2004 at Federal University of Sao Paulo for patients who underwent exploratory laparotomy for adnexal tumor or prophylactic oophorectomy during surgery for benign disease. None of the patients had received any preoperative therapy. Borderline tumors were excluded from this study.

The patients were divided in three groups: group A, malignant epithelial neoplasia (n=38); group B, benign epithelial neoplasia (n=28); and group C, normal ovaries (n=26). The study was approved by the Institutional Ethics Committee.

Immunohistochemistry. Sections were deparaffinized in three changes of xylene and rehydrated in a graded series of ethanol finishing in destilated water. For antigen retrieval slides were placed in 0.01 M citrate-buffer pH 6.0 and heated in a steamer for 30 min. Endogenous peroxidases were quenched by incubating in 3% H_2O_2, 20 min at room temperature. Sections were incubated overnight at 4˚C with antibody against Wnt1 (1:100), R&D Systems, Minneapolis, MN, USA; FZD1 (1:100) R&D Systems; Wnt5a (1:100) R&D Systems; FZD5 (1:100) R&D Systems; and ß-catenin (1:100) Santa Cruz Biotechnology, Santa Cruz, CA, USA. Subsequently, sections were incubated with biotinylated secondary antibody (LSAB, Dakocytomation) for 30 min, washed in PBS, and incubated with streptavidin-peroxidase conjugate (LSAB, Dakocytomation) for 30 min. Finally, the reaction was revealed using 3,3'-Diaminobenzidine tetrahydrocloride (Sigma) for 5 min. Slides were briefly counterstained in hematoxylin and dehydrated, and cover slips added. Negative and positive controls were made to run simultaneously. Positive controls were represented by mammary tissue. Negative controls were made by eliminating the primary antibody.

The presence of tumor tissue was confirmed previously in each core. Immunostaining was scored by two trained independent observers without prior knowledge of the clinicopathological parameters. Discordant cases were reviewed and agreed upon before data were statistically analyzed.

Immunoreactivity of Wnt1, FZD1, Wnt5a, FZD5 and ß-catenin was assigned a score based on the proportion of positive tumor cells over total tumor cells (percent positivity) ranging from 0 to 100%. The percentage of positive tumor cells was graded as follows: 0, none; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. Staining intensity was evaluated as 0, negative; 1, weak; 2, moderate; and 3, strong. The score was calculated multiplying the positivity percent rating by staining intensity (20). From this score, values ranging from 0 to 2 (none or weak reaction) were considered negative and values ranging from 3 to 9 (moderate or strong reaction) were considered positive.

Owing to too few cells in certain samples, 61 out of 460 (group A, 190; group B, 140; group C, 130; total, 460) samples were uninterpretable. For these specimens, a score of NA was given.

Statistical analyses. The software used was the Statistical Package for the Social Sciences (SPSS, Chicago, IL). The groups were compared to quantitative variables by ANOVA when normal distribution of variables or, otherwise, by Kruskal-Wallis test.

The associations between expression of biomarkers and clinicopathological parameters were tested with contingency tables and Pearson's Chi-square test. Survival of patients was estimated by Kaplan-Meier analysis and the covariates were analyzed by the log-rank test for univariate analysis. $p<0.05$ was considered statistically significant.

Table I. Malignant group.

Variables	(n=38)
Cytoreduction	n (%)
Optimal	22 (57.9%)
Suboptimal	16 (42.1%)
Stage	
I	10 (26.3%)
II	2 (5.3%)
III	16 (42.1%)
IV	10 (26.3%)
Hystology	
Serous	22 (57.9%)
Mucinous	7 (18.4%)
Endometrioid	4 (10.5%)
Clear cell	1 (2.6%)
Undifferentiated	4 (10.5%)

Results

The groups were homogeneous in terms of distribution of the following variables: age (p=0.145), body mass index (p=0.454), number of pregnancies (p=0.061), age at menarche (p=0.236), menopausal status (p=0.070), age at menopause (p=0.603), and cigarette smoking (p=0.443). Data regarding group A are shown in Table I.

For the expression of Wnt1, there was a significant association among the groups. The proportion of positive women in group A (29.4%) was significantly higher than group B (4.3%) and C (9.1%) (p=0.020). There was a significant association among the groups for the FZD1 expression ($p<0.001$). The proportion of positive scored patients in

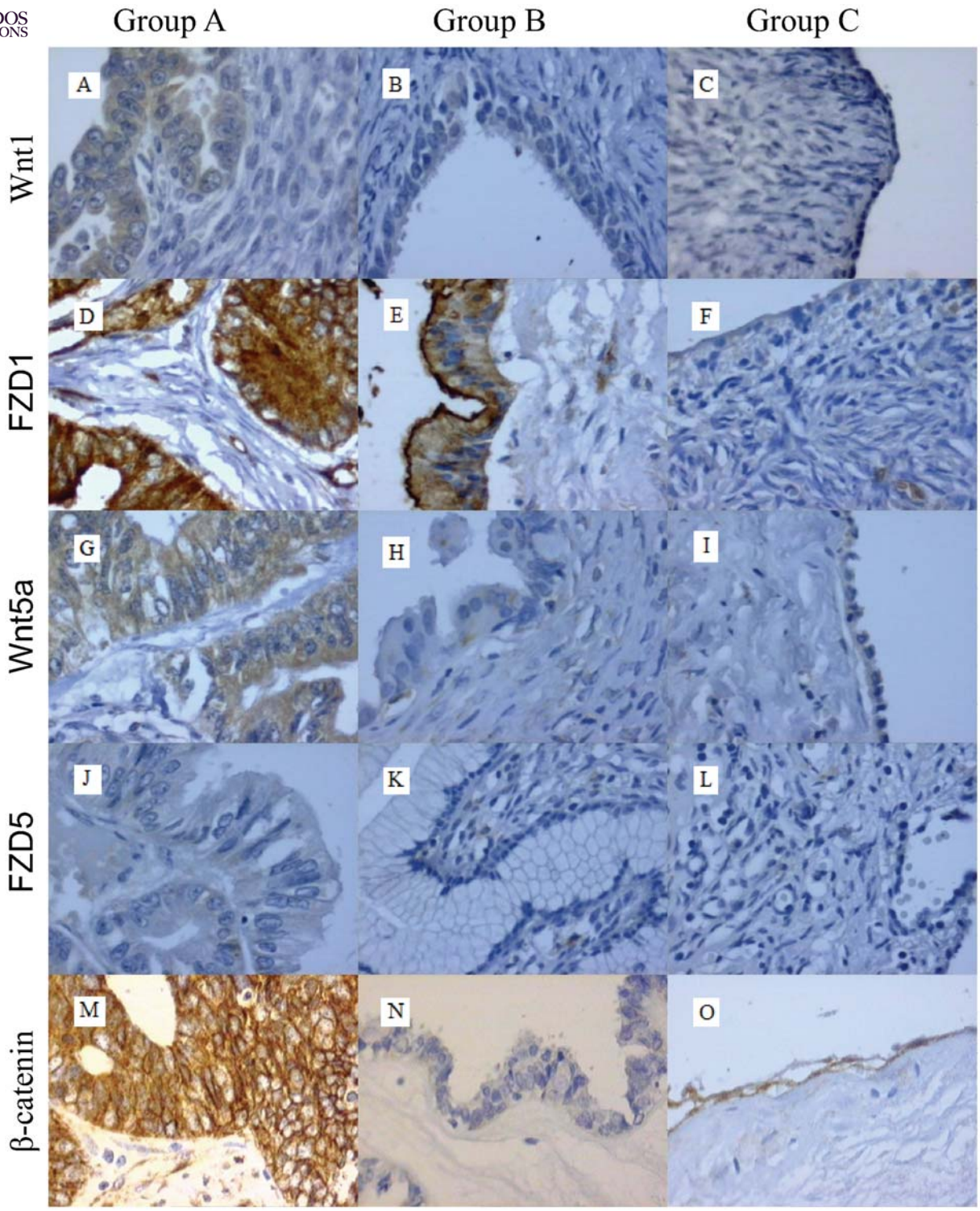

Figure 1. Immunostaining for Wnt1: strong staining for malignant tumor (A) and weak staining for benign tumor (B) and normal ovary (C). Immunostaining for FZD1: strong staining for malignant (D) and benign tumor (E) and weak staining for normal ovary (F). Immunostaining for Wnt5a: strong staining for malignant tumor (G) and weak staining for benign tumor (H) and normal ovary (I). Immunostaining for FZD5: weak staining for all groups (J-L). Immunostaining for ß-catenin: strong staining for malignant tumor (M) and normal ovary (O), and weak staining for benign tumor (N) (x400).

group C (54.5%) was significantly lower than the other groups (97.1% for group A and 90.0% for group B).

A significant association was observed among the groups for the Wnt5a expression ($p<0.001$). The proportion of Wnt5a positive women was significantly higher for group A (80.0%) compared to group B (25.0%) and C (27.3%). No significant association was observed between FZD5 expression and the patient group since the expression for groups A, B and C were 14.3, 4.0 and 8.7%, respectively ($p=0.380$).

The proportion of patients in group C with a positive score (95.8%) for ß-catenin staining was significantly higher than group B (52.4%) ($p=0.004$). Group A (74.3% of positive cases) did not significantly differ from the other groups (Figs. 1 and 2).

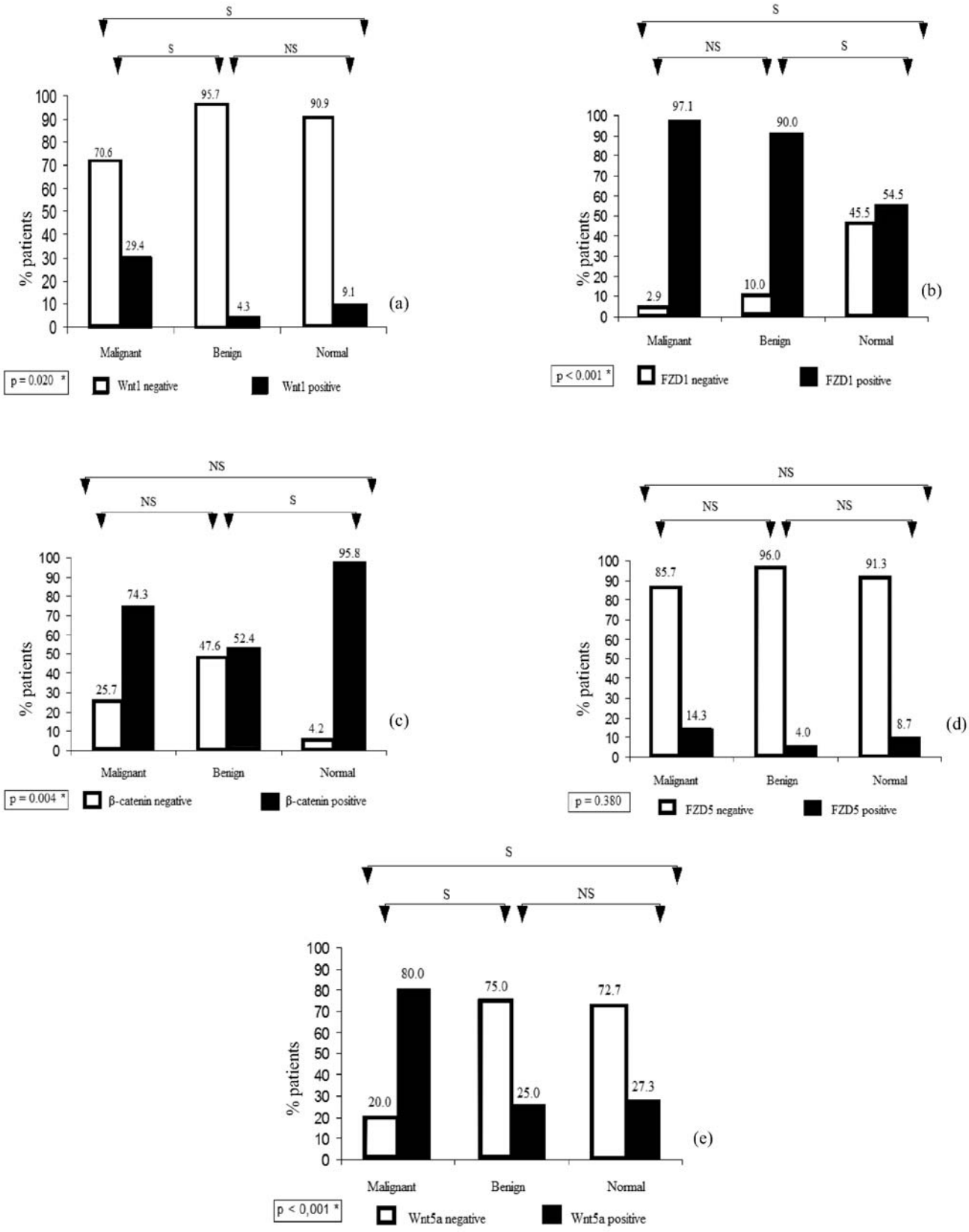

Figure 2. Expression of Wnt1 (a), FZD1 (b), ß-catenin (c), FZD5 (d) and Wnt5a (e). A significant proportion of patients from malignant group stained for Wnt5a.

There was not significant association of the survival curves according to the ß-catenin expression (p=0.062) in group A. Also in group A, no significant association of the survival curves was observed according to Wnt1 (p=0.497) and FZD5 (p=0.550) expression. The survival curves according to the FZD1 expression could not be calculated because only one patient in group A was negative for this protein.

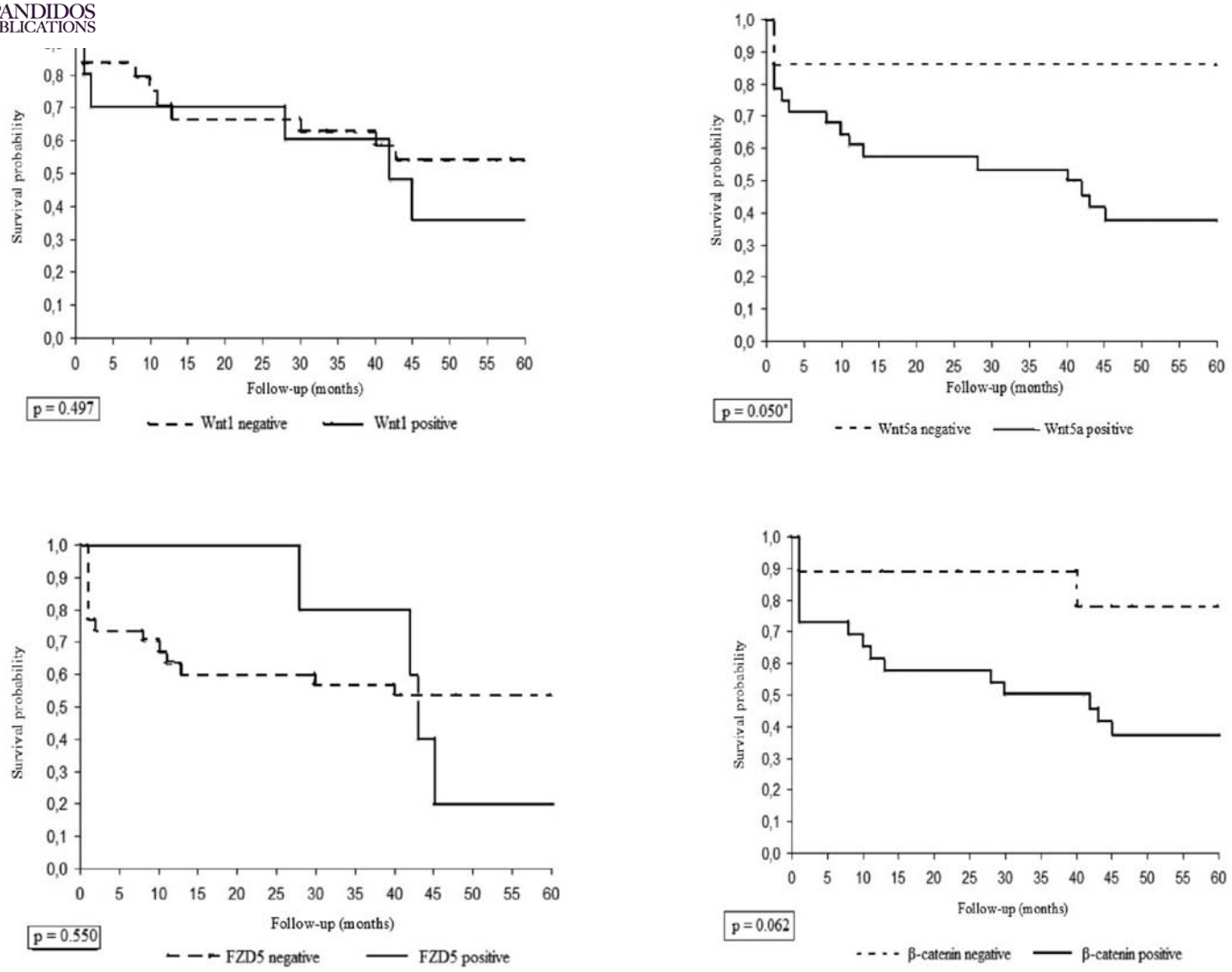

Figure 3. Kaplan-Meier overall survival curves for Wnt1, Wnt5a, FZD5 and ß-catenin in group A. The curve for FZD1 could not be calculated because only one patient in group A was negative for this protein.

Comparison of the survival curves in group A according to Wnt5a expression showed a significant difference between Wnt5a positive and Wnt5a negative patients, whereas the Wnt5a positive women showed worse results (p=0.050) (Fig. 3).

Discussion

Wnt1. Wnt1 is overexpressed in several types of human cancer. In our series, Wnt1 was overexpressed in ovarian carcinoma, which would be expected to lead to overexpression of ß-catenin, but it did not. It could be speculated that ß-catenin was inhibited by noncanonical pathways through diverse mechanisms, such as activation of PKC and/or Siah2 (18). It is an intriguing issue to be solved, since the expression of FZD1 was higher in malignant group as well.

Frizzled1. Frizzled proteins are found exclusively at the plasma membrane. FZD1 was found to be up-regulated (21) in breast cancer as well as in poorly differentiated colon cancer (22). However, the complexity of Wnt pathway extends to its receptors. When the co-receptor LRP5/6 interaction to FZD1 is considered, the transmission of the canonical Wnt signaling occurs as expected; otherwise, considering the co-receptor LRP1 interaction to FZD1, canonical signaling (23) is repressed. An interaction between Wnt5a and FZD1 might exist, however, it is known that FZD1 is activated by Wnt3a, Wnt3, Wnt1 and to a lesser extent Wnt2, but not by Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a or Wnt7b (24).

Wnt5a. We found a strong staining for group A compared to groups B and C and, furthermore, we demonstrated a prognostic role for Wnt5a. Staining for Wnt5a in malignant group correlated to worse prognosis. It is worth noting that only one Wnt5a negative patient died in group A. Several aspects could explain the positive Wnt5a expression and ovarian cancer.

Considering the three ß-catenin independent pathways. PCP pathway: It was found that alendronate inhibits lysophosphatidic acid-induced migration of human ovarian cancer cells by attenuating the activation of Rho (binding domain of Rhoketin), we suppose that Wnt5a signaling activates Rho, leading to the progress of ovarian carcinoma. Moreover, the activation of Rac leads to activation of JNK;

phosphorylation of c-jun by JNK acts synergistically with TCF/LEF on the promoter of the canonical target gene c-jun. Alternatively, Wnt5a can bind to the receptor tyrosine kinase Ror2 which results in Rho GTPase-independent activation of JNK (25).

WntCa^{2+}: Wnt5a signals through noncanonical pathways which involves stimulation of intracellular Ca^{2+} release and activation of PKC and CAMK2 (18). PKC takes an important role in this pathway since several studies have demonstrated that it is overexpressed in ovarian carcinomas and it is related to worst prognosis (26,27). Furthermore, down-regulation of PKC enhances the sensitivity of human ovarian carcinoma to various types of platinum compounds and PKC inhibitors can decrease the invasiveness of ovarian cancer. D'Souza and colleagues showed that PKC phosphorylates claudin-4 (a transmembrane proteins essential to the formation and maintenance of tight junctions) which would lead to disruption of barrier function in ovarian cancer cells (28). In melanoma cells, there is a direct correlation with Wnt5a expression, PKC activation and increased melanoma cell invasion. Disruption of the Wnt5a/Frizzled-5 pathways results in an inhibition of PKC activation and reduced invasiveness of melanoma cells, apparently due to motility function related to PKC (29).

Interestingly, it have been shown that Wnt5a can activate PKC as well PKC can activate Wnt5a (30). Thus, independently of PKC, Wnt5a/Ca^{2+} activates nuclear factor associated with T cells (NFAT), which is involved in tumorigenesis (25).

Protein kinase A pathway: Also D'Souza and colleagues suggested that PKA phosphorylates claudin3 leading to rupture of tight junctions and causing disruption of barrier function in ovarian cancer cells (31).

The overexpression of Wnt5a in some tumors is not a result of gene amplification or rearrangement, suggesting that the level of Wnt5a is being modulated by some further regulatory apparatus (29). Since Wnt5a was overexpressed in malignant neoplasms in our findings, it could be speculated that ß-catenin was inhibited by Wnt5a (17,18). Mikels and Nusse demonstrated that Wnt5a activates or inhibits ß-catenin pathway depending on the receptor context. They showed that Wnt5a can activate Wnt/ß-catenin signaling in the presence of FZD4 and LRP5 and can inhibit it in the presence of the transmembrane receptor Ror2 (32-34). Also, Liu *et al* demonstrated that the mechanism of endogenous receptor functionally distinguishes prototype canonical and non-canonical Wnts, since they linked noncanonical Wnt5a with the C-terminal half of Dickkopf-2 (Dkk2C) creating a Wnt5a/Dkk2C chimera that was capable of activating canonical signaling (35). Moreover, the function of Wnt5a as either a suppressor or promoter of malignant progression is beyond intracellular signaling and seems to be modulated by intercellular interactions (36).

Contrary to us, Dejmek *et al* found that the expression of Wnt5a in primary Dukes' B colon cancer constitutes a good prognostic marker for a longer survival (37). On the other hand, Kurayoshi *et al* found that the expression of Wnt5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion (38).

Frizzled5. Although FZD5 is considered by some authors a key molecule functioning as Wnt5a receptor, no significant association was observed by us between FZD5 expression and the patient group. This fact reflects the diversity of receptors and coreceptors that interacts with Wnt5a, such as FZD4, FZD2, Ror2 and others (39).

ß-catenin. ß-catenin is considered a central molecule of the Wnt signaling pathway and has a dual function in cell adhesion (mainly through controlling E-cadherin-mediated cell adhesion at the plasma membrane) and transcription. Both of these function are involved in human tumorigenesis. The role of ß-catenin in ovarian tumorigenesis is not clear yet. Rask *et al* found significant increase of ß-catenin in ovarian cancer compared to normal ovary (40). To explain the fact that ß-catenin is not mutated (and consequently, it is not stabilized) in most ovarian cancers, it was suggested that other factors that could influence the expression of ß-catenin, such as overexpression of frequently rearranged in advanced T-cell lymphomas-1 (FRAT1) (41), overexpression of GSK3 and reduction of APC (40).

It is known that ß-catenin has an important function in colorectal tumorigenesis (42). However, it does not seem to be the same for ovarian cancer. Most of the authors have shown that ß-catenin mutation is found practically only in endometrioid ovarian type carcinomas and are rare in serous, clear cell and mucinous carcinomas. It is noteworthy that ß-catenin is used to differentiate primary ovarian mucinous carcinoma from colorectal adenocarcinoma metastatic to the ovary, considering that ß-catenin is overexpressed in colorectal adenocarcinoma. Furthermore, only the nuclear sublocalization of ß-catenin is regarded as an indicator of dysregulation (43,44). In our specimens, we had only four endometrioid ovarian carcinomas from 38 patients (10%), and none from the three groups studied stained a nuclear sublocalization of ß-catenin.

Davies *et al* found that ß-catenin is consistently expressed in normal ovarian surface epithelium and benign tumors, and reduced or absent in ovarian carcinomas (45), which is in agreement to our findings whereas the malignant group did not differ from the others in terms of ß-catenin expression. Even more, the proportion of patients in group C (normal) with positive score for ß-catenin was significantly higher than group B (benign). Unfortunately, most studies of the surface epithelium are limited by its fragility; it is usually denuded by allowing the surface to dry intraoperatively or by touching or rubbing it during removal or gross pathologic examination (46).

We found a tendency to worst prognosis for ß-catenin positive patients in group A, however it did not reach the significance level. Kildal *et al* found that cytoplasmic or membranous ß-catenin staining were not of prognostic importance. Interestingly, they found that nuclear ß-catenin staining was associated with improved survival, and occurred preferentially in endometrioid carcinoma. Similar results were described by Gamallo *et al*, they found that nuclear ß-catenin indicates good prognosis in contrast to patients who expressed only membranous ß-catenin (47), mostly in endometrioid ovarian carcinomas. For breast cancer patients, Dolled-Filhart *et al* also found worse outcome with decreased expression of ß-catenin (48).

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are a promising research field for new therapeutic es in cancer. It is known that certain drugs act through Wnt pathways. Although, most studies on the relationship of ovarian cancer and Wnt pathway is focused on the canonical/ß-catenin pathway. Our findings suggest that the pathways related to Wnt5a also have a very important role in ovarian tumorigenesis and warrant further investigation. Wnt5a was found to be a predictor of poor prognosis for ovarian cancer.

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