

Characterization of Notch Signalling Pathway Members in Normal Prostate, Prostatic Intraepithelial Neoplasia (PIN) and Prostatic Adenocarcinoma

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Abstract Prostate Cancer (PCa) holds the second place in terms of cancer-related mortality rate among men. The Notch signalling pathway regulates the proliferation and differentiation in embryonic and adult tissues and determines the cell fate. The body of knowledge in the present literature is currently controversial about the effect of the Notch pathway on prostatic cancer. Therefore, the present study aimed to examine the immunolocalization and expression levels of Notch1-4, Jagged1-2, Delta, HES1 and HES5 from among the members of the Notch signalling pathway in tissues of normal, prostatic intraepithelial neoplasia (PIN) and malignant prostate. The current study included a sample of 20 patients with localised prostatic adenocarcinoma, 18 patients with high grade PIN (H-PIN) and 18 normal prostatic tissue. Immunolocalisations of Notch1, 2, 3, 4, Jagged1, 2, Delta, HES1 and HES5 were identified through the immunohistochemical method. The findings of the present study showed that all in-scope members of the Notch signalling pathway were localised in PIN structures to a greater extent than in other tissues and from amongst these members, specifically Notch1, Notch4, Jagged1 and HES1 were at more significant levels. Consequently, the findings of the present study may

indicate that the Notch signalling pathway can play a role especially in the formation of PIN structures.

Keywords Immunohistochemistry · Notch signalling pathway · Prostate cancer · Prostatic intraepithelial neoplasia

Abbreviations

ADAM17	A disintegrin and metalloproteinase domain-containing protein 10 or 17
CSL	CBF1, Su(H) and LAG-1
DAB	Diaminobenzidine
DII1	Delta like 1
HES	Hairy/Enhancer-of-Split family
HEY	Hairy/Enhancer-of-Split related with YRPW motif-like protein
N ^{EC}	Notch extracellular domain
N ^{IC}	Notch intracellular domain
N TM	Notch transmembrane domain
PCa	Prostate cancer
PBS	phosphate buffered saline
PIN	Prostatic intraepithelial neoplasia
sc	Santa Cruz Biotechnology
NB	Novus Biologicals
BA	Vector Laboratories

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Introduction

Prostate cancer (PCa) is a mostly terminal malignant non-cutaneous disease with a high incidence among male individuals [1–4]. The prostate-specific antigen screening technique has facilitated the diagnosis of this disease to a great extent [5]. The incidence of PCa varies on the basis of race and it is often

diagnosed after the age of 65 [6]. PCa has been associated with various risk factors including age, race, family history and diet [7]. Numerous studies have been conducted with the aim of understanding the biological and molecular mechanisms involved in the development, progression and metastasis of PCa [8]. However, the molecular mechanisms underlying the development and progression of PCa have not yet been completely brought to light [9].

Notch is a member of the signalling pathway that plays an important role in homeostasis and development of tissue [10, 11]. So far, four Notch receptors (Notch 1–4) [12] and five Notch ligands (Jagged 1–2; Delta 1, 3, 4) have been identified in mammals [13–17] and the members of transcription factors [18] (for example Hairy/Enhancer-of-Split family (HES) and Hairy/Enhancer-of-Split related with YRPW motif-like protein (HEY)). The findings of the current literature on the association of the Notch signalling pathway with PCa are contradictory. The findings of previous studies on human and mouse prostate tissues point out to an uncertainty in conclusions as to whether the Notch signalling pathway plays an oncogenic or tumour-suppressive role. Various studies in the literature indicated that the Notch signalling pathway is involved in the embryonic and postnatal prostate development [19, 20]. Previous studies also identified that the Notch signalling was overexpressed in various PCa cell lines, including highly metastatic PCa cell lines, as compared with prostate cell lines derived from normal prostate tissue [21, 22]. In addition, it was reported that Notch1 mRNA expression decreased significantly in clinically localized PCa when compared to benign prostate controls, whereas no significant variation was observed in the mRNA expressions of Notch2, Notch3 and Notch4. Consequently, an emphasis was placed on the possibility of Notch1 acting as a potential tumor suppressor in PCa [20]. Another study in the literature reported that Notch1 protein was overexpressed in malignant prostatic tissue compared to benign controls [23]. In a fashion similar to the Notch receptor, the expression of Notch ligands at the protein level of Jagged1 was reported to increase in clinically localized prostatic tumors when compared to benign prostate controls [24]. An additional study indicated the potential role of Jagged2 and DII1 in prostate tumorigenesis [20]. As a result and in the light of previous studies, it is clear that there is a controversy as to whether the Notch signalling pathway suppresses or induces the development of tumors and this matter needs clarification. Therefore, the present study aimed to identify the immunohistochemical distribution of Notch receptors (Notch1–4), Notch ligands (Jagged1–2 and Delta) and Notch signalling transcription factors (HES1, HES5) in normal prostatic tissue, in the PIN structure, which represents the onset of PCa, and in tissues with PCa and to contribute to the clarification of the association between the Notch signalling pathway and the development of PCa.

Materials and Methods

Case Selection

The current study included a sample of 20 patients with localised prostatic adenocarcinoma, 18 patients with high grade PIN (H-PIN) and 18 normal prostatic tissue. The patients with low grade PIN and metastatic adenocarcinoma were excluded from the evaluation. The patients with Gleason score of 3 or lower were considered as “low grade” and the patients with Gleason score of 4 or higher were considered as “high grade”. All tissue samples were obtained from patients who underwent radical prostatectomy.

Four micrometer thick haematoxylin and eosin stained tissue sections from the surgical specimens fixed in 10 % formalin and embedded in paraffin were reviewed and representative tissue blocks were selected. Immunohistochemical analysis for Notch signaling pathway (Notch1–4, Delta, Jagged 1 and 2, HES 1, HES 5) was performed on 56 prostate tissue samples including localised PCa, H-PIN and normal prostatic tissue.

Immunohistochemistry

Formalin fixed paraffin embedded samples were cut into 5 μ m sections and placed on slides covered with poly-L-lysine. After deparaffinization, slides were boiled in citrate buffer (pH 6.0) for 10 min (respectively 5 and 5 min) for antigen retrieval and cooled for 20 min at room temperature. Then, sections were immersed in 3 % hydrogen peroxide for 20 min to block endogenous peroxidase. Slides were then incubated in a humidified chamber with UltraV block (Lab-Vision, Fremont, CA, USA) for 7 min at room temperature. Excess serum was drained and sections were incubated with Notch1 (Santa Cruz Biotechnology(sc); sc-6014; 1:50 dilution), Notch2 (sc-5545; 1:100 dilution), Notch3 (Novus Biologicals (NB); NB100-93, 550; 1:100 dilution), Notch4 (NB100-93,551; 1:200 dilution), Delta (sc-9102; 1:200 dilution), Jagged1 (sc-6011; 1:50 dilution), Jagged2 (sc-5604; 1:100 dilution), HES1 (NBPI-30, 411; 1:300 dilution), HES5 (Merck Millipore; AB5708; 1:400 dilution) primary antibodies for overnight at 4 °C in a humidified chamber. Next day the sections were washed three times for 5 min with phosphate buffered saline (PBS) and then incubated with biotinylated secondary antibodies [(anti-goat IgG; Vector Laboratories (BA); BA-5000; 1:500 dilution) and (anti-rabbit IgG; BA-1000; 1:500 dilution)] for 45 min at room temperature in a humidified chamber. Then slides were washed three times for 5 min with PBS and the resulting signal was developed with diaminobenzidine (DAB) (Dako; K3466). Sections were counterstained with hematoxylin, dehydrated, mounted in Kaisers glycerin gelatin (Merck; OB514196, NJ, USA) and examined by light microscopy (Zeiss, Oberkochen, Germany). Negative controls were

performed by replacing the primary antibody with the non-immune IgG in the same dilutions as the specific antibodies.

Image J Analysis

Photographs of adenocarcinoma, PIN and normal gland structures were taken with an Axioplan microscope (Zeiss, Oberkochen, Germany) and the immunohistochemical staining densities were evaluated through the use of the Image J (<http://imagej.nih.gov/ij/>) program. From each group of preparations immunostained with Notch1, Notch2, Notch3, Notch4, Delta, Jagged1, Jagged2, HES1 and HES5, five preparations were selected and included in the evaluation. Firstly, the appropriate threshold was arranged in such a way as to mask the whole stained area (containing haematoxylin + DAB) and the density was measured. Then, the threshold was set to mask only the DAP-positive areas to measure the colour density of the area. The degree of staining was established through the division of the density of the DAB positive area into the total staining density of haematoxylin and DAB and the product of the result of this division by 100. As a result, the average density was expressed as a percentage in the range of 0–100.

Semi-Quantitative Evaluations

The positive-stained immunoreactive cells and immunostained densities were also assessed semi-quantitatively with the markers addressed in all groups and the scoring was applied in the following manner: 0 = negative; (+) = weak positive; + = positive; ++ = strong positive; +++ = very strong positive.

Statistical Analysis

The immunohistochemistry datas obtained from ImageJ analysis were compared with Student's *t*-test. Comparisons were made among adenocarcinoma, PIN and normal gland structures. Probability values of less than 0.05 were considered significant. All statistical analyses were performed using Sigma Stat 3.5 (Statcon, Witzenhausen, Germany).

Results

Notch Receptors

Notch1

In normal prostate tissue, Notch1 immunostaining was observed in luminal secretory, basal and smooth muscle cells located in the stroma (Fig. 1a). In the same group, Notch1 immunostaining intensity was positive in luminal secretory

and basal cells and weak positive in smooth muscle cells (Fig. 1a). In PIN structure, Notch1 was strong positive in basal and luminal secretory cells and positive in smooth muscle cells (Fig. 1b). In adenocarcinoma group, Notch1 immunostaining was weak positive in luminal secretory cells and positive in smooth muscle cells located in prostatic stroma (Fig. 1c).

When comparisons were made among normal prostate, PIN and adenocarcinoma tissues, Notch1 immunostaining intensity of PIN tissues was higher compared to normal tissues and this increase was statistically significant ($P < 0.05$, Fig. 4). Notch1 immunostaining intensity of the adenocarcinoma tissues was significantly reduced compared to normal and PIN tissues and this decrease was statistically significant ($p < 0,05$; Fig. 4).

Notch2

In normal prostate tissues, Notch2 immunostaining was weak positive in luminal secretory cells and positive in basal cells but not observed in smooth muscle cells located in prostatic stroma (Fig. 1e). In PIN tissues, Notch2 immunostaining was positive in luminal secretory and basal cells and negative in smooth muscle cells (Fig. 1f). In adenocarcinoma tissues, Notch2 immunostaining was weak positive in luminal secretory and smooth muscle cells located in stroma (Fig. 1g).

When Notch2 immunostaining intensities were compared among normal prostate, PIN and adenocarcinoma structures, there was no statistically significant difference between the groups ($p > 0,05$; Fig. 4).

Notch3

In normal prostate tissues, Notch3 immunostaining was negative in luminal secretory cells, positive in basal cells and weak positive in smooth muscle cells located in prostatic stroma (Fig. 1i). In PIN structure, Notch3 expression was very strong positive in luminal secretory and basal cells, whereas smooth muscle cells were weak positive (Fig. 1j). In adenocarcinoma structure, Notch3 immunostaining was positive in luminal secretory cells and weak positive in smooth muscle cells (Fig. 1k).

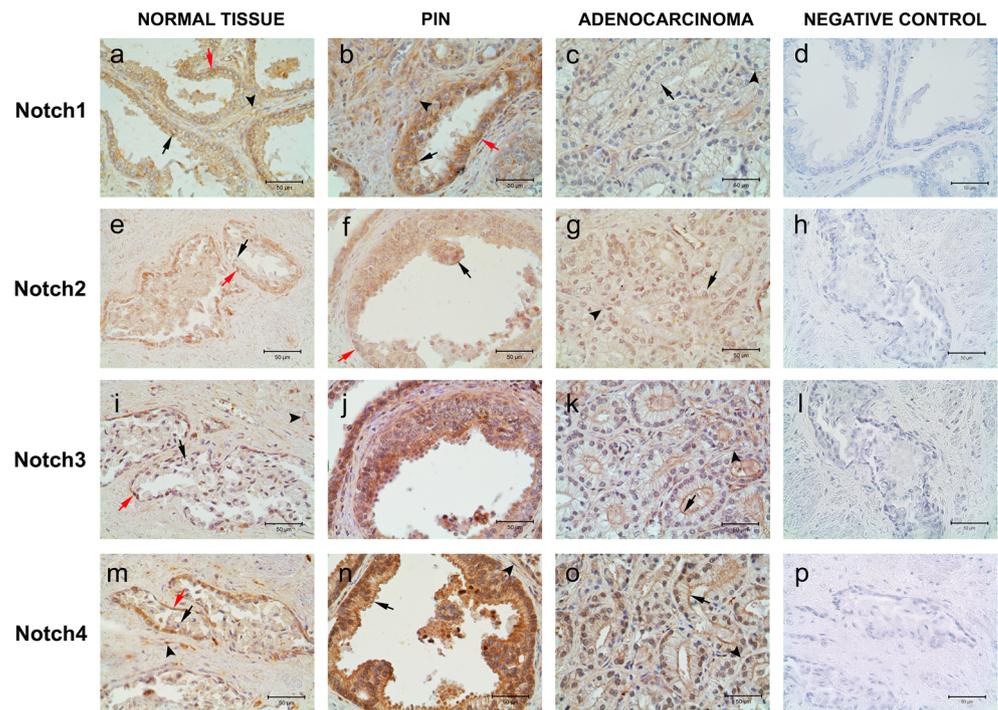
In PIN tissues, Notch3 immunostaining intensity was higher compared to normal and adenocarcinoma tissues and this increase was statistically significant ($p < 0.05$, Fig. 4).

Notch4

While Notch4 immunostaining was observed at low density in normal prostate and adenocarcinoma tissues, it was observed very strong especially in luminal secretory and basal cells of PIN tissues (Fig. 1m-o).

Notch4 immunostaining intensity in the PIN tissues were more intense compared to normal and adenocarcinoma tissues and this increase was statistically significant ($p < 0.05$, Fig. 4).

Fig. 1 Immunostainings of Notch receptors. Strong Notch 1, 3 and 4 immunostainings are observed particularly in PIN structures. 1a-d: Notch1; 1e-h: Notch2; 1i-l: Notch 3; 1 m-p: Notch4. Luminal secretory cells (black arrows), basal cells (red arrows), smooth muscle cells (arrow heads)



Notch Ligands

Jagged1

In normal prostate tissue, *Jagged1* expression was weak positive in luminal secretory cells and positive in basal cells (Fig. 2a). However, *Jagged1* was not expressed in smooth muscle cells (Fig. 2a). In PIN structures, *Jagged1* immunostaining was very strong especially in luminal secretory and basal cells (Fig. 2b). In adenocarcinoma tissues, *Jagged1* immunostaining intensity was strong positive in luminal secretory cells and weak positive in smooth muscle cells (Fig. 2c).

When comparisons were made among normal prostate, PIN and adenocarcinoma structures; *Jagged1* immunostaining intensity of PIN structure was determined to be more intense than normal tissue and adenocarcinoma structures. This intensity increase was statistically significant compared to normal prostate and adenocarcinoma tissues ($p < 0.05$, Fig. 4).

Jagged2

Jagged2 was weak positive in smooth muscle cells of normal prostate and adenocarcinoma tissues (Fig. 2e, g) and in all cell types of the PIN tissue (Fig. 2f).

When comparisons were made among normal prostate, PIN and adenocarcinoma tissues; *Jagged2* immunostaining intensity of PIN tissues were slightly denser than normal prostate and adenocarcinoma tissues. This increase was statistically significant ($p < 0.05$; Fig. 4).

Delta

Delta showed very low intensity in all studied groups (Fig. 2i-k).

When comparisons were made among normal prostate, PIN and adenocarcinoma tissues; there was no statistically significant difference for *Delta* immunostaining intensity ($p > 0.05$; Fig. 4).

Notch Signalling Transcription Factors

HES1

HES1 was found to be expressed in all cells of the studied groups except for epithelial cells of normal prostate tissues (Fig. 3a-c). Density increase in *HES1* expression was remarkable in PIN tissues, in particular luminal secretory and basal cells (Fig. 3b).

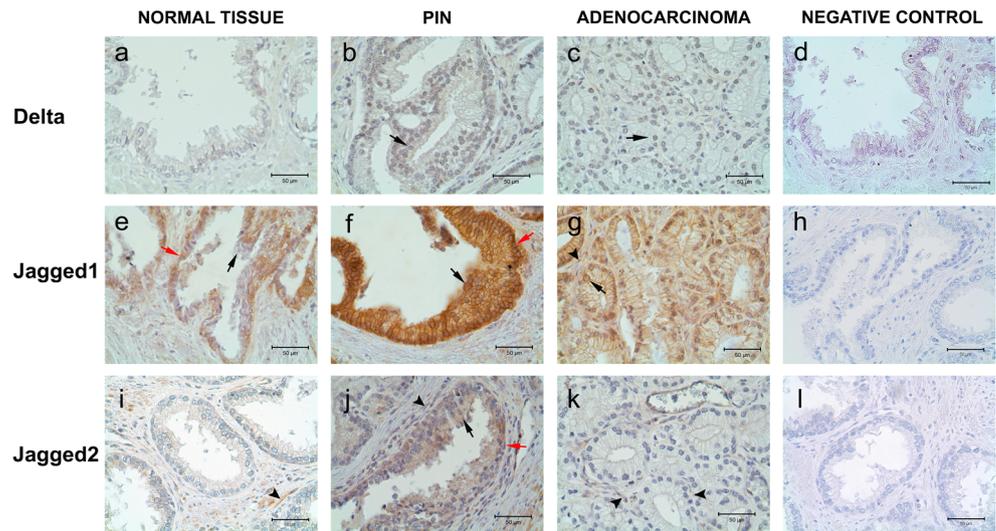
Increase of *HES1* immunostaining intensity in PIN tissues was statistically significant compared to both normal prostate and adenocarcinoma tissues ($p < 0.05$; Fig. 4).

HES5

HES5 was expressed less than *HES1* in PIN and adenocarcinoma (Fig. 3e-g). *HES5* intensity was higher remarkably in PIN tissues compared to normal and adenocarcinoma tissues ($p < 0.05$; Fig. 4).

There was no significant difference between the immunohistochemical staining patterns of low grade and high grade adenocarcinomas for Notch signaling pathway members.

Fig. 2 Immunostainings of Notch ligands. Strong Jagged-1 immunostaining was observed particularly in PIN structures. 2a-d: Jagged1; 2e-h: Jagged2; 2i-l:Delta. Luminal secretory cells (*black arrows*), basal cells (*red arrows*), smooth muscle cells (*arrow heads*)



In negative control sections, the immunostainings of all antibodies were not observed (Fig. 3d, h, l, p; 2d, h, l; 3d, h).

Discussion

Within the cell, Notch receptors are matured by glycosylation and by the slitting of precursor proteins through furin. When these receptors bind with their ligands on cell surface, which is the active form of Notch (N^{IC}), enters into the nucleus. N^{IC} that passed into nucleus, allows transcription of countless genes with CSL and other indirect mechanisms. N^{IC} is rarely are stored in the nucleus and is degraded quickly [25].

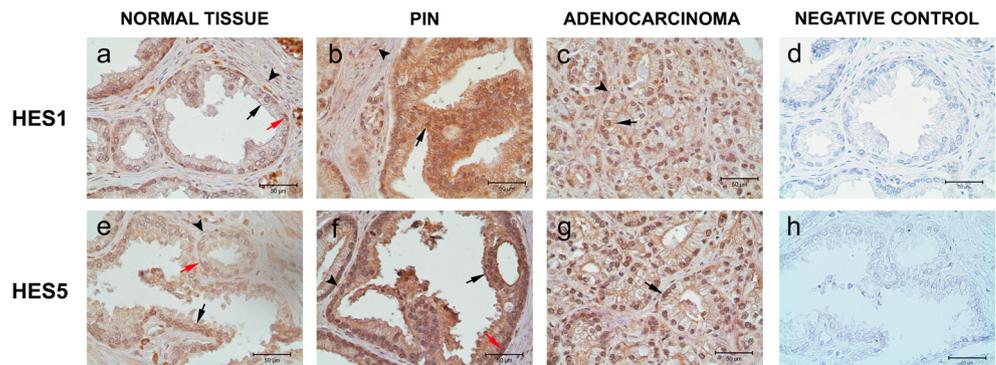
In a previous study that was about the relationship between Notch1 expression and PCa, Notch1 mRNA levels of tissues with prostate adenocarcinoma was significantly lower than normal [20]. In another study, it has been reported that the protein expression of Notch1 has increased significantly higher than normal in prostatic adenocarcinoma tissues [23]. It is understood that, the findings of earlier studies in this subject are conflicting. In our study though, it has been found

that while Notch1 is expressed lowest in adenocarcinoma structures, it is denser in PIN structures, compared to normal prostate tissue and areas with adenocarcinoma. Therefore, it is possible that Notch1 is mostly associated with the development of PIN structures.

In a study conducted earlier associated with Notch2, Notch2 expression in prostate adenocarcinoma tissues, were compared with normal tissues, but no differences were found between the two groups [20]. In our study, the Notch2 immunostaining was observed on all studied tissue groups at low levels. Our findings may suggest that Notch 2 has no role in the formation of PIN or adenocarcinoma structures.

In an earlier study regarding Notch3, it has been observed that Notch3 expression is increased in cholangiocarcinoma, and gall bladder carcinoma, and thus Notch3 might play a role in the development of cancer, and it is possible that it is oncogenic [26]. In another study, normal prostate tissue and prostate adenocarcinoma were compared and ultimately found to be similar in both types of tissues [20]. In our study, Notch3 was observed in weak levels observed in all tissue types, however, there was a similarity of expression in normal tissues and

Fig. 3 Immunostainings of Notch signalling transcription factors. Strong HES1 and HES5 immunostainings are observed particularly in PIN structures. 3a-d: HES1; 3e-h: HES5. Luminal secretory cells (*black arrows*), basal cells (*red arrows*), smooth muscle cells (*arrow heads*)



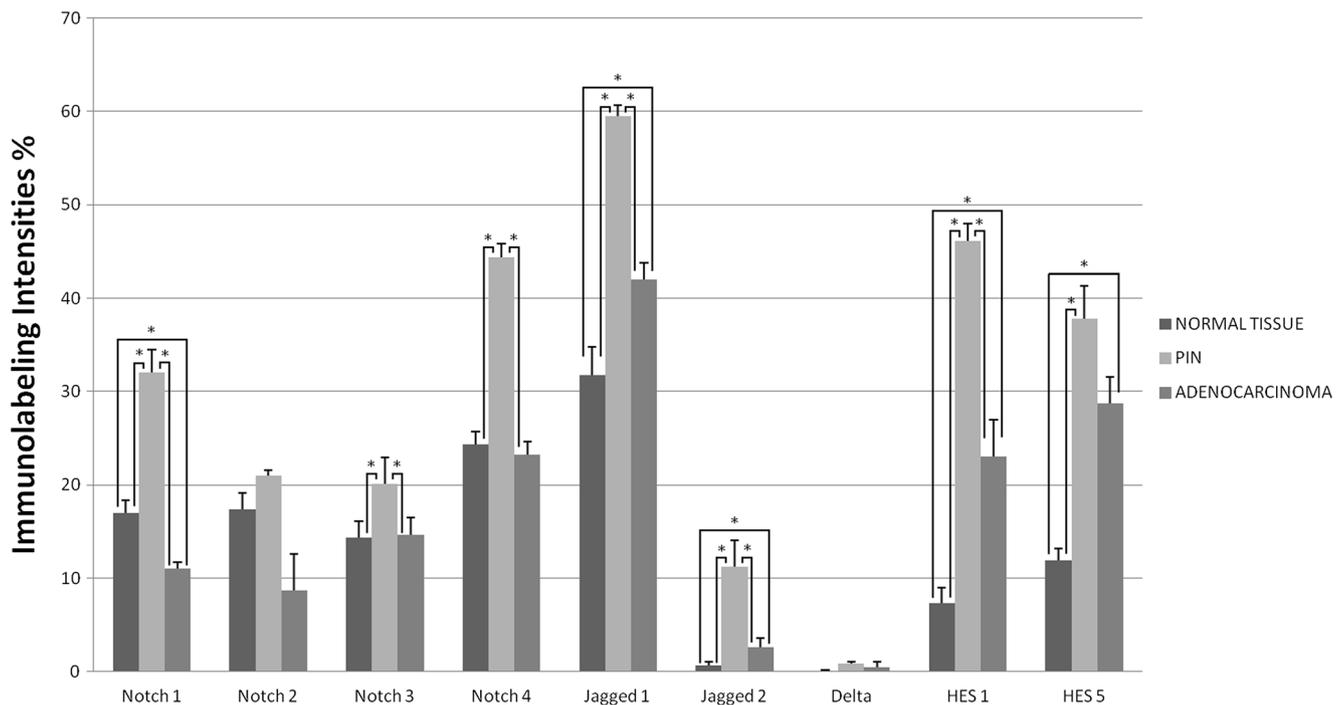


Fig. 4 Quantitative analyses of Notch1-4, Jagged1-2, Delta and HES1-5 immunolabeling intensities. Notch1, Jagged1, HES1 and HES5 immunostaining levels particularly in PIN structures were observed to be significantly increased compared to other regions

adenocarcinoma structures, and is increased significantly in PIN structures, compared to other tissues. Our findings can point out that Notch3 might play an active role in PIN structure development, and has nothing to do with the development of adenocarcinoma.

In previous studies done about the relationship between Notch4 and breast cancer, it was observed that Notch4 expression was increased in breast cancer, thus it was showed to be oncogenic [27, 28]. In contrast, in another study associated with Notch4 and prostate adenocarcinoma, Notch4 mRNA levels was found similar to the levels in normal tissues [20]. In our study, we observed that Notch4 immunostaining density significantly increased compared to the normal prostate tissue and adenocarcinoma. Moreover, the density of Notch4 immunostaining in PIN structures, was the highest compared to other members of Notch. This very intense expression of Notch4 in PIN structures, might show that Notch4 plays a more active role in the formation of this structure than other members of Notch signaling pathway.

In a study done in 2004, it was found that Jagged1 increases in prostatic tumors, compared to benign prostate controls, and this finding has shown the oncogenic role of Jagged1 [24]. In contrast, in a study conducted in 2006, Jagged1 mRNA levels were investigated in prostatic adenocarcinoma and normal control tissues, but no significant difference was observed between the two groups [20]. In another study conducted in 2013, it was observed that Jagged1 protein expression increases in the PIN structures compared to benign

controls, and peaks in PCa [8]. In our study, Jagged1 immunodensity was more intense than all other markers, in all the tissue types studied. On the other hand, Jagged1 immunodensity was marked in PIN structures, compared to normal and adenocarcinoma structures. Therefore, these findings could show that Jagged1 might play a role in the formation of PIN structures may be involved in the activation of this tissue.

Our findings regarding Jagged2 and Delta showed compliance with the findings of previous studies [20, 29]. As in previous studies, in our study, Jagged2 and Delta immunostaining density has been observed in low levels in all tissue types. According to these findings, Jagged2 and Delta ligands might not have any role in the formation and survival of PCa and PE structures.

Previous studies identified HES1 mRNA, one of the transcription factors of the Notch signalling pathway, on the PCa cell lines and indicated its oncogenic role [22, 29]. However, a specific study compared malignant PCa and normal prostatic tissue in terms of HES1 mRNA expression and failed to observe any differences between the two tissue types [20]. The present study identified a significant increase in the HES1 protein expression in the PIN structure when compared to the normal prostatic tissue and adenocarcinoma structure. According to these findings, the HES1-mediated Notch signaling may augment the formation of the PIN structure.

Although in previous studies, HES5 was identified to be upregulated in ovarian serous adenocarcinoma [30], no

previous study explored its expression in PCa. Therefore, our findings on HES5 is original. Our study established that HES5 protein expression demonstrated a significant increase in the PIN structure - although not to the same extent as HES1 - when compared to normal prostatic tissue and adenocarcinoma structure. Thus, HES5 may be functional in the formation of PCa, as well as the PIN structure that is considered to represent the onset of PCa.

Consequently, this study identified that all in-scope members of the Notch signalling pathway were overexpressed in PIN structures when compared to the other tissue types. Therefore, the Notch signalling pathway may be playing a role specifically in the formation of PIN structures. Moreover, in the course of the study, the highest rate of expression in the PIN structures was observed in Notch1 and Notch4 among Notch receptors, Jagged1 among Notch ligands and HES1 among the Notch signalling pathway transcription factors. Considering these findings, it is possible to state that the Notch signalling pathway may contribute specifically to the formation of PIN structures through Notch1 or Notch4, Jagged1 and HES1.

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Conflict of Interest The authors declare no conflict of interest.

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