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Evaluation of Notch1 gene expression in prostate carcinoma

Prostat karsinomunda Notch1 gen ekspresyonunun değerlendirilmesi

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Abstract

Aim: Prostate cancer is one of the most common cancers in the world and its incidence is rapidly increasing. Currently, several new treatment strategies that target specific signaling pathways as well as immunotherapy are being explored for prostate cancer. In the current study, we aimed to investigate the frequency and clinicopathologic features associated with Notch1 gene expression using immunohistochemistry and real-time-polymerase chain reaction in patients with prostate cancer.

Methods: This research was planned as a cohort study. Tissue samples of 60 patients with prostate cancer and 20 patients with benign prostatic hyperplasia were selected from the archives. Twenty patients with benign prostatic hyperplasia were used as the control group. The relative expression levels of Notch1 gene were determined by real-time-polymerase chain reaction while Notch1 protein expression was analyzed using immunohistochemistry.

Results: We found Notch1 gene expression at a significant level in 17 (28.3%) of 60 patients with prostate carcinoma. We found a significant correlation between high Gleason scores (P=0.007), high ISUP (International Society of Urologic Pathologists) grade group scores (P=0.008) and high prostate-specific antigen levels (P=0.018) for Notch1 gene expression.

Conclusions: We conclude that Notch1 is important in prostate carcinogenesis. Therefore, determining Notch1 will be an essential step in targeted therapies in patients with prostate carcinoma.

Keywords: RT-PCR, Immunohistochemistry, Notch1, Prostate cancer, ISUP, PSA

Öz

Amaç: Prostat kanseri dünyada en yaygın görülen kanserlerden biridir ve sıklığı giderek artmaktadır. Şu anda, prostat karsinomu için etkin bir tedavi yöntemi olmamakla birlikte immünoterapiyi ve spesifik sinyal yolaklarını hedefleyen yeni tedavi stratejileri araştırılmaktadır. Biz bu çalışmada prostat kanseri hastalarında immünohistokimya ve Real-Time polimeraz zincir reaksiyonu ile Notch1 gen ekspresyonunu ve klinikopatolojik özellikler ile ilişkisini araştırmayı amaçladık.

Yöntemler: Bu araştırma bir kohort çalışma olarak planlandı. Hastane arşivimizden 60 tane prostat kanseri ve 20 tane benign prostat hiperplazisi olan hastaların parafin blokları seçildi. Benign prostat hiperplazisi olan 20 hasta kontrol grubu olarak kullanıldı. Notchl gen ekspresyonu seviyeleri immünohistokimya ve Real-Time polimeraz zincir reaksiyonu kullanılarak analiz edildi.

Bulgular: Çalışmamızda 60 prostat karsinomu tanılı hastamızın 17'sinde (%28,3) Notch1 gen ekspresyonunu anlamlı düzeyde yüksek bulduk. Ayrıca yüksek Gleason skoru (P=0,07), yüksek ISUP skoru (P=0,008) ve yüksek prostat spesifik antijen düzeyi (P=0,018) ile Notch1 ekspresyonu arasında anlamlı bir korelasyon bulduk.

Sonuçlar: Prostat karsinogenezinde Notch1'in önemli olduğu sonucuna vardık. Bu nedenle, Notch1 prostat karsinomlu hastalarda hedefe yönelik tedavilerde önemli bir adım olacaktır.

Anahtar kelimeler: RT-PCR, İmmünohistokimya, Notch1, Prostat kanseri, ISUP, PSA

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Introduction

Prostate carcinoma (PCa) is the most commonly diagnosed malignancy in men and its incidence is rapidly increasing. Many factors are associated with pathophysiology and progression of PCa [1]. Hormonal imbalances, genetic factors, and many factors such as carcinogens in the diet and infectious agents are all sources that have been determined to contribute to prostate carcinogenesis [1-4]. The prostatic epithelium is composed of two types of cells: Luminal epithelial cells and basal epithelial cells [4]. Primary prostate tumors are almost always characterized by a luminal phenotype suggesting a luminal origin, but recently it has been indicated that both types of these epithelial cells are possible cells of origin for prostate carcinogenesis.

The Notch was first discovered in 1917 by Thomas Hunt Morgan [6]. Later, Kidd et al. discovered that the loss of Notch expression not only affected the neurodevelopmental phenotype of this Drosophila strain, but also the morphology of the eye, wing, and hair [7]. In later studies, it was found that the disorder of Notch expression is associated with some diseases, for example, T-cell acute lymphoblastic leukemia (T-ALL) patients, CADASIL syndrome (cerebral autosomal- dominant arteriopathy with subcortical infarcts and leukoencephalopathy) and Alagille syndrome [8-10]. The Notch signaling pathway plays a significant role both in embryonic development and the determination of cell fate in organ homeostasis [11]. The main function of the Notch pathway is the regulation of bi-directional cell fate decisions. Furthermore, it has been shown that Notch activation in cancer cells causes abnormal cell proliferation. Mammals have four Notch receptors, Notch1, Notch2, Notch3 and Notch4. There are also five Notch ligands including Jagged1, Jagged2, Delta 1, Delta 3 and Delta 4. In addition, the ligands for the Notch receptor are divided into two groups: Delta-like ligands (DLL1, DLL3, and DLL4) and Serrate-like ligands (JAG1 and JAG2). The Notch gene encodes a 300 kD single-pass transmembrane receptor. The Notch receptor consists of two parts, one intracellular and one extracellular. The large extracellular region contains 36 consecutive EGF (Epidermal Growth Factor) -like repeats and 3 cysteine-rich LIN 12 repeats. EGF repeats allow the Notch receptor to interact with ligands. The intracellular part of the Notch receptor is also called Notch Intracellular Domain (NICD) [12-14].

Many cancer pathways have been investigated to date [15,16]. Recently, studies have been examining the Notch pathway in terms of prostate carcinogenesis, with no full elucidation [17]. Therefore, in this study, we aimed to investigate the frequency and clinicopathologic features associated with Notch1 gene expression using immunohistochemistry and real-time-polymerase chain reaction in patients with prostate cancer.

Materials and methods

Tissue samples

Tissue samples from prostate carcinoma patients who underwent surgery between 2010 and 2017 were selected from the archives of Department of Pathology. The study was performed in accordance with the principles of Declaration of Helsinki and approved by the Ethics Committee of Akdeniz University (Date:30.11.2016, number:625). This study was supported by Akdeniz University Research Fund Accounting (BAP Project no: TTU-2017-2289). All our patients were sampled with suspicion of prostate carcinoma. The blocks with sufficient tumor tissue for the study were selected in historical order since 2017. Hematoxylin-Eosin stained preparations obtained from formalin-fixed, paraffin-embedded blocks were removed from the archive and re-examined. Sixty of our patients had prostate carcinoma; 33 samples were obtained with tru-cut biopsy, 16 with radical prostatectomy and 11 with prostate TUR. Twenty patients with benign prostatic hyperplasia were used as the control group. Notch1 gene expression was examined both immunohistochemically and by RT-PCR in 60 patients with prostate cancer and 20 with benign prostatic hyperplasia. The optimal blocks for immunohistochemistry and real-time-polymerase chain reaction (RT-PCR) were selected. The anamnesis, stage, diagnosis history, PSA values and treatment follow-up times for the cases were obtained from our hospital's automation system, patient follow-up files, and from the patients themselves or their relatives via telephone.

Immunohistochemistry

In retrospectively selected cases, sections with a thickness of 5 μm were obtained from the appropriate paraffin blocks. Immunohistochemistry was performed using Cell Signaling Technology rabbit monoclonal Notch1(D1E11) XP® antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:50 dilution for two hours. The staining procedure was performed on the DAKO Omnis autostainer (Agilent, Santa Clara, CA 95051, USA). Sections were examined using light microscopy (Zeiss, Oberkochen, Germany). Negative controls were performed by replacing the primary antibody with the nonimmune IgG in the same dilutions as the specific antibodies. Positive staining for Notch1 was observed in the cytoplasm and nucleus.

RNA Extraction and RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Total RNA was extracted from FFPE tissue using Purelink FFPE RNA Isolation Kit (Cat No: K156002; Invitrogen) according to the manufacturer's instructions. The amount of total RNA in each sample is measured using NanoDrop (Maestrogen, Taiwan). All samples are diluted to a concentration of 25 ng/μl. The reverse transcription reaction was performed using High Capacity cDNA Reverse Transcription Kit (Appliedbiosystems by Thermo Fisher Scientific). Complementary DNA was prepared from 25 ng of isolated total RNA, with 10xRT Random Primers, according to the manufacturer' s instructions. The program was as follows: 10 minutes at 25 ºC, 2 hours at 37 ºC, 5 minutes at 85 ºC and at 4 ºC thereafter on a polymerase chain reaction thermocycler (Applied Biosystems). The resulting cDNA was diluted to 1/5.

qPCR

The relative expression levels of Notch1 gene were determined by qPCR with TaqMan® Gene Expression Master Mix in Applied Biosystems 7900HT Real-Time PCR System and normalized to 18S. Primers and FAM-MGB hydrolysis probes were TaqMan® Gene expression assays on demand for Notch11 Hs01062014_m1 and 18S (Hs99999901_s1) (Applied biosystems by Thermo Fisher Scientific). All samples were

performed in duplicates. The PCR amplification program was as follows: 20 seconds at 95 ºC, 40 cycles of 1 second at 95 ºC and 20 seconds at 60 ºC. In addition, as the non-template control, ddH2O was analyzed for every plate. To assure that the amplification efficiencies for each real-time-PCR run and each gene were similar, the slope was adjusted for each sample. The data obtained from the qPCR was analyzed by the ΔΔCt-method.

Statistical analysis

Data were evaluated with the SSPS 22® program. Descriptive statistics were presented as mean (standard deviation) and median (minimum-maximum) for normally and non-normally distributed values, respectively. Nominal variables were shown as number (n) and percentage (%). As there were two groups, the significance of the difference between the groups was evaluated with the Student's t-test or the Mann Whitney U-test. Categorical variables were evaluated with the Pearson Chi-square test. A value of $P<0.05$ was considered statistically significant.

Results

Eighty cases, diagnosed at the Akdeniz University Medical Faculty Pathology Department between 2010 and 2017, were selected for this study. Of the patients with prostate adenocarcinoma, 20, 10, 14, 8 and 8 patients had Gleason scores of 6, 7, 8, 9 and 10, respectively. In the 80 cases we evaluated, the youngest and oldest patients were 46 and 81 years old, with a mean age of 65 years.

PSA values ranged between a minimum of 0.16 ng/ml and a maximum of 260 ng / ml. The Gleason score in our prostate adenocarcinoma cases ranged from a minimum of 6, to a maximum of 10. ISUP grade group were scored in 21 patients as ISUP-1, in eight patients as ISUP-2, in one patient as ISUP-3, in 14 patients as ISUP-4 and in 16 patients as ISUP-5. Notch1 gene expression was detected in 17 out of 60 patients by RT-PCR (Table 1). Thirty-three specimens were obtained with tru-cut biopsy, 16 with radical prostatectomy and 11 with prostate TUR material.

In immunohistochemical examination, cytoplasmic and nuclear staining in malignant gland epithelium was considered positive. In 17 of our 60 prostate carcinoma patients, a positive reaction was observed in the malignant gland epithelium. In our control group with benign prostatic hyperplasia, focal staining with Notch-1 was observed in the basal cells and smooth muscles in the stroma. In our control group, no staining was observed in the prostate gland epithelium. Immunohistochemically, going by the RT-PCR results, preexisting cytoplasmic and nuclear immunoreaction was observed in 17 of 60 patients (Figure 1, 2). A significant relationship was found between Notch1 gene expression, increase in Gleason scores ($P=0.007$), and ISUP grade groups (Figure 3).

The Notch1 gene expression of our patients with prostate adenocarcinoma were compared with those with benign prostatic hyperplasia, as evaluated with RT-PCR. Out of 60 patients with prostate adenocarcinoma, 17 had significantly higher levels of Notch1 gene expression than patients with benign prostatic hyperplasia.

In addition, in our study, 10 out of 17 patients with Notch1 gene expression were metastatic, and nine metastatic distant organs were detected. We could not perform a statistical evaluation because our data on metastasis were incomplete. We found that Notch1 gene expression was significantly associated with Gleason score, ISUP grade group increase and PSA elevation (Table 2).

Table 1: Notch-1 gene expression of 60 patients with prostatic adenocarcinoma compared to patients with benign prostatic hyperplasia (Bold texts: Notch-1 positive patients)

Patient number	Notch1 CT	18SrRNA CT	ΔCT	ΔΔCT (8.595)	Fold difference (2^-ΔΔCT)
1	31.98	21.64	10.34	1.745	0.298
2	27.64	15.43	12.21	3.615	0.082
3	**33.95**	**26.14**	**7.81**	**-0.785**	**1.72**
4	**36.146**	**29.16**	**6.99**	**-1.605**	**3.042**
5	29.22	14.78	14.44	5.845	0.017
6	**34.37**	**27.69**	**6.68**	**-1.915**	**3.771**
7	**34.85**	**27.88**	**6.97**	**-1.625**	**3.08**
8	**30.93**	**25.50**	**5.43**	**-3.165**	**8.96**
9	**33.89**	**26.42**	**7.47**	**-1.125**	**2.181**
10	35.11	24.58	10.53	1.935	0.262
11	29.44	16.01	13.43	4.835	0.035
12	35.69	26.76	8.93	0.335	0.79
13	33.97	21.83	12.14	3.545	0.09
14	29.98	16.18	13.8	5.205	0.027
15	**33.214**	**25.079**	**8.135**	**-0.46**	**1.376**
16	31.89	18.37	13.52	4.925	0.033
17	31.86	18.04	13.82	5.255	0.026
18	30.197	17.895	12.30	3.705	0.0389
19	34.38	25.15	9.23	0.635	0.64
20	**36.45**	**27.96**	**8.49**	**-0.105**	**1.08**
21	33.81	23.85	9.96	1.365	0.388
22	**29.89**	**22.14**	**7.75**	**-0.845**	**1.80**
23	**34.18**	**28.63**	**5.55**	**-3.045**	**8.25**
24	30.73	17.14	13.59	4.995	0.031
25	**34.11**	**26.13**	**7.98**	**-0.615**	**1.53**
26	**35.241**	**27.249**	**7.99**	**-0.605**	**1.52**
27	29.531	17.173	12.36	3.765	0.074
28	30.285	16.336	13.95	5.355	0.024
29	28.76	16.75	12.01	3.415	0.09
30	30.999	17.42	13.58	4.985	0.0316
31	29.536	16.135	13.40	4.805	0.058
32	34.501	24.496	10	1.405	0.378
33	31.473	17.895	13.58	4.985	0.032
34	29.86	15.97	13.89	5.295	0.03
35	31.861	20.559	11.30	2.705	0.153
36	31.793	18.893	12.9	4.305	0.051
37	30.1	17.87	12.23	3.635	0.081
38	33.63	23.63	10	1.405	0.38
39	34.43	24.38	10.05	1.455	0.36
40	**33.918**	**25.714**	**8.20**	**-0.395**	**1.315**
41	33.626	22.241	11.39	2.795	0.144
42	**35.059**	**27.177**	**7.88**	**-0.715**	**1.64**
43	31.431	19.075	12.36	3.765	0.074
44	**35.19**	**27.78**	**7.41**	**-1.185**	**2.27**
45	34.39	24.88	9.51	0.915	0.53
46	28.99	15.56	13.43	4.835	0.04
47	30.93	17.03	13.09	4.495	0.044
48	33.84	22.63	11.21	2.615	0.163
49	**35.61**	**27.62**	**7.99**	**-0.605**	**1.52**
50	31.08	15.99	15.09	6.495	0.011
51	30.0	18.29	11.71	3.115	0.12
52	29.099	19.392	9.71	1.115	0.461
53	30.338	15.53	14.81	6.215	0.013
54	30.508	17.225	13.28	4.685	0.0389
55	29.98	16.98	13	4.405	0.047
56	30.167	18.165	12	3.405	0.094
57	33.51	23.50	10.01	1.415	0.38
58	29.392	15.245	14.15	5.555	0.0213
59	29.60	18.76	10.84	2.245	0.22
60	**29.299**	**21.406**	**7.89**	**-0.705**	**1.63**

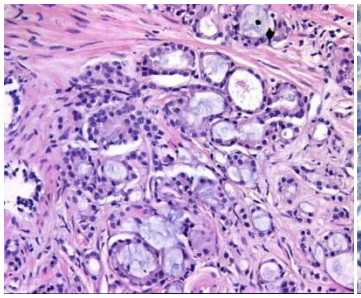

Figure 1: Prostate adenocarcinoma (H&E 20X)

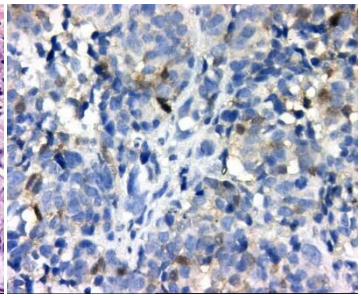

Figure 2: Prostate adenocarcinoma positive with Notch1 (40X)

Table 2: Mean age, PSA, ISUP and Gleason scores of scores in Notch-1 positive and negative patients

	Notch-1 positive (n=17)		Notch-1 negative (n=43)		*P*-value
	mean	SD	mean	SD	
Age	67	9	65	8	0.407
ISUP Grade Group	3.82	1.47	2.58	1.67	0.008
Gleason Score	8.35	1.41	7,23	1,32	0.007
Serum PSA Level	184.51	432.84	24.67	45.10	0.018

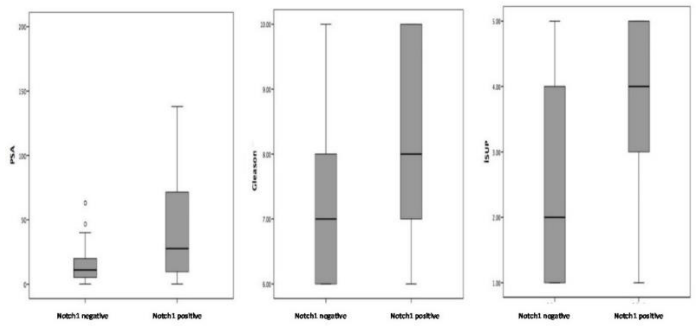

Figure 3: A significant relationship was found between Notch1 gene expression and Gleason score increase, ISUP score increase, and PSA elevation

Discussion

The aim of this study was to investigate the frequency and clinicopathologic features associated with Notch1 gene expression. We demonstrated that high Gleason scores, high ISUP grade group scores and high PSA levels significantly correlated with Notch1 gene expression.

It is well known that Notch signaling regulates normal and cancer development in many tissues including the prostate. The expression of Notch pathway elements clearly shows the regulation of Notch signaling pathway in prostate cancer in cases of established cancer cell lines, transgenic mouse models, and clinical tumor specimens.

The role of Notch ligands and Notch receptors in prostate tumorigenesis is not well defined. In a study in 2006, Notch1 mRNA expression has been reported to be significantly down-regulated in prostate cancers, thus suggesting a potential tumor suppressor role for Notch1 in prostate cancer [18]. This study also examined the expression of the remaining members of the Notch receptor family in prostate cancer but found no significant differences in mRNA levels for Notch-2-3-4 between benign and malignant prostate specimens [18]. Conversely, a study in 2007 revealed that Notch1 protein was overexpressed in malignant prostates compared to benign controls [19]. This suggests a potential carcinogenic role of Notch1 in prostate tumorigenesis. Notch1 protein expression, rather than Notch1 mRNA expression, is a more reliable indicator of Notch1 functionality. Researchers in a study showed that the Notch signal is associated with prostate development and cancer cell growth, and they correlated high grade localized PCa with the gene expression of the Notch signal. Because of the role of basal epithelial cells in the development of prostate carcinoma, they have suggested that PCa and basal epithelial cell relationship should be investigated and discussed [20]. A review of Notch pathway and prostate tissue revealed that Notch pathway was involved in prostate differentiation in benign prostate tissue and associated with lethal potential in prostate cancers [21]. The expression of Immunohistochemical Notch-1 in normal prostate tissue, prostatic intraepithelial neoplasia and PCa was investigated. As a result of this study, stronger staining was observed in prostatic intraepithelial neoplasia than prostatic adenocarcinomas [22].

Prostate cancer most frequently metastasizes to bone, brain, and lymph nodes. Therefore, the relationship between the metastasis of prostate cancers and Notch pathway was investigated [22-29]. As a result of these studies, metastatic prostate cancer was shown to have increased expression levels of the Notch1 receptor ligand, when compared to benign prostate tissue and localized prostate cancer [22-28].

Surgical treatment and chemotherapy are used in the treatment of prostate cancers. Although chemotherapy agents used in prostate carcinomas have exceptionally good response at first, it is known that a rapid resistance to chemotherapeutic agents develops soon. Therefore, resistance mechanisms against chemotherapeutic agents have been investigated. Since the notch pathway is known to play a role in prostate carcinomas, its effect on chemotherapy resistance has been researched. It is concluded that the notch pathway is effective in the progression, metastasis, and chemotherapy resistance of prostate cancer [24,28].

To fully understand the role of Notch1 in prostate carcinogenesis, it may be necessary to assess the possibility that primary tumor growth and metastasis progression may be affected differently by Notch signaling. There is also a need for further study, more mouse models, and cell lines in this regard. If the role of Notch1 in prostate tumorigenesis is better understood, therapeutic strategies for Notch1 can be developed. Therefore, this is an important and urgent need, given the proposed use of anti-Notch compounds for the treatment of cancer, including prostate cancer.

Limitations

Our study had some limitations. Due to our financial limitation, we were able to study a small number of patients and evaluate Notch1 only.

Conclusions

The current study showed that a high Gleason score, high ISUP scores, and high PSA levels and Notch1 gene expression are significantly correlated. Due to the small number of studies related to this topic and the inadequate data obtained, it is important to conduct further studies with wider series to determine the prognosis of PCa patients and manage their treatment.

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