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Predictive Value of CCR3 Expression on Diagnosis and Prognostic Classification of the Prostate Adenocarcinoma

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ABSTRACT

Objective: Chemokines are factors secreted from damaged or infected tissues to induce an inflammatory and immunological reaction. Both chemokines and their receptors are considered as critical actors of tumor formation. Our study aims to investigate CCR-3 expression, which is a chemokine receptor that has recently been found to be secreted by most cancer cells, in prostatic adenocarcinoma (PCa), and reveal any possible relation between the Gleason prognostic grade and CCR-3 levels. **Material Method:** The study included 25 patients with PCa who underwent prostatectomy, and 25 patients with benign prostate hyperplasia (BPH). CCR-3 was detected at mRNA level by the Real-Time PCR, and at protein level by immunohistochemical (IHC) staining in both PCa and BPH patients. Results from PCa and BPH groups were compared. In PCa group, any correlation between the CCR-3 expression and Gleason prognostic grade was also searched. **Results:** Although CCR-3 mRNA levels in PCa group were found to be significantly higher than the BPH group (p=0.001), the difference was not significant at the protein level (p=0.205). The difference between the CCR3 expression levels of patients with different Gleason prognostic grades was not significant at both mRNA and protein levels. A statistically significant positive correlation was found between the CCR-3 IHC staining and total PSA levels (p=0.001) in PCa patients. **Conclusion:** We concluded that the CCR-3 mRNA levels may be useful in the diagnosis of PCa.

Keywords: CCR-3, Prostate Cancer, Gleason Prognostic Grade, Chemokine.

Prostat Adenokarsinomunun Tanısında ve Prognostik Sınıflandırmasında CCR3 Ekspresyonunun Prediktif Değeri

ÖZ

Amaç: Kemokinler, enflamatuar ve immünolojik bir reaksiyonu indüklemek için hasarlı veya enfekte dokulardan salgılanan faktörlerdir. Hem kemokinler hem de reseptörleri, tümör oluşumunun kritik aktörleri olarak kabul edilir. Çalışmamız, prostat adenokarsinomunda (PCa) son zamanlarda çoğu kanser hücresi tarafından salgılandığı tespit edilen bir kemokin reseptörü olan CCR-3 ekspresyonunu araştırmayı ve Gleason prognostik derecesi ile CCR-3 seviyeleri arasındaki olası ilişkiyi ortaya koymayı amaçlamaktadır. **Gereç ve Yöntem:** Çalışmaya, prostatektomi yapılan PCa'lı 25 hasta ve iyi huylu prostat hiperplazisi (BPH) olan 25 hasta dahil edildi. Her iki hasta grubunda da CCR-3 ekspresyonu hem Real-Time PCR ile mRNA düzeyinde, hem de immünohistokimyasal (IHC) boyama ile protein düzeyinde saptandı. PCa ve BPH gruplarının sonuçları karşılaştırıldı. PCa grubunda CCR-3 ekspresyonu ile Gleason prognostik derecesi arasında herhangi bir ilişki de araştırıldı. **Bulgular:** Her ne kadar PCa hastalarında CCR-3 ekspresyonu mRNA düzeyinde anlamlı olarak yüksek bulunduysa da (p=0.001), fark protein düzeyinde anlamlı değildi (p=0.205). Yine, farklı Gleason prognostik derecelerine sahip hastaların CCR3 ekspresyon seviyeleri arasındaki fark hem mRNA hem de protein seviyelerinde anlamlı değildi. PCa hastalarında CCR-3 IHC boyaması ile toplam PSA düzeyleri (p=0.001) arasında istatistiksel olarak anlamlı pozitif korelasyon bulundu. **Sonuç:** CCR-3 mRNA düzeylerinin PCa tanısında faydalı olabileceği sonucuna varıldı.

Anahtar Kelimeler: CCR-3, Prostat Kanseri, Gleason Prognostik Derecesi, Kemokin.

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INTRODUCTION

Prostate cancer (PCa) is the second most common type of cancer in men (Castillejos-Molina and Gabilondo-Navarro, 2016). The only way to confirm the diagnosis of the disease is by a prostate biopsy in patients with abnormal findings on digital rectal examination or high prostate-specific antigen (PSA) levels (Munjal and Leslie, 2021). Age and family history stand as the main risk factors for the disease. Androgen and estrogen hormones, metabolic syndrome, smoking and diet are the other risk factors (Bostwick et al., 2004). Although no causal relationship between chronic inflammatory process and PCa has been shown so far, it is still suggested as a risk for the development of the disease (Munjal and Leslie, 2021).

Chemokines are the factors secreted from the damaged or infected tissues to induce the inflammatory and immunological reactions. Approximately 50 different types of chemokines are reported in the literature. Recently, it has been shown that most cancer cells secrete chemokines (Balkwill, 2004). The effect of chemokines on cancer is highly complicated. While many chemokines show anti-tumor activity through the activation of the immune system cells or the suppression of the angiogenesis, others found to increase the cancer growth and metastasis by direct activation of the cancer cell proliferation, migration and angiogenesis (Araújo et al., 2020).

Chemokines create their effects by binding their specific receptors. Chemokine receptors have seven transmembrane domains and, of which 23 different types are currently known, are classified as typical or atypical chemokine receptors according to their G protein binding. Nineteen of them are G-protein bound and are classified as typical chemokine receptors. The remaining four are classified as atypical chemokine receptors because, although they have the same membrane structure as typical ones, they are not G protein bound. Consequently, they cannot induce classical intracellular signaling and act as scavengers targeting chemokines for lysosomal degradation (Legler and Thelen, 2018).

In tumor formation process, chemokines and their receptors are considered as critical actors. Both tumor proliferation and metastasis are facilitated by chemokines and their receptors. However, we do not know much about the role of neither chemokines nor their receptors in the formation and growth of malignant PCa. Recently, C-C chemokine receptor type 3 (CCR-3), which is a receptor for C-C type chemokines and binds to several of them; namely CCL4, CCL5, CCL7, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28, is found to be highly expressed in eosinophil and basophils, and has been shown to be expressed in melanoma, glioblastoma and kidney cancers, and has also shown to be expressed in PCa (Lee et al., 2010; Tian et al., 2016; Vela et al., 2015; Zhu et al., 2014).

The aim of this study is to determine the CCR-3 expression level in PCa and to compare it with its expression level in BPH molecularly and immunohistochemically in order to answer whether this molecule may be used in diagnosis and/or differential diagnosis of the PCa. Evaluation of the presence of any correlation between the CCR3 levels and prognostic Gleason classification in PCa is also aimed to investigate the predictive value of its expression level in prognosis of PCa patients.

MATERIALS AND METHODS

In this study, 25 PCa patients and 25 BPH patients were evaluated in terms of age, total PSA level and CCR-3 expression. CCR-3 expression levels of PCa patients in different Gleason prognostic grades were also compared.

Specimen selection and retrospective data collection

This study included 10% neutral buffered formaldehyde (10% NBF) fixed and paraffin embedded prostate specimens obtained from 50 patients, between January 2016 and December 2017.

Half of the patients (n=25) were diagnosed with PCa and the other half (n=25) with BPH by the examination of hematoxylene and eosin (H&E) stained slides of their surgical specimens. The data about the age, total PSA level, and Gleason prognostic grade of patients were collected from the patient files retrospectively. Gleason prognostic grades of the PCa patients were determined as described by Epstein et al. (2016a).

Immunohistochemistry

In order to determine CCR3 levels, IHC staining was applied to the 5 µm thick paraffin sections of the PCa and BPH patients. Briefly, after being hydrated and incubated in 3% hydrogen peroxidase (H2O2) for 15 minutes, sections were washed by phosphate buffered saline (PBS). In order to prevent nonspecific staining, blocking solution was applied to them. Next, sections were incubated by anti-CCR-3 antibody (1:100, ab36827, Abcam, Boston, USA) at 370 C for 1 hour. After washing away of the unbound primary antibody, secondary antibody (iVIEW DAB Detection Kit, 05266157001, Roche Diagnostics GmbH, Germany) was applied to the sections for 30 min. Fresh 3, 3'-diaminobenzidine (DAB) chromogen (GBI Labs, Mukilteo, WA, USA) was applied to the washed sections for 1-2 minutes. After the removal of the chromogen, nuclear counter stain, hematoxylene, was applied, and slides were dehydrated and cleared. Mounted slides were evaluated under light microscopy (Nikon Eclipse CI) by a blind pathologist who verified and scored the CCR3 IHC staining intensity of each sample by a scale from 0 to 3 (0; negative, 1; low, 2; moderate and 3; high staining intensity).

Real time PCR

After the completion of IHC staining, RNA was isolated from the paraffin embedded prostate tissues of the 25 PCa and 25 BPH patients. Additionally, paraffin blocks from 10 patients, that have shown totally normal prostate histology on H&E-stained sections, were also used for RNA isolation to constitute the control group of the real-time PCR experiments.

Total RNA of all samples was purified with the use of the RNeasy FFPE Kit (QIAGEN, Germany) as described by the manufacturer. After RNA isolation, cDNA synthesis was performed by Hyperscript First strand Synthesis kit (Cat no: 601-005, Lot no: FS015B04002, USA). The Real-Time PCR (GeneAllSybr Green Master Mix, Cat No: 801-520, Lot No: QP116G25001) was conducted by StepOnePlusTM Real-Time PCR System (Applied Biosystem).

Real-Time PCR was repeated at least three times for each sample. To trigger reactions, their own genes and actin beta (ACTB) were used. 7500 Fast Real-Time Sequence detection system Software (Applied Biosystems, Foster City, CA) was used to quantify the levels of gene expressions. The threshold cycle (Ct) was utilized to define gene expressions. In addition, ACTB was considered as a reference gene functioning as an internal reference to make the RNA expressions normal, measured as $2^{-\Delta\Delta CT}$. We present the primer sequences below:

ACTB (103 bp)

5'CCTGACTGACTACCTCATGAAGATCCTC3' (forward),5'CGTAGCACAGCTTCTCCTTAATGTCA C3' (reverse).

CCR-3 (180 bp)

5'GGTTTTATCACACAGGCTTG3'(forward), 5'AGCTCTTCCTGAATTTATCT3'(reverse)

Statistical Analyses

SPSS v.24.0 (SPSS Inc., Chicago, Illinois, USA) package program was used for statistical analysis of the data. Student t test and ANOVA were used to compare the continuous variables of PCa and BPH patients. Mann Whitney U and Kruskal Wallis tests were used when the data was not normally distributed. Bonferroni post hoc test was performed when necessary. The correlation analysis was performed by Spearman's rho test. p values less than 0.05 were considered as statistically significant.

RESULTS

The mean age of PCa patients was found as 64.88±4.52, while it was 66.12±6.19 in BPH patients. No significant difference in terms of age was found between them (p=0.423) (Table 1). While the mean total PSA level was 24.68±21.90 ng/mL in PCa patients, it was 7.23±8.86 ng/mL in BPA group. Total PSA level was significantly higher in PCa (p=0.001) (Table 1).

Table 1. Age and total PSA level of BPH and PCa patients.

Data	BPH		PCa		p
	M	SD	M	SD	
Age (year)	66.12	6.19	64.88	4.52	0.423
Total PSA level (ng/mL)	7.23	8.86	24.68	21.91	**0.001**

Student t test was used. **M:** Mean, **SD:** Standard deviation

IHC stained tissue sections from BPH patients (Figure 1a) showed weaker staining when compared with the sections from PCa patients (Figure 1b). The mean staining intensity scores of BPH and PCa were 1.48±0.65 and 1.80±0.87 respectively. Although staining intensity was found higher in PCa, the difference between them was not significant (p=0.205) (Figure 1c). Similarly, when the IHC staining intensities of the PCa patients in different Gleason prognostic grades were compared, the difference was again not significant (p=0.20, Figure 1d). The correlation between the IHC staining for CCR-3 and total PSA levels was also evaluated and a significant positive correlation was found demonstrating that CCR-3 expression goes up with increasing total PSA level (p=0.001) (Table 2). The other correlation analyzed was the one between the IHC staining for CCR-3 and Gleason prognostic grade of the patient, which was insignificant (p=0.375) (Table 2).

Table 2. Correlation between the IHC CCR-3 staining intensities and total PSA level and Gleason prognostic grade in PCa patients.

Correlation (CCR-3 expression)	Rho*	p
Total PSA	0.448	**0.001**
Gleason prognostic grade	0.185	0.375

*Spearmens Rho test was used.

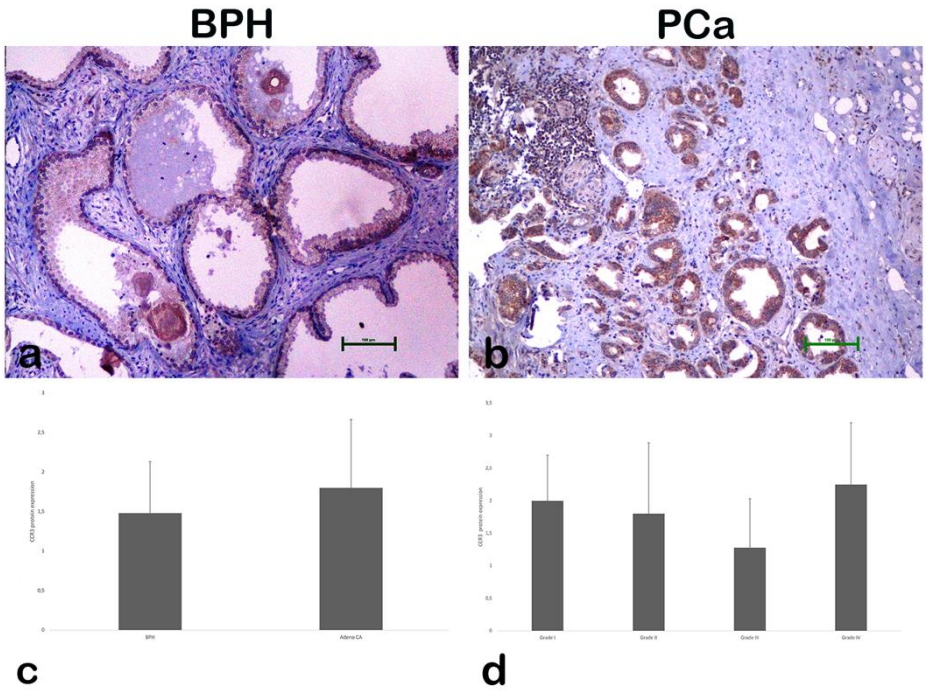

Figure 1. CCR-3 IHC staining of a) BPH tissue sample b) PCa tissue sample c) Comparison of IHC staining intensities of BPH and PCa groups d) Comparison of IHC staining intensities of PCa patients having different Gleason prognostic grades. Black and green bars in figures 1a and 1b represents 100 micrometre.

When mRNA levels of PCa patients were compared with BPA patients and control group, CCR-3 was found to be significantly higher in PCa patients (p=0.001) (Figure 2a). Comparison of the CCR-3 mRNA levels obtained from the PCa tissues of patients in different Gleason prognostic grade groups showed that the difference between Gleason scoring and CCR-3 mRNA levels of patients in different groups was insignificant (Fig 2b). This situation made us think that CCR-3 expression level could not contribute to determining the prognosis of the patient.

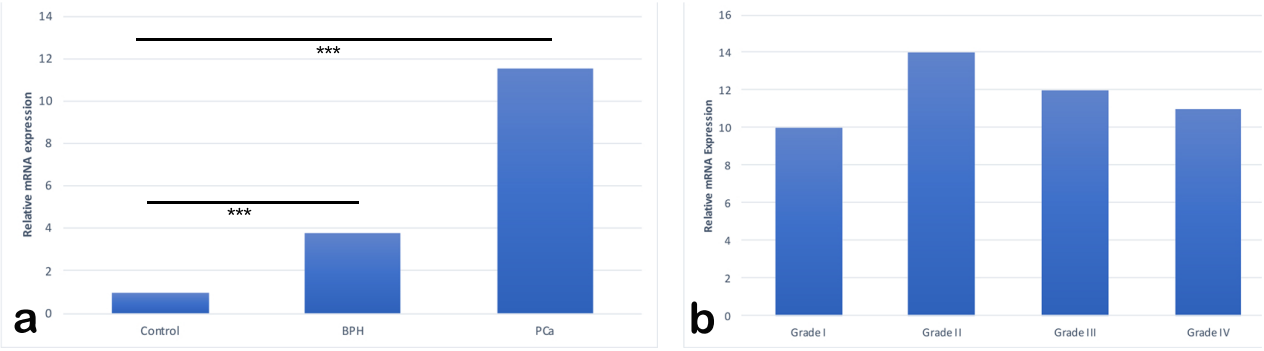

Figure 2. a) Comparison of CCR-3 mRNA levels measured by qRT-PCR and normalized to ACTB levels in BPH and PCa patients, b) Comparison of CCR-3 mRNA levels measured by qRT-PCR and normalized to ACTB levels in different Gleason prognostic grades.

DISCUSSION

In this study, the diagnostic and prognostic importance of CCR-3 expression in PCa patients was investigated. CCR-3 expression levels were determined and compared with prognostic parameters such as Gleason prognostic grade and total PSA levels in PCa patients. CCR-3 expression at mRNA

level was significantly higher in PCa patients. There was no relationship between Gleason prognostic grade and CCR-3 expression. The high total PSA level was found to be correlated with the increased CCR3 expression. These findings suggest that CCR-3 expression may contribute to the diagnosis of PCa.

Chemokines, which are peptide signaling cytokines, are known to guide cell migration. Inflammation, infection, tissue repair, cancer progression, and organogenesis of lymph nodes are the other examples of important physiological and pathological events in which chemokines have been shown to play a role (Guan, 2015).

Laurent et al., in their study published in 2016; demonstrated that CCR3 was expressed in PCa and was not expressed in normal prostate epithelium, with the IHC. They also showed that there is a correlation between the CCR3 level and the Gleason score. In our study, we examined CCR3 expression not only with IHC methods but also with qPCR. Again, in addition to normal and PCa tissues, we included samples of BPH in our study. We showed that there is CCR3 expression in the prostate gland in BPH. This expression was significantly less at the mRNA level than in PCa. Contrary to Laurent et al. (2016), we could not reveal a relationship between CCR3 levels and Gleason score in our study. We think that this may be due to the small number of our subjects. Laurent et al. (2016) showed that in prostate cancer, expression of the CCR3 receptor is associated with the occurrence of aggressive disease with local dissemination and a higher risk of biochemical recurrence (Laurent et al., 2016).

For cancer to spread, cancer cells must transform normal stromal cells, thereby shifting the microenvironment to a metastasis-supporting state (Alizadeh et al., 2014). Zhu et al. (2014) showed that eotaxin-1 promotes prostate cancer cell invasion and migration, as a result of increasing MMP-3 expression via the CCR-3-ERK pathway. In the same study, they also showed that mesenchymal stromal cells increase the invasive potential of PCa cells via increased eotaxin-3/CCR-3 expression (Zhu et al., 2014). Other studies have also proven that, there are some other cells in which CCR3 activates the ERK pathway such as smooth muscle cells and large cell lymphoma cells (Markwick et al, 2012, Miyagaki et al., 2011). The importance of the ERK pathway in proliferation, invasion and metastasis, which are important stages of tumor progression, is well known (Reddy et al., 2003).

In 2014, a new terminology was adopted by the World Health Organization (WHO) for the urinary tract tumors and male genital organs through using Gleason prognostic grade, a new grading system, between grades 1-5 (Mottet et al., 2017). The current modified Gleason classification, which forms the basis of new classification of groups, has little similarity to the original Gleason system. With this new classification, it is aimed to correct the accurate classification of PCa patients and to reduce the excessive treatment of cancer (Epstein et al., 2016b). Gleason prognostic grade was associated with the risk of death and metastasis of PCa in terms of tumor management strategies including definitive treatment, conservative treatment and androgen blockade (Leapman et al., 2017). In our study, there was no significant relationship between CCR-3 expression and Gleason prognostic grade which is important for prognostic and treatment management.

It is known that leukocyte infiltration occurs in many tumors. Although the degree of inflammation may vary, it exists in almost every tumor (Coussens & Werb, 2002). Even if the inflammatory reaction is considered to be protective, the inflammatory cells may also show tumor-promoting activity by producing growth factors or by causing additional DNA damage (Zhao et. al., 2021). For example, cyclooxygenase-2 (COX-2) enzyme, which is responsible for the conversion of arachidonic acid to prostaglandins, is produced by inflammatory stimuli and this enzyme is high in colon cancer and other cancers (Kumar et. al., 2014). Acetylsalicylic acid (ASA), a nonsteroidal anti-inflammatory drug, has been shown to inhibit COX-2, thereby reducing the release of prostaglandins responsible for different forms of tissue damage. With this effect, ASA prevents the formation of tissue and/or cell damage in many diseases with similar antioxidant properties. Clinical and preclinical studies show that ASA can reduce oxidative damage and have a role in cancer prevention (Xu et al., 2012; Demirel and Kılçıksız, 2011). Perhaps a possible link between CCR3 and COX2 pathways may be a new topic for future studies.

Life-style changes such as smoking cessation, weight loss and regular exercise are recommended for males with prostate cancer. However, recent studies have suggested that regular use of aspirin may prevent the development of prostate cancer (Cuzick et al., 2014). CCR-3 expression in PCa tumors has been reported to have potential benefits in terms of preventing the local spread, high biochemical recurrence risk and the emergence of aggressive disease (Laurent et al., 2016). In their study, Lee et al. (2010) demonstrated that CCR-3 was more expressed in malignant melanoma. It is also claimed that the presence of CCR-3 protein may increase the aggressive potential of malignant cutaneous tumors and may facilitate proliferative effect in tumor cells. In another study, it has been shown that CCR-3 and its ligand eotaxin/CCL11 expression play a role in the spreading and binding of CD30+ malignant T cells (Kleinhans et al., 2003). On the other hand, in a study investigating CD4+ (TH1 and TH2) cell groups in the blood of gastric cancer patients in terms of chemokine receptor expression, it was stated that CCR5 in TH1 and CCR3 in TH2 were extremely limited for clinical evaluation due to the low CCR3 expression (Andalib et al., 2013). There was a significant difference in

terms of CCR-3 expression between BPH and PCa patients in our study. CCR-3 expression was significantly increased in PCa patients.

The study has some limitations. First of all, the number of subjects is small, and we think that this is the main reason why significant differences when CCR3 expression was examined at the mRNA level were not observed at the protein level. In addition, we think that another limitation is not questioning the chronic pathologies of the subjects such as obesity, hypertension and diabetes. In summary, we think that it is necessary to conduct new studies that include more subjects and examine the comorbidities of the subjects. Again, we believe that it will be meaningful to evaluate serum CCR3 levels in new studies to be planned.

CONCLUSION

In conclusion, CCR-3, a member of the chemokine family, was found to have increased expression in PCa patients. No significant CCR-3 effect was detected in terms of Gleason prognostic grade which has a role in predicting tumor morphology and prognosis. On the other hand, increased CCR-3 expression which is correlation to increased total PSA is thought to be used to identify potential therapeutic strategies and to predict conditions favorable to tumor and secondary to inflammation.

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Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

Author Contributions

Plan, design: ASA, EA; **Material, methods and data collection:** ASA, EA, **Data analysis and comments:** ASA, EA, FB; **Writing and corrections:** ASA, EA, FBS.

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