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Shi Deng

Sichuan University West China Hospital

Yin sheng He

Sichuan University West China Hospital

pan zhao

Sichuan University West China Hospital

Peng Zhang (✉ zhangpenghuaxi@163.com)

Sichuan University West China Hospital

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Title page

The role of resistin gene polymorphisms in bladder cancer

Shi Deng¹, Sheng yin He², Pan Zhao³, Peng Zhang⁴

¹ Department of Urology, institute of Urology, West China Hospital, Sichuan University, Chengdu 610041, PR China, E-mail: dengshidiy@163.com

² Department of Urology, institute of Urology, West China Hospital, Sichuan University, Chengdu 610041, PR China, E-mail: shengyinhehuaxi@163.com

³ Department of Urology, institute of Urology, West China Hospital, Sichuan University, Chengdu 610041, PR China, E-mail: zhaopanhuaxi@163.com

⁴ Department of Urology, institute of Urology, West China Hospital, Sichuan University, Chengdu 610041, PR China, E-mail: zhangpenghuaxi@163.com

Correspondence to: Professor Peng Zhang, Department of Urology, West China Hospital, Sichuan University, 37# Guoxuexiang Street, Chengdu, China 610041, E-mail: zhangpenghuaxi@163.com

Telephone number: +86-18980605795

Fax: fax: +86-28-85422451

Abstract

Background: Published studies have demonstrated that resistin, a recently discovered adipokine, is connected to insulin resistance, type 2 diabetes mellitus, obesity, inflammation, and atherosclerotic vascular disease. A comprehensive study of the adipocytokine family and tumor pathogenesis indicates an intimate relationship between resistin and the incidence, progression, and metastasis of gastric cancer, esophageal cancer, choriocarcinoma, colorectal cancer, pancreatic cancer, and biliary tract cancer. To date, the connection between resistin and bladder cancer has not been thoroughly investigated and remains unclear.

Methods: Overall, 322 patients with bladder cancer and 366 normal controls were included in the study. Two SNPs of the resistin gene, rs1862513 (also known as -420 C/G) and rs10401670 (3'UTR C/T) were genotyped across the entire cohort. Next, the association between the two SNPs and the incidence, risk factors, and prognosis of bladder cancer, were analyzed.

Results: The frequency of T allele and CT/TT genotype of rs10401670 was significantly lower in bladder cancer patients ($P=0.03$, $OR=0.79$ and $P = 0.018$, $OR = 0.68$, respectively) compared to normal controls. No differences were found with regards to the rs1862513 genotype frequency and the distribution of allele frequency between the two groups. Stratified analyses showed that the CT heterozygous genotype of rs10401670 was associated with bladder cancer at an earlier age ($OR=1.97$, $95\% CI=1.14-3.40$) and the CG heterozygous genotype of rs1862513 was correlated with high incidence of bladder cancer in smokers ($OR=1.73$, $95\% CI=1.05-2.87$). Multiple Cox regression analysis showed that for bladder cancer patients, the presence of a CG heterozygous genotype of rs1862513 was associated with a decrease in the risk of recurrence in MIBC patients ($P = 0.04$, $OR= 0.49$). Additionally, the rs1040167 CT/TT genotype ($P = 0.03$, $OR= 2.45$), especially the TT homozygous genotype ($P = 0.02$, $OR= 3.00$) was associated with high risk of death. These results indicate that the rs1040167 CT/TT and TT homozygous genotype may be a risk factor for overall survival of bladder cancer patients.

Conclusions: Our results suggest that resistin genotype serves as a risk factors for the

occurrence and prognosis of bladder cancer, and could be be a potential biomarker for this devastating disease.

Keywords: Bladder cancer, resistin, Polymorphisms, prognosis

Introduction

Bladder cancer is a common malignancy that seriously affects the survival and quality of life of those that are affected. As per the World Health Organization in 2012, bladder cancer is the ninth most frequently diagnosed malignant tumor and the thirteenth most common cause of cancer-related death around the world. Every year, approximately 430,000 new patients are diagnosed with bladder cancer and 165,000 die due to the disease [1]. In the United States, there will be approximately 80,470 new diagnoses and 17,670 deaths related to bladder cancer in 2019 [2]. In China, roughly 78,100 people developed bladder cancer in 2014, which accounted for 2.05% of all malignant tumors[3]. The TNM staging of bladder cancer indicates the disease can be split into two entities, non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC) [4]. At time initial diagnosis, nearly 75% of new cases belong to NMIBC, while the remaining 25% are MIBC[5]. After the combined treatment of transurethral resection of bladder tumor (TURBT) and perfusion chemotherapy, most individuals with NMIBC have a favorable prognosis. However, approximately 10% of all patients progress to MIBC and/or distant metastasis[6]. The five-year survival rate for MIBC patients is only 69% and rapidly drops to 6% in metastatic patients[5]. Early diagnosis and treatment can significantly improve the survival of malignant tumors. It has been reported that the five-year survival for earlier diagnosis and treatment of early-stage bladder cancer was three times higher than that of patients diagnosed at an advanced stage[7]. At present, various methods such as cystoscopy, bladder imaging and urinary cytology are used for diagnosis of bladder cancer, and many of them are able to achieve an accurate diagnosis. However, so far, none of these methods can predict the outcome of bladder cancer treatment.

The cause of bladder cancer is complex and may be closely related to many risk factors, such as genetic mutations, lifestyle, and environmental and chemical exposures. Despite the fact that many people are exposed to the high-risk factors, only a small percentage of them will eventually develop bladder cancer. These results

suggest that genetic differences may be the main factor associated with occurrence of disease [8]. Nowadays, scientists are increasingly starting to recognize that genetics have a critical function in the pathogenesis of bladder cancer, particularly genes such as *CD44*, *CRCC1*, and *PDCD6* [9-11]. The introduction of genetic sequencing might help predict the subset of patients that will develop bladder cancer, as well as their prognosis.

Resistin, an adipokine that consists of 108 amino acids with a molecular weight of 12.5 kDa, is mainly secreted by monocytes and macrophages[12]. Since the discovery of resistin in 2001, research has focused on its role in obesity, lipid metabolism, and diabetes [13-15]. Latest studies indicate that resistin functions in tumor development across multiple systems. Nakajima *et al.* found that, compared to normal controls, serum resistin levels in colon cancer patients were increased, especially in female patients. Additionally, serum resistin levels were also associated with colon cancer stage [16]. In breast cancer, researchers observed that high resistin levels were associated with increased malignant biological behavior and decreased survival rate, which suggests that resistin is not only associated with the incidence but also prognosis of the disease [17]. A similar phenomenon was observed in endometrial cancer as higher serum resistin levels were associated with increased morbidity [18]. Resistin is encoded by the *RETN* gene on chromosome 19p3.2, which consists of four exons and three introns. Some gene polymorphisms have been found in the promoter region, introns, and 3' untranslated regions of the *RETN* gene. The genetic polymorphisms also vary across different ethnicities[15,19-21]. Studies suggest that multiple SNPs of the *RETN* gene are closely associated with GDM, T2D, obesity, colon cancer, polycystic ovary syndrome, and other diseases. Additionally, resistin and related SNPs are associated with multiple biological behaviors and multi-systemic diseases [21-24]. However, as far as we know, the link between *RNTN* SNPs and the incidence, development, and prognosis of bladder cancer have not been indicated by previous studies.

Materials and Methods

Study subjects

Overall, 322 bladder cancer patients at the West China Hospital of Sichuan University from March 2009 to July 2016 were recruited for this study. All patients, who administered surgical treatment without neoadjuvant therapy, were validated as bladder cancer by the pathology department at West China Hospital. Follow-up of all participants was successfully conducted by telephone, letters or outpatient system to evaluate their postoperative status. The follow-up period started from the day of the pathology report to either July 2016, when the patient developed metastasis or suffered from tumor-related death. The control population represented the general population, and were recruited from the health examination center at the hospital. Overall, 366 normal individuals who had no tumor history, structural heart disease, liver or kidney disease, diabetes, or immune system disease, were included as the control group. All participants in both groups were Han residents. This study was granted approval by the West China Hospital ethics committee, and each individual provided their informed consent.

DNA isolation and genotyping

This study investigated genotyping of two SNPs: rs1862513 (−420 C/G) and rs10401670 (3'UTR C/T). Individual genomic DNA was extricated from EDTA-treated peripheral blood samples (200 µL) through the use of DNA isolation kit by Biotek (Peking, China).

RETN polymorphism genotyping was conducted using the TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA). Assay IDs C__1394112_10 and C__1394125_10 were used for rs1862513 and rs10401670, respectively. Once the real-time PCR was completed, SNPs were marked with a fluorescent VIC dye to mark the allele C probe of rs1862513 and the allele C probe of rs10401670, whereas the rest were labelled with fluorescent FAM dye. Real-time PCR utilizing the TaqMan probe was conducted as per manufacturer's guidelines. Methods for real-time PCR included 95°C for 10 min, then 45 cycles at 92°C for 15

seconds, and 1 min at 60°C. Repeated assays were performed in approximately 10% of randomly selected samples. All results showed 100 % concordance.

Statistical Analysis

All statistics were carried out with SPSS 23.0. The incidences of the two tag SNP genotypes were determined by direct counting. Hardy–Weinberg equilibrium was verified by a X^2 test. Odds ratio (OR), and the matching 95% confidence interval (95% CI), were used to analyze the differences in allele and frequency. The genotypic variation was analyzed by genetic models in the SNP Statistics. $P < 0.05$ represented statistical significance. Univariate analysis was conducted for sex, age, smoking status, stage of tumor and grading, and genotypes and prognostic factors. Cox regression analysis was performed through the Hazard ratios (HR) and 95 % CIs.

Results

SNP allele frequency and genotypes of patients with and without bladder cancer

Genotyping of the two SNPs were performed in 322 patients and 366 normal controls. The genotypes of each SNP were identified using DNA sequencing analysis. The genotype frequencies of all participants were similar to those predicted by the Hardy-Weinberg equilibrium. Table 1 describes the allele frequencies and genotype distribution of resistin SNPs in patients and normal controls. The genotype frequency distribution of rs10401670 in both groups showed significant differences (CT/TT genotype: 60.2% for patients vs. 68.8% for normal control subjects, $P = 0.018$, OR = 0.68, 95 % CI = 0.50-0.93 in the dominant genetic model). The allele T in rs10401670 showed significantly lower frequency in bladder cancer patients (37.1%) compared to controls (42.9%) ($P=0.03$, OR=0.79, 95 % CI =0.63–0.98). For rs1862513, there were no significant differences among both groups for genotype frequencies and distribution of allele frequency.

Resistin marks SNPs and patient features

The effect of resistin SNPs on the occurrence of bladder cancer were investigated by the method of stratified analyses. Based on patient demographics such as smoking, gender, and tumor grade, genotype distributions of resistin tag SNPs were stratified in both the bladder cancer and control group. Results are summarized in Table 2. For rs10401670, CT heterozygous subjects are associated with a higher likelihood of suffering from bladder cancer at an early age (OR=1.97, 95 % CI=1.14–3.40). For rs1862513, smoking combined with CG heterozygous genotype increases the likelihood of bladder cancer compared to controls (OR=1.73, 95 % CI=1.05–2.87).

Resistin tag SNPs and patients' outcome

Univariate analysis showed that rs10401670 and rs1862513 were not associated with overall or progression-free survival of bladder cancer. However, Cox regression analysis showed different results (Table 3). In MIBC patients, the rs1862513

genotype was associated with recurrence during the postoperative follow-up. The recurrence of bladder cancer in patients with CG heterozygous genotype for the rs1862513 locus was significantly lower compared to CC/GC homozygote patients ($P = 0.04$, $OR = 0.49$, $95\% CI = 0.25-0.98$). Nevertheless, overall survival of MIBC patients was associated with rs10401670, but recurrence-free survival was not. Compared to CC homozygous patients, patients with allele T (CT/TT genotype) showed a high risk of death ($P = 0.03$, $OR = 2.45$, $95\% CI = 1.12-5.36$). In the recessive model, the risk was increased. The TT homozygous genotype patients were more likely to die compared to the CC/CT genotype ($P = 0.02$, $OR = 3.00$, $95\% CI = 1.17-7.74$).

Discussion

Gene polymorphism is a discontinuous genetic variation that are present across individuals of different forms or types within a single species. It may be an adaptive performance in the evolutionary process. For humans, it may lead to individuals having different clinical manifestations of the same disease, and have different responses to similar treatments. Several previous studies have demonstrated that gene polymorphisms, such as PDCD6, XRCC1, and CD44, have a close association with the occurrence and development of bladder cancer [9-11].

We discovered that allele T and CT/TT genotype of rs10401670 is related to decreased incidence of bladder cancer. In addition, the CT heterozygous genotype of rs10401670 was associated with developing bladder cancer at an earlier age and CG heterozygous genotype of rs1862513 increases the risk of developing bladder cancer in smokers. Further studies revealed that in MIBC, the GC genotype of rs1862513 may play a prognostic role in recurrence and the CT/TT genotype of rs10401670, especially the TT homozygote, may serve as a predictor of death. As far as we know, the relationship between resistin and bladder cancer has not previously reported.

Resistin may mediate tumor initiation and progression in a variety of ways. Studies have shown that resistin activates NF- κ B signaling, which regulates the expression of TNF- α and IL-12 [25]. Song *et al.* confirmed that resistin activates the PI3K/Akt pathway, and upregulates VEGF and matrix metalloproteinase via the MAPK (ERK1/2 and p38) pathway, followed by neoangiogenesis [26]. Studies involving pancreatic cancer and choriocarcinoma also suggest that resistin promotes VEGF synthesis, induces epithelial cell proliferation and neoangiogenesis, upregulates the expression of matrix metalloproteinase, reduces the synthesis of tissue metal proteinase inhibitors, and increases tumor invasiveness[27,28]. As progression of bladder cancer is associated with poor prognosis, early diagnosis of this malignant disease is critical [7]. The current clinical diagnosis of bladder cancer largely depends on imaging examination, cystoscopy or cytology. However, as cystoscopy is an invasive approach, it is not acceptable to many patients [29], and urine cytology is not

sensitive to bladder cancer detection, particularly for low-grade tumor [30]. Thus, other new methods are needed for early diagnosis and surveillance post-surgery. *MCEMPI* gene encodes a single transmembrane domain protein that is mainly expressed by monocytes and mast cells. The second intron of this gene is where the SNP rs10401670 is located. [15]. The 3'UTR C/T (rs10401670) is a rare resistin SNP.

In fact, to date, only two reports of resistin rs10401670 have been available. Hivert *et al.* found that the SNP rs10401670 was associated with serum resistin and fasting plasma glucose levels [15]. Similarly, a study of 1269 children by Ortega *et al.* revealed that the SNP rs10401670 was not only associated with resistin levels, but also related to TC and low-density lipoprotein[14]. These results reveal that rs10401670 is closely related to human metabolism. In our study, we found that the proportion of rs10401670 T allele (P = 0.03, OR = 0.79, 95% CI = 0.63-0.98) and CT/TT genotype (P = 0.018, OR = 0.68, 95% CI=0.50-0.93) in the controls was substantially higher in comparison to patients, suggesting that the rs10401670 T allele and CT/TT genotype could decrease the chance of developing bladder cancer.

The risk of developing bladder cancer generally increases with age, reaching a peak at approximately 70 years, and rarely develops before the age of [31]. Wan *et al.* found that, when compared to elderly patients, the clinical stage and tumor grade was generally low in younger patients [32]. Gupta *et al.* also confirmed that young patients show good prognosis following TURBT and postoperative intravesical chemotherapy [33]. Epidemiological data suggests that the frequency of bladder cancer in males was higher compared to females and may be associated with a harmful lifestyle. However, contrasting studies found that the incidence of bladder cancer in older females was higher than males, which may be associated with changes in hormonal levels [34,35]. Therefore, age has a significant function in the onset and prognosis of bladder cancer. Our data indicates that the proportion of bladder cancer patients with the rs10401670 CT heterozygous genotype before the age of 70 was 1.79 times higher compared to patients over 70 years of age. This indicates that the population carrying the rs10401670 CT heterozygous genotype may heighten the chance of suffering from bladder cancer at an early age. Therefore, early examination of bladder cancer,

particularly in postmenopausal women, should help improve the detection rate and decrease the chance of suffering from bladder cancer. Additionally, smoking is an important environmental risk factor in the occurrence of bladder cancer [36]. It is estimated that approximately one-third of all females and 50% of all males diagnosed with bladder cancer in Europe are smokers [37]. Although the incidence of bladder cancer decreases after smoking cessation, the incidence is still significantly higher compared to non-smokers. Zeegers *et al.* demonstrated that frequency of bladder cancer in smokers was nearly 2.57 times higher than non-smokers, and after smoking cessation, the frequency of bladder cancer was still 1.73 times higher than non-smokers[36]. Compared to non-smokers, smokers afflicted with bladder cancer manifest clinically unfavorable pathological features including earlier onset, higher invasive behaviour, larger tumor size, and higher tumor grade [38,39]. We found that the possibility of bladder cancer was 1.73 times increased in smokers with the CG heterozygous genotype of rs1862513 compared to non-smokers, suggesting that patients with this specific genotype should limit their exposure to tobacco.

According to the reports published by American Institute of Cancer Research and the World Cancer Research Fund (<http://www.aicr.org/learn-more-aboutcancer/infographic-obesity-and-cancer.html>), approximately one-third of Americans are obese, and about 120,400 patients are diagnosed with various types of obesity-related cancers each year[40]. After reviewing 31 case studies that associate obesity with bladder cancer, Noguchi found that obesity increased the risk, progression, relapse and mortality of bladder cancer [41]. Obesity increases fatty acid levels and circulating immune cells, leading to chronic inflammation, thus contributing to tumor cell proliferation, invasiveness, and therapeutic resistance [42,43]. As an adipokine, resistin is closely associated with obesity. Moreover, resistin mediates multiple inflammatory and tumor responses [44]. Signal transducer and activator of transcription 3 (STAT3), a transcription factor, is present in an active state of malignant tumors of multiple systems, and are closely related to tumor cell growth, apoptosis, and drug sensitivity. Deshmukh *et al.* found that resistin promotes growth and invasion of breast cancer cells by directly and indirectly increasing STAT3 or

IL-6[43]. Ezrin is a member of the protein family of ezrin, radixin and moesin. It regulates a variety of key cellular functions, such as cell morphology, adhesion, cell division, and transmembrane signal transduction pathways, and is closely involved in tumor metastasis[45-47]. Lee found that resistin influences ezrin by increasing the intracellular calcium, phosphorylation of protein phosphatase 2A (PP2A), and phosphorylation of PKC α , thereby promoting distant metastasis of breast cancer[48]. Thus, resistin is involved in tumor metabolism in a variety of ways, and increases tumor malignancy and adversely affects survival. Our results reveal that in MIBC patients, rs1862513CG heterozygous genotype might reduce the risk of recurrence, while T alleles of rs10401670, especially TT homozygote, may increase the risk of death. Thus, our results suggest that rs1862513 and rs10401670 SNPs of resistin have a vital function in prognosis and metastasis of bladder tumor patients. However, the detailed molecular mechanisms have yet to be elucidated.

Conclusions

In summary, to our knowledge, we are the first to report a relationship between resistin gene polymorphism and bladder cancer. Our findings indicate that rs10401670 is correlated with likelihood of bladder cancer. Additionally, rs10401670 and rs1862513 are associated with general pathological risk factors in patients, for example, age and smoking. Further analysis suggests that the SNPs rs10401670 and rs1862513 can be used as risk factors for prognosis of bladder cancer patients. Unfortunately, this study does have some limitations. First of all, multiple genes are potentially associated with incidence, advancement, and prognosis of bladder cancer and our analysis only assessed the role of resistin. Second, bladder malignancy caused by genetic polymorphisms might vary from race to race, and the participants in our study were only Han residents. Therefore, our results might not be applicable to other races. Furthermore, the sample size in our study was limited. Large sample studies are needed to confirm the results in the future.

List of abbreviations

NMIBC: non-muscle-invasive bladder cancer **MIBC**: muscle-invasive bladder cancer

TURBT: transurethral resection of bladder tumor **STAT3**: signal transducer and activator of transcription 3 **PP2A**: phosphorylation of protein phosphatase 2A

Declarations**Availability of data and materials**

The data used in the current study are not shared, please contact author for data requests.

Authors' Contribution

SD gathered patients' information, and drafted the manuscript. **SYH** carried out the genotyping of these SNPs. **PZ** performed the statistical analysis. **PZ** conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was performed in compliance with the principles of good clinical practice outlined in the Declaration of Helsinki and federal guidelines, and approval from the West China Hospital Ethics Committee was acquired. Informed consent was obtained from each participant in the study.

Consent for publication

Consent for publication was obtained from every individual whose data are included in this manuscript.

Competing interests

The authors declare that they have no competing interests.

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