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Genetic variant of *MAML2* in the *NOTCH* signaling pathway and the risk of bladder cancer

A STROBE-compliant study

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Abstract

The NOTCH signaling pathway plays a crucial role in cell phenotype and transformation. Single nucleotide polymorphisms (SNPs) may regulate gene expression to trigger bladder cancer susceptibility. Here, we aimed to explore the relationships between genetic variants in the NOTCH pathway and bladder cancer progression.

We screened SNPs located in NOTCH pathway genes using the 1000 Genomes Project dataset (CHB). A case-control cohort study including 580 bladder cancer cases and 1101 controls was conducted to genotype the candidate SNPs. The expression quantitative trait locus (eQTL) and bioinformatics analyses were performed to explore the biological function of the SNPs' host gene and their relationship. Kaplan–Meier analysis was performed to assess the association between host gene expression and bladder cancer patient prognosis.

The rs7944701 in the intron of mastermind-like 2 (*MAML2*) had the strongest signal and was related to bladder cancer risk (OR = 1.329, 95% CI = 1.115–1.583, $P = .001$). eQTL analysis showed that rs7944701 with a C allele was negatively associated with mastermind-like 2 (*MAML2*) expression (TT versus TC/CC). Bioinformatics analysis indicated that *MAML2*expression was lower in bladder cancer tissues than in non-tumor tissues ($P = 5.46 \times 10^{-3}$). Additionally, bladder cancer patients with high *MAML2* expression had a significantly poorer prognosis (HR = 1.53, 95% CI = 1.29–1.82, $P = .010$).

The rs7944701 in *MAML2* was strongly associated with bladder cancer susceptibility in a Chinese population. This genetic variant and its host gene could be a potential novel biomarker for individuals suffering from bladder cancer.

Abbreviations: BLCA = bladder urothelial carcinoma, CI = confidence interval, eQTL = expression quantitative trait locus, GEO = Gene Expression Omnibus, HR = hazard ratio, HWE = Hardy-Weinberg equilibrium, KEGG = Kyoto Encyclopedia of Genes and Genomes, LD = linkage disequilibrium, MAF = minor allele frequency, MAML2 = mastermind-like 2, OR = odds ratio, SD = standard deviation, SNP = single nucleotide polymorphisms.

Keywords: bladder cancer, genetic variant, molecular epidemiology, NOTCH pathway, single nucleotide polymorphisms

1. Introduction

Bladder cancer remains one of the most common malignant cancers in the world, and it has high mortality and recurrence rates. In China, there are currently over 80,500 patients diagnosed with bladder cancer. Although the management of bladder cancer treatment has improved, the incidence and mortality still have not decreased in recent years.[1] Approximately 25% of diagnosed bladder cancer patients presenting with muscle invasion have poor outcomes despite radical surgery and chemotherapy and even radiotherapy.[2] As a previous study reported, risk factors for bladder cancer are complex and include acquired carcinogen exposure (such as tobacco smoking and occupational factors) and genetic variants.[3] Although cystoscopy remains a gold standard for bladder cancer diagnosis, its wide application is limited because of its invasiveness and high cost. In several years, plenty of methods and markers have been discovered and been attempted to predict and diagnose bladder cancer, such as routine systemic inflammatory markers,[4] neutrophil-to-lymphocyte ratio,[5] circulating tumor cells,[6] non-coding RNAs[7] and Glasgow Prognostic Score.[8] Therefore, a convenient way to early diagnose and manage bladder cancer and even recurrence after surgery is urgently needed.

The NOTCH pathway plays a leading role in cell fate.[9] It also has been studied in different malignant diseases and the Notch inhibitors possess anti-proliferative effects on cancer, thereby serving as a new treatment for cancer.[10] The NOTCH pathway itself seems to have a tumor suppressive function and inactivation

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promotes bladder cancer progression.[11] Disorder of the NOTCH pathway promotes cancer stem cell phenotype and epithelial-mesenchymal transition, thus increasing chemoresistance.[12] All of these factors provide a survival advantage for tumors and contribute to enhanced malignancy and a poor prognosis for patients. However, the genetic mechanism of how the NOTCH pathway is involved in bladder cancer remains unclear.

Single nucleotide polymorphisms (SNPs), as common genetic variations, occur in human evolution. Because of their changes in the structure of the genes, the biological functions of these genes will be influenced. Multiple studies demonstrate that many functional SNPs are related to the risks of bladder cancer and regulate genetic functions in pathways.[13–15] To the best of our knowledge, a systematic understanding of how SNPs implicated in the NOTCH signaling pathway contribute to bladder cancer development is still lacking. The aim of this study was to explore the underlying function and relationship of SNPs located in the NOTCH signaling pathway in bladder cancer.

2. Materials and methods

2.1. Study subjects

Firstly, the subject characteristics (580 cases and 1101 cancer-free controls) were updated according to previous association studies.[16,17] Briefly, a total of 580 bladder cancer patients were enrolled in Nanjing who was diagnosed by at least 2 pathologists. Meanwhile, local urologists collected the particular clinical information of each case. The 2004 WHO/ISUP classification of bladder cancer was used to confirm the tumor grade and stage. Additionally, 1101 controls were matched to the cases based on gender and age (±5 years). There was no genetical relationship between cases and controls. Besides, there were other 37 bladder cancer cases collected for the associated research. These 37 cases were also diagnosed by 2 pathologists and matched to the 580 cases population. The Institutional Review Board of the Affiliated Hospital of Nanjing University of Chinese Medicine approved this study.

2.2. SNP screening

The genes included in the NOTCH pathway were found using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps (www.kegg.jp/kegg/pathway.html). KEGG is an online database resource that helps researchers to explore mechanisms and functions in biological system research.

The schematic flow for selecting the candidate SNPs is shown in Figure 1. First, there were 36,960 SNPs selected in gene regions (including 2 kb upstream) based on the 1000 Genomes Project

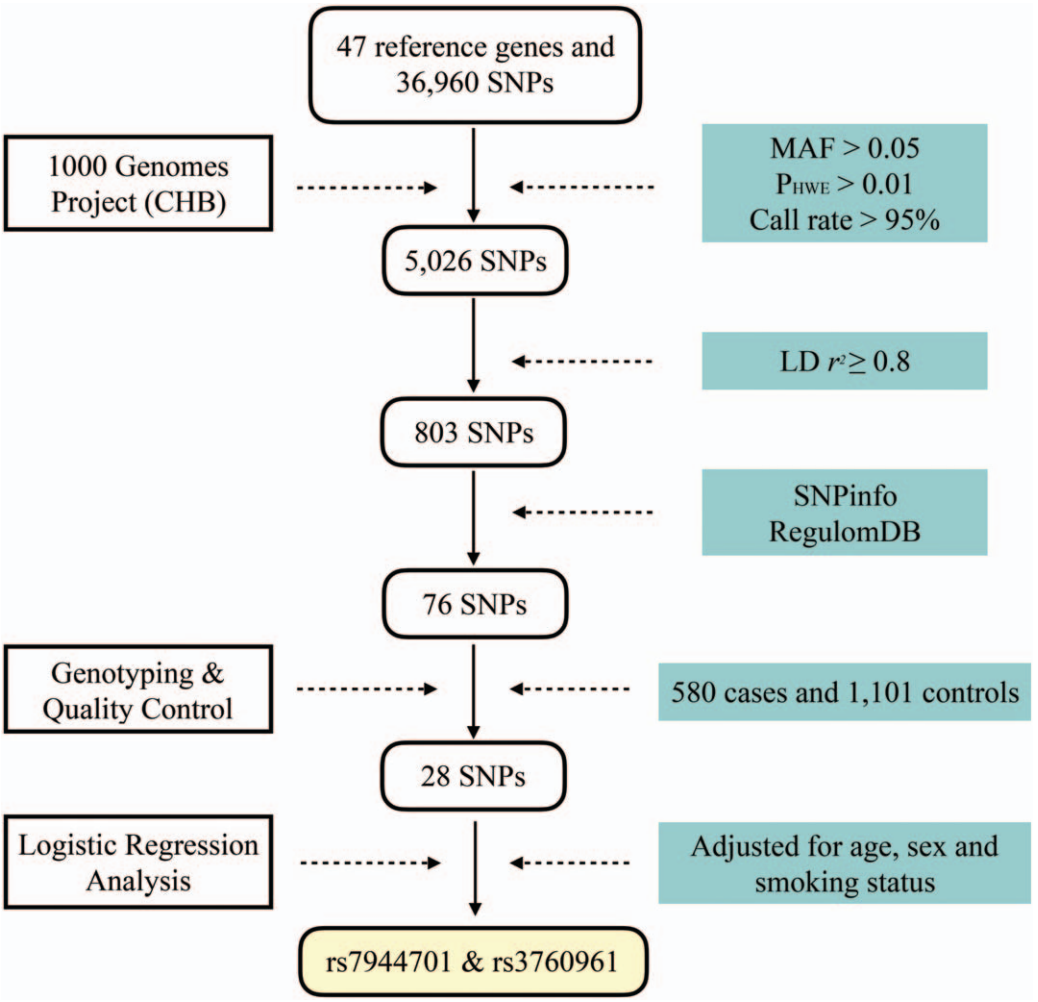

Figure 1. Flow chart for selecting single nucleotide polymorphisms in NOTCH pathway genes.

Chinese in Beijing (CHB) data (March 2012). Subsequently, a total of 5026 SNPs were extracted after quality control procedures were applied using the following criteria:

(1) minor allele frequency (MAF) ≥0.05,
(2) call rate >95%, and

Hardy-Weinberg equilibrium (HWE) ≥0.01.

Second, CLUMP analysis was applied to filter the candidate SNPs with linkage disequilibrium (LD) $r^2 \geq 0.8$. Otherwise, we used RegulomeDB and SNPinfo Web Server to predict the SNP function. SNPinfo Web Server (https://snpinfo.niehs.nih.gov) is a set of web-based tools to predict the functional characteristics of SNPs. RegulomeDB (http://www.regulomedb.org/) is a database based on public datasets that is used to annotate SNPs and predict regulatory elements. Additionally, we retained SNPs with RegulomeDB scores greater than or equal to 3 and equipped with SNPinfo function.

2.3. SNP genotyping

Briefly, genomic DNA was extracted from EDTA-venous blood by the Qiagen Blood Kit (Qiagen). Illumina Human Omni Zhong Hua Bead Chips and Human Omni Express chips were chosen to perform genotyping for all samples. A selection process was established to filter the SNPs as follows: MAF ≥ 0.05, call rate >95% and HWE ≥ 0.01. Meanwhile, we performed a logistic regression analysis to calculate the outcomes and adjusted for gender, age and smoking status.

After confirming the candidate SNPs, we performed genotyping for 37 bladder cancer cases by ourselves (Supplementary Table 1, http://links.lww.com/MD/D578). DNA of 37 bladder cancer patients were extracted from EDTA-venous blood by the Qiagen Blood Kit (Qiagen) and genotyped by TaqMan allelic discrimination assay. A 384-well ABI 7900HT real-time PCR system (Applied Biosystems, Foster City, CA) and SDS 2.4 software (Applied Biosystems) were used to measure and analyze allelic discrimination. The primers and probes sequences are shown in Supplementary Table 2, http://links.lww.com/MD/D579.

2.4. RNA extraction, RT-PCR and expression quantitative trait locus (eQTL) analysis

RNA samples were isolated from 37 bladder tissues (paired to the above samples) by using Trizol reagent. PrimerScrpit RT Master Mix (Takara Bio Inc., DaLian, Co., Ltd) and RNA were mixed for reverse transcription and heated to 80°C for 5 seconds after 15 minutes at 37°C. Then, ddH_2O was utilized to dilute the cDNA to one tenth. EvaGreen qPCR Master Mix (ABM Inc, Canada) and an ABI Prism 7900HT quantitative real-time PCR instrument were used for the reaction system, and GAPDH was used as a control. The primers are shown in Supplementary Table 2, http://links.lww.com/MD/D579. Combined with allelic discrimination and host gene expression, eQTLs were calculated for the candidate SNPs.

2.5. Bioinformatics analysis

We used the online software Xena (http://xena.ucsc.edu) to conduct a Kaplan–Meier analysis of overall survival with regard to bladder urothelial carcinoma (BLCA) target genes based on the TCGA database. Xena can be used to analyze and visualize functional genomics datasets based on public genomic or phenotypic datasets. In addition, UALCAN (http://ualcan.path.uab.edu/index.html), an interactive web tool for analyzing cancer transcriptome data, was used to analyze BLCA target gene expression based on major cancer stages.[18] Gene Expression Omnibus (GEO) was used to download extra datasets and to verify the target gene expression in bladder cancer, and we chose GSE42089,[19] GSE38265[20] and GSE37815,[21] which had particular clinical and phenotype information in this phase.

2.6. Statistical analysis

The goodness-of-fit χ^2 test was performed to evaluate the HWE of the genotype frequencies in controls. Relationships between SNPs and bladder cancer susceptibility were assessed with odds ratios (ORs) and 95% confidence intervals (CIs) derived from multivariate logistic regression analysis with adjustment for gender and age. All statistical analyses were performed using R software (version 3.2.3). Two-sided $P < .05$ was considered significant.

3. Results

3.1. Characteristics of the study population

The frequency distributions of demographic characteristics in the case group and control group are shown in Table 1. There were no significant differences between cases and controls in gender and age (gender: $P = .909$; age: $P = .753$). Moreover, the smoking status was significantly different between the 2 groups ($P < .001$).

3.2. Association of candidate SNPs and bladder cancer susceptibility

As shown in Figure 1, there were 47 candidate genes involved in the NOTCH signaling pathway. We screened 36,960 SNPs based on the 1000 Genomes Project (CHB) and obtained 5026 SNPs

Table 1

Frequency distributions of selected variables between bladder cancer cases and controls.

	Cases (n = 580)		Controls (n = 1101)		
Variables	**N**	**%**	**N**	**%**	***P***
Age (mean ± SD)	64.71 ± 12.09		64.46 ± 12.06	0.6887	
≤60	199	34.31	357	32.43	.4348
>60	381	65.69	744	67.57	
Gender					
Male	481	82.93	905	82.20	.7072
Female	99	17.07	196	17.80	
Smoking status					
Never	313	54.25	717	65.12	**<.0010**
Ever	264	45.75	384	34.88	
Tumor grade					
Low	287	52.47			
Intermediate	174	31.81			
High	86	15.72			
Tumor stage					
Non-muscle invasive	377	71.27			
Invasive	152	28.73			

[1]Two-sided χ^2 test for the frequency distributions of selected variables between cases and controls.
[2]SD = standard deviation.

Table 2

Association between the selected SNPs and bladder cancer risk in case-control study.

Gene	SNP	Allele (major/minor)	MAF		HWE	OR (95% CI)
			Case	Control		
MAML2	rs7944701	T/C	0.2579	0.2103	0.01315	1.329 (1.115–1.583)
TLE2	rs3760961	A/G	0.3914	0.4309	0.9022	0.8491 (0.7342–0.982)

[1]Based on the NCBI database, build 37; [2]MAF: minor allele frequency in cancer-free controls; [3]Hardy-Weinberg equilibrium (HWE) among control subjects; [4]OR: odds ratio; [5]CI: confidence interval; [6]The logistic regression analysis was adjusted for age, gender and smoking status.

after the quality control procedures were applied. Moreover, two different web tools were used to predict the functional characteristics, and the interaction of their results was obtained (Supplementary Figure 1, http://links.lww.com/MD/D577). A total of 76 SNPs remained for genotyping and after quality control procedures were applied. After a logistic regression analysis adjusted for age, sex and smoking status, only rs7944701 and rs3760961 had *P* values below .05 (Table 2). Both SNPs were distributed in the intron region. In the additional study, we chose the rs7944701 T > C SNP located in *MAML2* with the lowest *P* value (OR = 1.329, 95% CI = 1.115–1.583, $P = .001$).

3.3. rs7944701 is an eQTL for MAML2

To explore the association between rs7944701 and *MAML2* further, we analyzed the rs7944701 allelic discrimination and MAML2 expression in 37 bladder-adjacent normal tissues. When rs7944701 carried the C allele, *MAML2*was significantly differentially expressed ($P = .034$) (Fig. 2A). Therefore, rs7944701 is an eQTL for *MAML2*, and allelic changes would influence *MAML2* expression even though it is an intronic variant.

3.4. Association of target genes with bladder cancer

SNP rs7944701 is located in the first intron of the *MAML2* gene. We assessed the expression of the target gene *MAML2* in bladder cancer. In the TCGA BLCA data, there were 412 tumor tissues and 19 normal tissues included. We observed that *MAML2* expression in tumor tissues was clearly lower ($P = 9 \times 10^{-4}$; Fig. 2B). Besides, three GEO datasets, including non-tumor bladder tissues and bladder cancer tissues, were all human, and expression profiling was performed by array. We explored these data and found a remarkable statistical discrepancy in MAML2 expression between non-tumor controls and tumor cases. As shown in Figure 2C, *MAML2* expression was higher in non-tumor tissues than in tumor tissues in GSE37815, GSE38265,

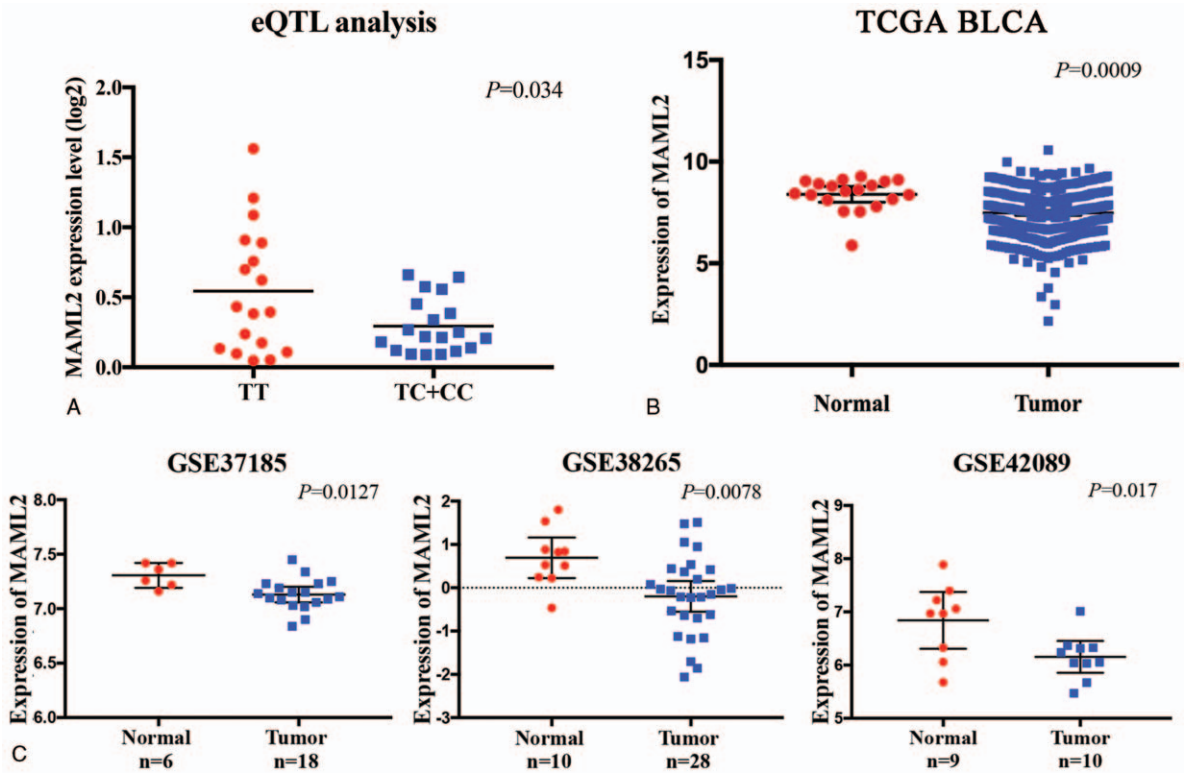

Figure 2. Expression of MAML2 in bladder cancer and normal tissues and eQTL analysis. (A) rs7944701 is an expression quantitative trait locus, for MAML2; (B) MAML2 expression was significantly different between normal tissues and tumor tissues based on bladder urothelial carcinoma; (C) The expression of MAML2 was compared based on GSE42089, GSE38265, and GSE37815. MAML2 = mastermind-like 2

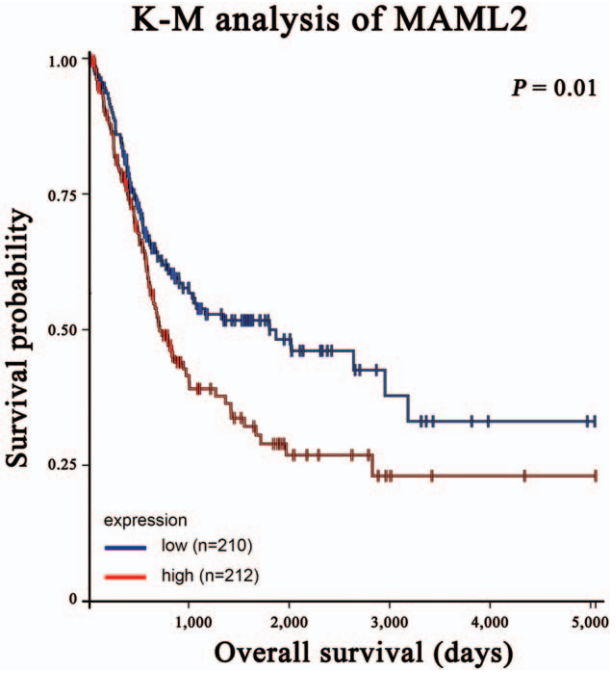

Figure 3. Kaplan–Meier curve of overall survival in bladder cancer patients.

and GSE42089 ($P=.0127$, $P=7.8\times10^{-3}$and $P=1.7\times10^{-3}$, respectively).

Subsequently, after dividing the BLCA cohort into two groups, high and low MAML2 expression, according to the median expression. The high MAML2 expression was correlated with lower overall survival (hazard ratio (HR) = 1.53, 95% CI = 1.29–1.82, $P=.010$; Fig. 3).

4. Discussion

The NOTCH pathway, as a highly conserved system, is a key element in the regulation of embryogenesis, nervous system development and function, and even tumorigenesis.[9] Rampias et al revealed that the NOTCH pathway was a new tumor suppressor in bladder cancer.[22] They unraveled that mutations in FGFR3 and RAS enriched in the NOTCH signaling pathway were associated with bladder cancer risk. Notably, mutations in the pathway could promote tumorigenesis by phosphorylating ERK1/2. Additionally, another study observed a remarkable association between bladder cancer and Notch2. Hayashi et al reported that the overexpression of Notch2 promoted tumor growth, invasion, and metastasis.[23] Notch 2 operates as an oncogene that promotes bladder cancer growth and metastasis through EMT, cell-cycle progression, and maintenance of stemness.[23] Notch2 activation correlated with adverse disease parameters and worse prognosis by immune histochemistry. Thus, inhibition of Notch2 is a rational novel treatment strategy for invasive bladder cancer. Another study had indicated that Notch1 and Notch2 have opposite effects on the progression of bladder cancer could give rise to potential therapeutic approaches aimed at blocking or restoring the Notch pathway.[24] According to these previous studies, the NOTCH pathway may regulate the incidence and progression of bladder cancer in an especially complex way. Nevertheless, these results are inconsistent. In this study, we comprehensively evaluated the association between 36,960 SNPs and 47 candidate NOTCH pathway genes. Based on the 1000 Genomes Project (CHB), we found several functional genetic variants associated with bladder cancer involved in the NOTCH pathway. In the following genotyping and statistical analysis, we found that 2 genetic variants, rs7944701 and rs3760961, had remarkable significance in bladder cancer. We focused on rs7944701 with the lowest *P* value in the additional study. Interestingly, the host gene *MAML2* had a strong association with bladder cancer prognosis.

The SNP rs7944701 is located in *MAML2*, which is a member of the mastermind-like family of nuclear protein coding genes, defined as transcriptional coactivators of NOTCH receptors.[25] Through binding with the NOTCH intracellular domain, the MAML protein activates downstream genes of NOTCH and has high expression in the salivary gland, placenta, and skeletal muscle.[26] The *MAML2* gene was also detected in hidradenoma and leukemia.[27,28] Some studies have reported that CRTC1/3-MAML2 protein fusion is a key promoting element in hidradenoma development, and *MAML2* gene fluorescence in situ hybridization (FISH) could be a useful method to diagnose hidradenoma.[29] These results suggest that MAML2 or the MAML2 fusion protein is not a specificity marker. However, we found that few studies focused on the association between *MAML2* and bladder cancer. In the present study, we were the first to find that *MAML2* has significant differential expression in bladder cancer individuals.

To explore the connection between MAML2 and bladder cancer, we used UCSC Xena based on the TCGA bladder cancer data. These results indicated that patients with high expression of *MAML2* had remarkably worse prognoses, suggesting that MAML2 acts as a promoter in the progression of bladder cancer. Relying on these findings, we revealed that *MAML2* was a low expression gene in bladder cancer patients. MAML2 had an inhibitory function in the differentiation and proliferation processes of bladder cancer. Moreover, there were also significant differences in MAML2 expression between normal bladder tissues and bladder cancer tissues. In three GEO datasets, the expression of *MAML2* was dramatically higher in normal tissues than in tumor tissues. Thus, we speculated a specific bond exists for identifying the expression of *MAML2* to predict the progression of bladder cancer and even bladder cancer patients' prognosis.

Additionally, we found that the strongest signal was represented by an intronic SNP called rs7944701, which was related to the susceptibility of bladder cancer in the Chinese population. Several studies have discovered many SNPs located in noncoding regions that are associated with cancer risks and progression. Jiang et al demonstrated that MYC enhancer with rs6983267-G allele could be activated and enhanced colorectal cancer cell proliferation.[30] Recent case reported by Tian et al also revealed that the intronic SNP, rs7959129, could facilitate a promoter-enhancer interaction and synergistically increase colorectal cancer risk.[31] Pattison et al showed that the intronic SNP rs8102137 was a strong genetic variant correlating with bladder cancer risk.[32] In their further research, they found that another intronic SNP, rs200996365, was in high LD with rs8102137 and was located within the *KLF5* region, which is a transcriptional activator of *CCNE1*. These findings indicated that the high-risk intronic variant indirectly enhanced cell proliferation and the incidence of bladder cancer. Thus, we speculated that the intronic rs7944701 would affect its host gene *MAML2* expression or function and would sequentially influence

the regulation of the NOTCH pathway involved in bladder cancer. In order to prove our hypothesis, we performed an eQTL analysis for rs7944701. To the best of our knowledge, this study is the first eQTL analysis of rs7944701. The results illuminated that the rs7944701 C allele was negatively associated with *MAML2* expression.

5. Conclusion

We demonstrated that the novel *MAML2*variantrs7944701, in the NOTCH/MAML2 signaling axis, was correlated with bladder cancer risk. For the first time, we revealed that rs7944701 was an eQTL for MAML2 and enhanced the susceptibility to bladder cancer. We also provided evidence that the TC/CC variant decreased the expression of *MAML2*. Simultaneously, *MAML2* was expressed at significantly low levels in tumor tissue, and remarkably high expression levels indicated better overall survival. In the future, the exact mechanism of the NOTCH/MAML2 signaling axis in bladder cancer needs further exploration. In our research, in addition to rs7944701, there was another SNP with a strong signal, rs3760961, which has significant value in bladder cancer. Further studies are warranted to elucidate the mechanism of involvement of NOTCH pathway genes in bladder cancer development and carcinogenicity. Additionally, study of MAML family genes as functional mRNAs is also important in future research.

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Validation: Qingling Zhang, Jingyuan Tang.
Visualization: Qian Lu.
Writing – original draft: Yang Shen.
Writing – review & editing: Yang Shen, Jingyuan Tang.

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