Genetic variants in microRNAs predict bladder cancer risk and recurrence

Meilin Wang¹,²,³,* Haiyan Chu¹,²,* Pu Li⁴, Lin Yuan⁵, Guangbo Fu⁶, Lan Ma², Danni Shi², Dongyan Zhong¹, Na Tong², Chao Qin⁴, Changjun Yin⁴, Zhengdong Zhang¹,²,³,**

¹State Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, Nanjing, China

²Department of Molecular & Genetic Toxicology, the Key Laboratory of Modern Toxicology of Ministry of Education, School of Public Health, Nanjing Medical University, Nanjing, China

³Department of Occupational Medicine and Environmental Health, Jiangsu Key Laboratory of Cancer Biomarkers, Prevention and Treatment, Cancer Center, Nanjing Medical University, Nanjing, China

⁴Department of Urology, The First Affiliated Hospital of Nanjing Medical University

⁵Department of Urology, Jiangsu Province Hospital of TCM, Nanjing, China

⁶Department of Urology, The Huai-An First Affiliated Hospital, Nanjing Medical University, Nanjing, China

*The authors contributed equally to this work.

**Correspondence to: Zhengdong Zhang, State Key Laboratory of Reproductive Medicine, Institute of Toxicology, Nanjing Medical University, 140 Hanzhong Road, Nanjing 210029, China. Tel: +86 25 86862926; Fax: +86 25 86527613; Email: drzdzhang@gmail.com
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Abstract

MicroRNAs (miRNAs) play important roles in numerous cellular processes, including development, proliferation, apoptosis, and carcinogenesis. Because altered expression and function of miRNAs has been observed in bladder cancer, we investigated whether genetic variations in miRNAs are associated with bladder cancer risk and prognosis. Using bioinformatics tools, we selected five single nucleotide polymorphisms (SNPs) located in miRNAs and used these to evaluate miRNA-disease associations in a two-stage model, consisting of 1019 bladder cancer cases and 1182 controls (683 cases and 728 controls in the training set, and 336 cases and 454 controls in the test set). We found the miR-146a rs2910164 C allele was associated with significantly decreased risk of bladder cancer in both the training and test sets, as well as the combined set (OR = 0.80, 95%CI = 0.71-0.90, \( P = 2.92 \times 10^{-4} \)). Furthermore, the rs2910164 GC/CC genotypes conferred a significantly reduced risk of recurrence, compared with the GG genotype \( (P = 0.016) \). Functional analysis revealed the miR-146a rs2910164 C allele inhibited cell proliferation and significantly downregulates expression of IRAK1 and TRAF6 in bladder cancer cells. Additional examination of 64 bladder cancer tissues showed that individuals carrying the C allele had increased expression levels of miR-146a compared with those carrying the G allele \( (P = 0.010) \). Taken together, our findings demonstrate that miR-146a rs2910164 plays an important role in the risk and recurrence of bladder cancer, suggesting it may represent a biomarker for risk prevention and therapeutic intervention. Further larger and prospective cohorts are needed to validate our findings.
Introduction

Bladder cancer is a common malignancy with a complicated, multifactorial etiology, involving both genetic and environmental factors. Tobacco smoking and occupational exposure to chemical carcinogens are the strongest risk factors for bladder cancer (1). At diagnosis, approximately 70% of bladder cancers present with non-muscle invasive bladder cancer tumors, while the remaining cases have invasive tumors (2). Most patients with non-muscle invasive tumors are treated by transurethral resection, with up to 70% of these cases developing at least one recurrence within 5 years (3). Therefore, many studies have investigated molecular biomarkers for prediction of risk and recurrence of bladder cancer.

Although some single nucleotide polymorphisms (SNPs) have been identified in genome-wide association studies (GWAS) as susceptibility loci for bladder cancer risk (4-6) and successfully replicated in our previous studies (7, 8), such loci explain only a small portion of the total risk; few of these SNPs discovered by GWAS involve microRNA (miRNA) genes.

MiRNAs are a class of small non-coding RNAs (~22 nt), which normally function as negative regulators of mRNA expression of the target genes at the posttranscriptional level (9). Accumulating evidence has shown that miRNAs play critical roles in regulating a variety of biological processes, such as organ development, cell proliferation, cell differentiation, and apoptosis (10). Moreover, it has been shown that miRNAs participate in human carcinogenesis as either tumor suppressors or oncogenes (11, 12). miRNAs are aberrantly expressed in human cancer, and their profiles could be used to classify human cancers (13). Specifically, miRNA expression profiles and specific miRNAs have been shown to be
associated with bladder cancer tumorigenesis (14-16).

The biological functions of most miRNAs are not fully understood. More recently, it has been proposed that the presence of genetic variants in miRNA genes, their processing mechanisms and target binding sites, may collectively affect cancer risk and prognosis (17). miRNA SNPs, as common genetic variants, may alter miRNA expression and maturation (18).

In the present study, we investigated the effects of genetic polymorphisms in miRNA genes on the risk and prognosis of bladder cancer. Through further functional analysis, we have identified a novel and functional SNP, rs2910164, in miR-146a that may contribute to the development and recurrence of bladder cancer.

Materials and Methods

Our overall study design and working model for investigating association between SNPs in miRNAs and bladder cancer risk and prognosis is summarized in Figure 1.

Study population

This study was approved by the institutional review board of Nanjing Medical University and all subjects signed a written informed consent form. Details regarding subject recruitment in this two-stage study have been previously described (19). All cases were histologically confirmed to have bladder transitional cell carcinoma at the time of enrollment in our ongoing study. Briefly, the training set including 683 cases with bladder cancer and 728 control subjects that were recruited from the First Affiliated Hospital of Nanjing Medical University between January 2003 and May 2011. As a test set, an additional 336 cases and 454 controls were recruited from the Huai-An Affiliated Hospital of Nanjing Medical
University starting from May 2005. All controls were recruited from those who accompanied the cases, who were seeking for health care in the same hospitals, and who were genetically unrelated cancer-free individuals. Cases and controls were excluded if they had symptoms suggestive of bladder cancer, such as hematuria. All subjects were interviewed, and a 5 ml venous blood sample was obtained from each subject. A subset of cases in the test set were selected for additional follow-up, including 199 (199/226, 88.1%) non-muscle invasive bladder cancer cases. Follow up consisted of telephone calls every three months following the initial diagnosis. Of these follow-up cases, 27 (27/226, 11.9%) were excluded due to incomplete follow-up data.

**SNP selection and genotyping**

We queried all known human miRNA available in the public miRBase database (version 10.0) (Supplementary Table 7). We performed extensive searches of SNP databases including HapMap (release #24), dbSNP, and Patrocles, based on two criteria: (a) SNPs located in the pre-miRNA or mature sequences, and (b) minor allelic frequency (MAF) ≥ 5% in the Chinese population. Among them, 14 SNPs in miRNAs matched both criteria (Supplementary Table 1), and no additional SNPs were found. Furthermore, the Gibbs binding free energy (ΔG, KJ/mol) for each pair of common and variant alleles was computed using miRanda software (20). The difference of the free energies between the two alleles was calculated as ΔΔG. Because the energy parameter ΔΔG could impact binding of each SNP with the target miRNA, we ranked the values of ΔΔG, and chose the upper tertile of the distribution as a cut-off level. Finally, we selected five SNPs in miRNAs that have a ΔΔG ≥ 2.60 KJ/mol and MAF ≥ 5% in the database (miR-146a rs2910164, miR-196a2 rs11614913, miR-605 rs2435561, miR-618...
rs2682818, and miR-923 rs47960429).

The TaqMan allelic discrimination method was used to genotype the five selected SNPs. The sequence of primers and probes for each SNP is shown in Supplementary Table 6. The samples were read and analyzed from the ABI Prism 7900HT Sequence Detection System using SDS 2.2 software (Applied Biosystems, Foster City, CA). The average genotype call rates for all SNPs were 99.6%. About 10% of the samples were randomly selected for confirmation by repeat genotyping, and the results were 100% concordant.

MiRNA expression vector construction, cell transfection, and detection

To investigate the effect of SNP on expression levels of miR-146a, the pri-miRNA, consisting of a 340bp DNA fragment, was amplified from a genomic DNA sample with a miR146a rs2910164 G allele, and cloned into pEGFP-N3 expression plasmid vector containing a green fluorescent protein marker (Clontech, Palo Alto, CA). Using the G allele construct as a template, site-directed mutagenesis was used to generate plasmids containing the C allele. The amplified fragments were then sequenced to confirm that there were no nucleotide errors.

Human T24 and EJ bladder cancer cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). These two cell lines were selected because they are derived from high grade and invasive tumors that have been extensively characterized (19, 21). The cells were grown at 37°C in the presence of 5% CO₂ in a humidified incubator. For transfections, T24 and EJ bladder cancer cells were transfected with vector DNA containing either the G or C allele of miR146a rs2910164, and using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Transfection efficiency was verified by fluorescence microscopy. The
pEGFP-N3 vector without an insert was used as a negative control.

Cells were collected 48 hours after transfection, and miRNAs were extracted using the miRVana miRNA isolation kit (Applied Biosystems). The expression levels of miR-146a were determined using TaqMan MicroRNA Assays (Applied Biosystems). All reactions were done in triplicate. The expression of miR-146a relative to RNU6B was determined using the $2^{-\Delta\Delta C_T}$ method.

**Cell proliferation assay and cell cycle analysis**

Cell culture was continued for 24, 48 and 72 hours and assessed using the Cell Counting Kit 8 (Dojindo, Kumamoto, Japan). The absorbance value of each well was determined at 450nm using the Infinite M200 spectrophotometer (Tecan, Germany). Each time point was assayed in triplicate. Each experiment was repeated six times.

Forty-eight hours after transfection, cells were harvested and fixed in 70% ice cold ethanol followed by RNase A treatment, stained with 50 μg/ml of propidium iodide for DNA content analysis by flow cytometry on a FACS Calibur system (Beckman Coulter, San Jose, CA). Results were expressed as a percentage of cells in each cell cycle phase.

**Identification of the potential mRNA targets of miR-146a**

Potential miR-146a targets were predicted and analyzed using three computer-aided algorithms, including TargetScan, PicTar, and miRanda. Because a single computer-aided algorithm can generate a high number of false positives, the combination of these three approaches was used to provide a more accurate assessment of real miRNA targets. We used TargetScan Release 6.1 as the primary source of target identification; this provided a total of 224 conserved targets, from which we selected the top 10 targets with the highest aggregate $P$
value (Supplementary Table 5). We then used PicTar and miRanda software to predict the miR-146a targets based on the parameters of PicTar score >5.0 and mirSVR score <-1.50, respectively. As a result, four genes (I\textit{RAK}1, \textit{TRAF}6, I\textit{GS}F1, and \textit{NOVA}1) were predicted by at least two of these three software packages. However, further identification of two target genes (\textit{I\textit{RAK}1} and \textit{TRAF}6) was based on their potential roles in other cancers as reported previously (22).

\textbf{Construction of reporter plasmids and luciferase reporter assay}

To construct luciferase reporter plasmids, \textit{I\textit{RAK}1} and \textit{TRAF}6 3'-UTR fragments (i.e., 677bp and 1368bp) were inserted at the \textit{Xba}I site, downstream of the luciferase gene in the pGL3-promoter vector (Promega, Madison, WI) (Figure 4A). The nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analyses.

Forty-eight hours after transfection in T24 and EJ cells, luciferase activity in lysates was measured with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and normalized against the activity of the PRL-TK. Independent triplicate experiments were performed for each plasmid construct.

\textbf{Quantitative real-time RT-PCR assay}

A total of 64 bladder cancer tissues were obtained from surgically removed specimens of individual cases. All of the tissues were frozen in liquid nitrogen upon collection. The hematoxylin and eosin (H&E) stained sections prepared using the cancerous tissues were microscopically evaluated by the pathologists. In this study, only tissues containing over 85% of malignant cells were selected. Detailed clinical information is shown in Supplementary Table 4. Total RNA were isolated from tissues using mirVana miRNA Isolation Kit (Applied
Biosystems). The expression levels of miR-146a were determined using TaqMan MicroRNA Assays (Applied Biosystems). *IRAK1* and *TRAF6* mRNA expression levels were evaluated using the comparative CT method. The transcription level of *GAPDH* was used as an internal control. Real-time PCR was performed on an ABI 7900HT Real-Time PCR System equipped with a 384-well reaction block (Applied Biosystems).

**Statistical analysis**

Differences in the distribution of selected demographic variables between bladder cancer cases and controls in the training and test sets were evaluated using the Student t-test (for continuous variables) or \( \chi^2 \)-test (for categorical variables). Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit \( \chi^2 \)-test. Unconditional univariate and multivariate logistic regression analyses were performed to obtain estimates for associations between miRNA SNPs and bladder cancer risk by computing odds ratios (ORs) and their 95% confidence intervals (CIs). Bonferroni correction for multiple testing was applied. The heterogeneity between subgroups was assessed with the \( \chi^2 \) based Q test. In the test set, the associations between miRNA-146a rs2910164 and occurrence outcomes in bladder cancer cases were estimated using the method of Kaplan-Meier and assessed using the log-rank test. Cox proportion hazards models were used to adjust for potential confounders. The Spearman test was used to analyze the correlation between miR-146a expression and *IRAK1* and *TRAF6* mRNA expression levels. A \( P < 0.05 \) was considered statistically significant. All tests were two-sided and were performed using SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC).
Results

Characteristics of Cases

The distribution of selected characteristics between bladder cancer cases and control subjects in the two-stage study are shown in Table 1. There were no differences in the distribution of age and sex between cases and controls in the training set and test set ($P = 0.875$ for age and $P = 0.842$ for sex in the training set, and $P = 0.639$ for age and $P = 0.557$ for sex in the test set, respectively). However, there were more smokers among the cases than among the controls, and there were more subjects that smoked greater than 20 pack-years in the cases than in the controls in both the training and test sets ($P < 0.001$), suggesting that smoking may play a role in the etiology of bladder cancer. Furthermore, there were no significant differences in the distributions of age, sex, smoking status, tumor grade, and stage between the two sets.

Effects of selected SNPs in miRNAs and bladder cancer risk

We evaluated genotype frequencies of the five miRNA SNPs among cases and controls and their associations with bladder cancer risk in the training set. The primary information and allele frequencies observed are listed in Table 2. All genotype distributions of control subjects were consistent with those expected from Hardy-Weinberg equilibrium. As shown in Table 2, ORs and their 95% CIs were used to test the association of each SNP with bladder cancer risk in heterozygous, homozygous, dominant and additive models, and two SNPs (rs2910164 in miR-146a and rs2682818 in miR-618) were significant ($P = 0.004$ for miR-146a rs2910164 and $P = 0.013$ for miR-618 rs2682818 in the additive model). But this significance
disappeared after Bonferroni correction for miR-618 rs2682818 \((P = 0.064)\). Furthermore, in the test set, only miR-146a rs2910164 showed a significant association with bladder cancer risk \((P = 0.030)\), consistent with the results from the training set analysis. In the combined set analyses, the rs2910164 C allele in miR-146a was associated with 20% decreased risk of bladder cancer, compared with the G allele \((\text{OR} = 0.80, 95\% \text{CI} = 0.71-0.90, P = 2.920 \times 10^{-4})\).

When we performed stratified analyses by age, sex, smoking status, grade, and stage (Supplementary Table 2 and Figure 1), we observed a decreased bladder cancer risk for the rs2910164 C allele; this was more pronounced among different subgroups especially among male subjects \((P < 0.001)\). However, no significant heterogeneity was detected in the subgroups.

**Effects of miR-146a rs2910164 and bladder cancer recurrence**

We further investigated whether there was any association between miR-146a rs2910164 genotypes and the recurrence of non-muscle invasive bladder cancer in the test set that had clinical follow-up data. Among the 199 bladder cancer cases, 74 developed recurrence during the follow-up time of 72.0 months. As shown in Figure 2, the median recurrence-free survival time was 47.0 months for cases with the GC/CC genotypes, and 27.0 months for cases with the GG genotype. Survival analysis showed that the difference in the two groups was statistically significant \((\text{log-rank test, } P = 0.016)\). Compared with the rs2910164 GG genotype, a significant overall 42% decrease in recurrence risk was observed for individuals carrying at least one rs2910164 C allele \((\text{HR} = 0.58, 95\% \text{ CI} = 0.36-0.94)\) (Supplementary Table 3). In stratified analysis by age, sex, pack-years of smoking and grade, the decreased
risk was more evident in never smokers (HR = 0.41, 95% CI = 0.21-0.80) and cases with G1/G2 grade tumors (HR = 0.58, 95% CI = 0.35-0.97).

**Effects of miR-146a rs2910164 on the miRNA expression levels**

To assess the impact of miR-146a rs2910164 on the expression levels of miR-146a, we measured expression levels between the vectors containing the G or C allele by TaqMan miRNA assay. As shown in Supplementary Figure 2, expression levels of miR-146a with the C allele had significant increases of 3.12-fold higher in T24 cells ($P = 0.010$), and 3.86-fold higher in EJ cells ($P = 0.034$), compared with those with the G allele, suggesting that the rs2910164 polymorphism could influence the expression of miR-146a.

**Effects of miR-146a rs2910164 on cell proliferation and cell cycle**

In an attempt to determine the functional role of miR-146a rs2910164 in bladder cancer carcinogenesis, we assessed miR-146a cell proliferation with the rs2910164 G or C allele vector in the bladder cancer cell lines. As shown in Figures 3A and B, the CCK8 assay revealed that the expression of miR-146a with the G or C allele led to a significant decrease in cell growth of T24 and EJ cells, indicating that miR-146a exerts a growth-inhibiting function in bladder cancer cells. Furthermore, cells transfected with the C allele showed a greater decrease in cell proliferation at 48 and 72 hours, compared with the G allele in T24 cells ($P < 0.05$). Similar proliferation rates were observed in EJ cells ($P < 0.05$).

Given that miR-146a is known to inhibit bladder cancer cell proliferation, we next sought to determine whether miR-146a has an impact on the cell cycle progression of bladder cancer...
cells (Figure 3C). The cell cycle distribution of T24 and EJ cells showed that cell counts in
the G1 phase were significantly increased in response to the vector with rs2910164 C allele in
T24 \((P = 0.010)\) and EJ \((P = 0.022)\) cells, compared with those with the G allele, whereas the
cell population in S phase was moderately reduced \((P = 0.058\) in T24 cells and \(P = 0.010\) in
EJ cells) (Figure 3D and E). Therefore, miR-146a with rs2910164 C allele may confer a
growth-inhibiting function, due to its inhibition of cell cycle progression at G1/S transition in
bladder cancer cells.

**Potential target genes of miR-146a**

Using bioinformatics methods, two genes were selected as candidate targets of miR-146a. As
shown in Figure 4B, the 3’-UTR of IRAK1 and TRAF6 have two or three presumed sites in
conserved regions that can bind to the seed region of miR-146a. To investigate whether
miR-146a rs2910164 may influence the activity of target genes, we constructed luciferase
reporter vectors by using pGL3-promoter vector, spanning the 3’-UTR of IRAK1 and TRAF6
(Figure 4A), and used them for transient transfections with bladder cancer cell lines. As
shown in Figures 4C and D, vectors transfected with miR-146a rs2910164 C allele had a 24% to
55% decrease in relative luciferase activities, compared to those with the G allele in T24 \((P
= 0.032\) for IRAK1 and \(P = 0.028\) for TRAF6) and EJ \((P = 0.012\) for IRAK1 and \(P < 0.001\) for
TRAF6) cells.

We next determined whether miR-146a could regulate IRAK1 and TRAF6 mRNA
expression levels in bladder cancer tissues. Our results showed that miR-146a was inversely
corrected with IRAK1 and TRAF6 mRNA expression levels \((R = -0.263, P = 0.036\) for IRAK1,
and $R = -0.780$, $P = 0.013$ for TRAF6).

**Effects of miR-146a rs2910164 on the expression in bladder cancer tissues**

We also evaluated the association between miR-146a rs2910164 and miRNA expression levels in 64 bladder cancer tissues detected by real-time quantitative RT-PCR. Of these bladder cancer tissues, the distribution of the GG, GC, and CC genotypes was 20, 31, and 13, respectively (Supplementary Figure 3). After adjustment for sex, age, and smoking status, the rs2910164 C allele was significantly associated with increased expression of miR-146a ($P = 0.010$).

**Discussion**

In the present study, by examining SNPs in comprehensive miRNAs and their associations with bladder cancer susceptibility, we identified miR-146a rs2910164 to be associated with bladder cancer risk and recurrence. We found that a change from G to C change in SNP rs2910164 substantially alters the expression levels of miR-146a as well as its targeted genes IRAK1 and TRAF6. We also observed *in vivo* evidence the miR-146a rs2910164 C allele is associated with higher expression levels of miRNA compared with the G allele.

Alterations in the expression of miRNA genes are known to contribute to the pathogenesis of many cancers, which can be caused by various mechanisms, including deletions, amplifications or mutations involving miRNA loci, epigenetic silencing or the dysregulation of transcription factors that target specific miRNAs (12). Saunders et al. surveyed the publicly available SNPs data in the context of miRNA and suggested that the occurrence of SNPs in miRNA sequences is relatively rare (18). However, accumulating
evidence suggests that SNPs in miRNA genes are likely to affect the expression of the miRNA and therefore may contribute to the susceptibility to common human diseases (17). Hu et al. (23) and Hoffman et al. (24) reported that miR-196a2 rs11614913 affected the processing of the pre-miRNA into its mature, regulatory form, which is associated with risk of many kinds of cancer (25-27). For bladder cancer, Yang et al. first explored the role of SNPs in miRNA-related genes in bladder cancer predisposition and found that polymorphisms in miRNA processing pathway genes were involved in bladder cancer susceptibility (28).

In the present study, we performed a genome-wide analysis of SNPs located in miRNA genes, and further evaluated their associations with bladder cancer risk. From the initial 14 screened SNPs in miRNAs, we observed that miR-146a rs2910164 was associated with significantly decreased risk of bladder cancer in two independent cohorts, which is consistent with previous findings for this disease (29-34). However, some studies have reported opposite findings in other cancers. Specifically, Jazdzewski et al. first observed that miR-146a rs2910164 was associated with increased predisposition to papillary thyroid carcinoma due to altered processing that resulting lower expression of the mature sequence (22). Yang et al. (28) and Mittal et al. (35) failed to replicate the significant association of miRNA-146a rs2910164 with bladder cancer in Caucasians, which may have been due to small sample sizes and thus requires further validation. Furthermore, in a mini-meta analysis, we pooled the eligible published data on miRNA-146a rs2910164 and cancer risk and found that rs2910164 had a different effect on cancer risk; that is the C allele had a protective effect among Asians but conferred an increased risk among Caucasians (Supplementary Figure 4),
which was confirmed in a subsequent meta-analysis (36). In addition, the frequency of the
rs2910164 C allele differs by ethnicity (i.e., 0.237 in Caucasians, 0.633 in Japanese, and
0.500 in Africans). Furthermore, the Gibbs free energy was -42.40 kcal/mol for the
rs2910164 G allele, and -39.60 kcal/mol for the C allele, suggesting a less stable secondary
structure for the C allele. It has been suggested that genetic variants in miRNAs could change
the conformation of the secondary structure and thereby alter the expression of mature
miRNAs. Thus, miR-146a rs2910164 may affect miRNA biogenesis and function, and
contribute to susceptibility for bladder cancer.

As deregulated cell proliferation is a key mechanism for neoplastic progression (37), it is
biologically plausible that miR-146a rs2910164 may modulate the risk of bladder cancer. Our
results from the CCK8 assay and growth curves both indicated that miR-146a produced
significant growth inhibition in bladder cancer cells in vitro. Moreover, the analysis of
biological functions for miR-146a rs2910164 by cell proliferation and cell cycle assay
suggested the C allele was more likely to lead to an inhibition of tumorigenesis compared
with the G allele. This was further supported by the finding that over-expression of miR-146a
could inhibit migration and invasion of cancer cells (38). Furthermore, the cell cycle assay
showed that the inhibition of expression by the miR-146a rs2910164 C allele may be due to
significantly increased cell cycle arrest at G1. Therefore, our results suggest that miR-146a
may be involved in carcinogenesis and the development of bladder cancer as a tumor
suppressor gene, and the rs2910164 C allele provides increased suppressor function
compared to the G allele. In concordance with the expression results in cells, endogenous
miR-146a expression levels in bladder cancer tissues were found to be up-regulated by the
miR-146a rs2910164 C allele compared with the G allele. Some investigators reported that rs2910164 C allele was associated with higher miR-146a expression levels in cervical cancer (34) and gastric cancer tissues (38), but lower expression levels in prostate cancer (30), compared with the G allele. Although the detailed mechanism by which miR-146a rs2910164 has an effect on cancer risk is not fully clear, its effect on carcinogenesis may be organ-specific, reflecting site-dependent differences in the etiological factors.

SNPs in miRNA genes could potentially impact various biological processes by influencing target genes. For example, Duan et al. found that miR-125a reduced miRNA-mediated translational suppression via Lin-28 (39). Integrating bioinformatics and experimental assays, we identified IRAK1 and TRAF6 as two direct functional downstream targets of miR-146a in bladder cancer cells. It has been known that NF-κB activation could be mediated through IRAK1 and TRAF6 (40). We found that expression levels of IRAK1 and TRAF6 were significantly inhibited by the miR-146a rs2910164 C allele compared with the G allele. Therefore, miR146a rs2910164 C allele triggered inhibition of invasion and migration could be mediated through the down regulation of IRAK1 and TRAF6, coupled with subsequent inactivation of NF-κB. Furthermore, others have observed that miR-146a could block cancer cell invasion and metastasis through repression of IRAK1 and subsequent inactivation of NF-κB activation (41-43). A recent study suggested that miRNA-146a downregulates NF-κB activity via targeting TRAF6 and functions as a tumor suppressor (44). Therefore, miR146a rs2910164 could affect the negative regulation in IRAK1 and TRAF6 that induces the NF-κB pathway to influence susceptibility to bladder cancer.

There is a clear need for noninvasive diagnostic markers that may replace
urethrocystoscopy. These markers should be highly sensitive with a highly specific predictive value, so that urethrocystoscopy may be avoided in instances of a negative screening result (45). To date, several molecular markers have been reported to be associated with bladder cancer recurrence (46-48). Our results now identified miR-146a rs2910164 as associated with the recurrence of bladder cancer, and which may be a valuable molecular marker to predict bladder cancer prognosis, although the detectable effect size in our study was relatively small and could contribute to a false positive association. By the same token, miR-196a2 rs11614913 has been found to be a prognostic biomarker for both lung cancer (23) and head and neck cancer (49). To our knowledge, this is the first study to provide evidence that miR-146a rs2910164 may play an important role in the prediction of bladder cancer recurrence.

One strength of the current study is the relatively large sample size (1019 bladder cancer cases and 1182 control subjects) among our two-stage case-control bladder cancer association analyses, affording sufficient statistical power to detect subtle differences. Another strength of the present study is the use of a systematic search for the genome-wide SNPs in miRNAs, rather than a single or candidate miRNA gene. Although recent GWAS have identified a large number of robust associations between specific chromosomal loci and bladder cancer risk, many of which are located in desert regions, such studies have potentially missed biologically relevant but statistically weak, association signals (50). Our results suggest that SNPs in miRNAs associated with bladder cancer are needed for better coverage of miRNAs in future GWAS microarrays. Furthermore, our genetic association results were consistent with functional analysis. Thus, our finding is biologically plausible in the light of the putative
function of the SNP in miRNAs.

In conclusion, we provided evidence for the first time that miR-146a rs2910164 may contribute to the risk and recurrence of bladder cancer in a Chinese population. These data suggest that miR-146a may serve as a biological marker and as a therapeutic target in bladder cancer. Larger prospective studies are needed to confirm our findings.

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References


Table 1. Distribution of selected variables between bladder cancer cases and cancer-free control subjects

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<td>Controls (N=728)</td>
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<td>117 17.1</td>
<td>120 16.5</td>
<td>66 19.6</td>
</tr>
<tr>
<td>&gt;20</td>
<td>197 28.8</td>
<td>159 21.8</td>
<td>100 29.8</td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>327 47.9</td>
<td>177 52.7</td>
<td>504 49.5</td>
</tr>
<tr>
<td>G2</td>
<td>246 36.0</td>
<td>109 32.4</td>
<td>355 34.8</td>
</tr>
<tr>
<td>G3</td>
<td>110 16.1</td>
<td>50 14.9</td>
<td>160 15.7</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>Non-muscle invasive (pT x-pT x)</td>
<td>445 65.1</td>
<td>226 67.3</td>
</tr>
<tr>
<td>Invasive (pT x-pT x)</td>
<td>238 34.9</td>
<td>110 32.7</td>
<td>348 34.2</td>
</tr>
</tbody>
</table>

$^a$Training set and test set merged.
Table 2. Association of five miRNA polymorphisms with bladder cancer risk

<table>
<thead>
<tr>
<th>MiRNAs</th>
<th>SNPs</th>
<th>Allele (Major/minor)</th>
<th>Risk allele</th>
<th>MAF</th>
<th>$P_{HWE}^{a}$</th>
<th>OR (95%CI) $^{b,c}$</th>
<th>Additive model</th>
<th>$P^{d}$</th>
<th>$P^{e}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Heterozygous</td>
<td>Homozygous</td>
<td>Homozygous/Homozygous</td>
<td>pottery</td>
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<tr>
<td></td>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Training set</td>
<td></td>
<td></td>
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<tr>
<td>miR-146a</td>
<td>rs2910164</td>
<td>G/C</td>
<td>G</td>
<td>0.416</td>
<td>0.470</td>
<td>0.363</td>
<td>0.74 (0.59-0.95)</td>
<td>0.72 (0.57-0.90)</td>
<td>0.80 (0.69-0.93)</td>
</tr>
<tr>
<td>miR-196a2</td>
<td>rs11614913</td>
<td>T/C</td>
<td>C</td>
<td>0.468</td>
<td>0.466</td>
<td>0.196</td>
<td>1.16 (0.90-1.48)</td>
<td>0.99 (0.73-1.33)</td>
<td>1.10 (0.87-1.39)</td>
</tr>
<tr>
<td>miR-605</td>
<td>rs20435561</td>
<td>A/G</td>
<td>G</td>
<td>0.276</td>
<td>0.266</td>
<td>0.934</td>
<td>1.01 (0.81-1.26)</td>
<td>1.15 (0.76-1.74)</td>
<td>1.04 (0.84-1.28)</td>
</tr>
<tr>
<td>miR-618</td>
<td>rs2682818</td>
<td>C/A</td>
<td>C</td>
<td>0.263</td>
<td>0.305</td>
<td>0.071</td>
<td>0.99 (0.80-1.25)</td>
<td>0.47 (0.31-0.71)</td>
<td>0.88 (0.72-1.09)</td>
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<tr>
<td>miR-923</td>
<td>rs47960429</td>
<td>G/C</td>
<td>G</td>
<td>0.384</td>
<td>0.405</td>
<td>0.119</td>
<td>0.89 (0.70-1.12)</td>
<td>0.80 (0.58-1.12)</td>
<td>0.87 (0.70-1.08)</td>
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<tr>
<td>Test set</td>
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<tr>
<td>miR-146a</td>
<td>rs2910164</td>
<td>G/C</td>
<td>G</td>
<td>0.408</td>
<td>0.468</td>
<td>0.701</td>
<td>0.72 (0.52-0.99)</td>
<td>0.67 (0.45-1.01)</td>
<td>0.70 (0.51-0.94)</td>
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<tr>
<td>miR-618</td>
<td>rs2682818</td>
<td>C/A</td>
<td>C</td>
<td>0.294</td>
<td>0.301</td>
<td>0.256</td>
<td>0.95 (0.70-1.28)</td>
<td>0.60 (0.35-1.04)</td>
<td>0.87 (0.65-1.16)</td>
</tr>
<tr>
<td>Combined set</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>miR-146a</td>
<td>rs2910164</td>
<td>G/C</td>
<td>G</td>
<td>0.413</td>
<td>0.469</td>
<td>0.341</td>
<td>0.74 (0.61-0.89)</td>
<td>0.66 (0.52-0.84)</td>
<td>0.71 (0.59-0.85)</td>
</tr>
</tbody>
</table>

$^{a}$HWE among control subjects.
$^{b}$Reference genotype or allele is major homozygote or allele.
$^{c}$Adjusted by age, sex and pack-year of smoking.
$^{d}$Additive model.
$^{e}$After Bonferroni correction.

HWE: Hardy-Weinberg equilibrium; MAF: minor allele frequency; OR: odds ratio; CI: confidence interval.
Figure legends

Figure 1. Flow diagram of study design.

Figure 2. Kaplan-Meier survival curves for recurrence among bladder cancer cases based on miR-146a rs2910164 genotypes.

Figure 3. Effect of miR-146a rs2910164 genotypes on cell proliferation and cell cycle in T24 and EJ cells. (A, B) Forced expression of miR-146a inhibited cell growth in vitro. Cell growth activity was measured using CCK-8 cell proliferation assay kits. Cells from T24 and EJ cells were transfected to express either the miR-146a G allele, miR-146a C allele or control vector. Each value represents the mean of five replicates, and the relative cell growth activity is shown. (C) Representative histograms depicting cell cycle profiles of indicated cells in Vector, G allele, and C allele, respectively. Cells were stained with PI and analyzed by flow cytometry. Various phases of the cell cycle in (D) T24 cells and (E) EJ cells. The results are means of triplicate independent experiments.

Figure 4. Effect of the miR-146a rs2910164 in promoter transcriptional activity. (A) Schematic representation of reporter plasmids containing the 3’-UTR of IRAK1 and TRAF6 genes, which was inserted downstream of the luciferase reporter gene in pGL3-promoter plasmid. (B) 3’-UTR of IRAK1 and TRAF6 genes have many presumed sites in conserved regions that can bind with the seed region of miR-146a. The two constructs were transiently transfected into (C) T24 and (D) EJ cells, respectively. The luciferase activity of each construct was normalized against the internal control of PRL-TK. The data indicate the mean values with the standard deviation (SD) from three independent experiments.
Figure 1

miRBase

ΔDG ≥ 2.8 kcal/mol
MAF ≥ 5%

Five miRNAs SNPs

rs2910164 and rs2682818

miR-146a rs2910164

Functional study

IRAK1/TRAF6
Target mRNA

NF-κB

Cell proliferation
Cell cycle mRNA expression

Training set (683 cases and 728 controls)

Test set (336 cases and 454 controls)

Follow-up study (from test set) (199 bladder cancer cases)

Risk and prognosis Prediction
Figure 2

![Cumulative Recurrence Graph]

- No. at risk:
  - GG: N = 78
  - GC/CC: N = 121

- Time (months):
  - GG: 61, 15, 10, 5, 2, 1, 0, 0
  - GC/CC: 100, 47, 34, 15, 6, 3, 1, 0

- P = 0.016
Genetic variants in microRNAs predict bladder cancer risk and recurrence

Meilin Wang, Haiyan Chu, Pu Li, et al.

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