Genetic Variants in miRNAs Predict Bladder Cancer Risk and Recurrence

Melin Wang, Haiyan Chu, Pu Li, Lin Yuan, Guangbo Fu, Lan Ma, Danni Shi, Dongyan Zhong, Na Tong, Chao Qin, Changjun Yin, and Zhengdong Zhang

Abstract

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 located in miRNAs and used these to evaluate miRNA–disease associations in a two-stage model, consisting of 1,019 bladder cancer cases and 1,182 controls (683 cases and 728 controls in the training set and 336 cases and 454 controls in the test set). We found that 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% confidence interval (CI) = 0.71–0.90, P = 2.92 × 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 that miR-146a rs2910164 C allele inhibited cell proliferation and significantly downregulated 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 show 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. Cancer Res; 72(23): 6173–82. ©2012 AACR.

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 nonmuscle invasive bladder cancer tumors, whereas the remaining cases have invasive tumors (2). Most patients with nonmuscle invasive tumors are treated by transurethral resection, with up to 70% of these cases developing at least 1 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 (SNP) 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 miRNA genes.

miRNAs are a class of small noncoding 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 biologic 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 cancers (13). Specifically, miRNA expression profiles and specific miRNAs have been shown to be associated with bladder cancer tumorigenesis (14–16).

The biologic 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 Fig. 1.

Study population
This study was approved by the Institutional Review Board of Nanjing Medical University (Nanjing, China), and all subjects signed a written informed consent form. Details about subject recruitment in this 2-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 (Nanjing, China) 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 (Nanjing, China) 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 was selected for additional follow-up, including 199 (199/226, 88.1%) nonmuscle invasive bladder cancer cases. Follow-up consisted of telephone calls every 3 months following the initial diagnosis. Of these follow-up cases, 27 (27/226, 11.9%) were excluded because of 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 S7). We conducted extensive searches of SNP databases including HapMap (release #24), dbSNP, and Patrocles, based on 2 criteria: (i) SNPs located in the pre-miRNA or mature sequences, and (ii) minor allelic frequency (MAF) 5% or more in the Chinese population. Among them, 14 SNPs in miRNAs matched both criteria (Supplementary Table S1), and no
additional SNPs were found. Furthermore, the Gibbs binding free energy ($$\Delta G$$, kJ/mol) for each pair of common and variant alleles was computed using miRanda software (20). The difference of the free energies between the 2 alleles was calculated as $$\Delta \Delta G$$. Because the energy parameter $$\Delta G$$ could impact binding of each SNP with the target miRNA, we ranked the values of $$\Delta \Delta G$$, and chose the upper tertile of the distribution as a cut-off level. Finally, we selected 5 SNPs in miRNAs that have a $$\Delta \Delta G$$ of 2.60 kJ/mol and MAF 5% or more 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 5 selected SNPs. The sequence of primers and probes for each SNP is shown in Supplementary Table S6. The samples were read and analyzed from the ABI Prism 7900HT Sequence Detection System using SDS 2.2 software (Applied Biosystems). 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 340 bp DNA fragment, was amplified from a genomic DNA sample with a miR-146a rs2910164 G allele, and cloned into the pEGFP-N3 expression plasmid vector containing a GFP marker (Clontech). 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 2 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 miR-146a rs2910164, and using Lipofectamine 2000 (Invitrogen) 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$$^{-\Delta \Delta CT}$$ 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). The absorbance value of each well was determined at 450 nm using the Infinite M200 spectrophotometer (Tecan). Each time point was assayed in triplicate. Each experiment was repeated 6 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 (PI) for DNA content analysis by flow cytometry on a FACS Calibur system (Beckman Coulter). Results were expressed as a percentage of cells in each cell-cycle phase.

Identification of the potential miRNA targets of miR-146a

Potential miR-146a targets were predicted and analyzed using 3 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 3 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 S5). We then used PicTar and miRanda software to predict the miR-146a targets based on the parameters of PicTar score more than 5.0 and mirSVR score less than −1.50, respectively. As a result, 4 genes (I(R)AK1, TRAF6, IGSF1, and NOVA1) were predicted by at least 2 of these 3 software packages. However, further identification of 2 target genes (I(R)AK1 and TRAF6) was based on their potential roles in other cancers as reported previously (22).

Construction of reporter plasmids and luciferase reporter assay

To construct luciferase reporter plasmids, I(R)AK1 and TRAF6 3’-untranslated region (UTR) fragments (i.e., 677 bp and 1,368 bp) were inserted at the XbaI site, downstream of the luciferase gene in the pGL3-promoter vector (Promega; Fig. 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) and normalized against the activity of the plasmid Renilla luciferase-thymidine kinase (PRL-TK). Independent triplicate experiments were carried out for each plasmid construct.

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 more than 85% of malignant cells were selected. Detailed clinical information is shown in Supplementary Table S4. 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). I(R)AK1 and TRAF6 miRNA expression levels were evaluated using the comparative CT method. The transcription level of GAPDH was used as an internal control.
Real-time PCR was conducted 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 χ² test (for categorical variables). Hardy–Weinberg equilibrium (HWE) was tested using a goodness-of-fit χ² test. Unconditional univariate and multivariate logistic regression analyses were conducted to obtain estimates for associations between miRNA SNPs and bladder cancer risk by computing ORs and their 95% confidence intervals (CI). Bonferroni correction for multiple testing was applied. The heterogeneity between subgroups was assessed with the χ²-based Q test. In the test set, the associations between miR-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 proportional hazards models were used to adjust for potential confounders. The Spearman test was used to analyze the correlation between miR-146a expression and IRAKI and TRAF6 mRNA expression levels. A P < 0.05 was considered statistically significant. All tests were 2-sided and were conducted using SAS software (version 9.1.3; SAS Institute, Inc.).

Results
Characteristics of cases
The distribution of selected characteristics between bladder cancer cases and control subjects in the 2-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 2 sets.

Effects of selected SNPs in miRNAs and bladder cancer risk
We evaluated genotype frequencies of the 5 miRNA SNPs among cases and controls and their associations with bladder cancer risk in the training set. The primary information and

<table>
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*Training set and test set merged.
allele frequencies observed are listed in Table 2. All genotype distributions of control subjects were consistent with those expected from HWE. 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 2 SNPs (rs2910164 in miR-146a and rs2682818 in miR-618) were significant \( (P = 0.004 \text{ for miR-146a, } P = 0.013 \text{ for miR-618}) \) 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 \( \text{OR} = 0.80, \text{95% CI} = 0.71–0.90, P = 2.920 	imes 10^{-4} \). When we conducted stratified analyses by age, sex, smoking status, grade, and stage (Supplementary Table S2 and Fig. 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 nonmuscle 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 Fig. 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 2 groups was statistically significant (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 1 rs2910164 C allele \( \text{HR} = 0.58, \text{95% CI} = 0.36–0.94; \text{Supplementary Table S3}) \). 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 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 Fig. S2, 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 of 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 Fig. 3A and B, the Cell Counting Kit (CCK)-8 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...

![Figure 3. Effect of miR-146a rs2910164 genotypes on cell proliferation and cell cycle in T24 and EJ cells. A and 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 3 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 T24 cells (D) and EJ cells (E). The results are means of triplicate independent experiments.](image-url)
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 (Fig. 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 of 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; Fig. 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, 2 genes were selected as candidate targets of miR-146a. As shown in Fig. 4B, the 3'-UTR of IRAK1 and TRAF6 have 2 or 3 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 (Fig. 4A), and used them for transient transfections with bladder cancer cell lines. As shown in Fig. 4C and D, vectors transfected with miR-146a rs2910164 C allele had a 24% to 55% decrease in relative luciferase activities, compared with those of 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 correlated 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 mRNA 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 Fig. S3). 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 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 that 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 and colleagues (23) reported that miR-146a rs2910164 C allele is associated with higher expression levels of miRNA compared with the G allele.

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 and colleagues (23) and Hoffman and colleagues (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 and colleagues 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 conducted 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 2 independent cohorts, which is consistent with previous findings for this disease (29–34). However, some studies have reported opposite findings in other cancers. Specifically, Jadzewsiki and colleagues first observed that miR-146a rs2910164 was associated with increased predisposition to papillary thyroid carcinoma due to altered processing that resulted in lower expression of the mature sequence (22). Yang and colleagues (28) and Mittal and colleagues (35) failed to replicate the significant association of miR-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 miR-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 Fig. S4), 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 G allele was significantly associated with lower expression levels of miR-146a compared with the G allele.

SNPs in miRNA genes could potentially impact various biologic processes by influencing target genes. For example, Duan and colleagues found that miR-125a reduced miRNA-mediated translational suppression via Lin-28 (39). Integrating bioinformatics and experimental assays, we identified IRAK1 and TRAF6 as 2 direct functional downstream targets of miR-146a in bladder cancer cells. It has been known that NF-kB 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, miR-146a rs2910164 C allele–triggered inhibition of invasion and migration could be mediated through the downregulation of IRAK1 and TRAF6, coupled with subsequent inactivation of NF-kB. Furthermore, others have observed that miR-146a could block cancer cell invasion and metastasis through repression of IRAK1 and subsequent inactivation of NF-kB (41–43). A recent study suggested that
miR-146a downregulates NF-κB activity via targeting TRAF6 and functions as a tumor suppressor (44). Therefore, miR-146a 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 (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 (1,019 bladder cancer cases and 1,182 control subjects) among our 2-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 biologic marker and as a therapeutic target in bladder cancer. Larger prospective studies are needed to confirm our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Wang, Z. Zhang

Development of methodology: M. Wang, H. Chu, P. Li, L. Ma, D. Shi, D. Zhong

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Li, L. Yuan, G. Fu, Q. Cen, C. Yin

Analysis and interpretation of data (e.g., statistical analysis, biosubjects: M. Wang, H. Chu, N. Tong, Z. Zhang administration, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Ma, D. Shi, D. Zhong

Study supervision: Z. Zhang

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