Functional genetic variations in the IL-23 receptor gene are associated with risk of breast, lung and nasopharyngeal cancer in Chinese populations

Jian Zheng1,†, Lan Jiang1,†, Liyuan Zhang1,†, Lei Yang1, Jieqi Dong1, Yonghe You1, Na Li1, Hongchun Wu1, Wei Li1, Jiachun Lu1 and Yifeng Zhou1,2

1Laboratory of Cancer Molecular Genetics, Medical College of Soochow University, Suzhou 21502, China, 2Department of Radiotherapy & Oncology, The Second Affiliated Hospital of Soochow University, San Xiang Road No. 1055, Suzhou 215004, China and 3The Institute for Chemical Oncology, The Second Affiliated Hospital of Soochow University, San Xiang Road No. 1055, Suzhou 215004, China and 4The Institute for Chemical Oncology, The Second Affiliated Hospital of Soochow University, San Xiang Road No. 1055, Suzhou 215004, China and 5The Institute for Chemical Oncology, The Second Affiliated Hospital of Soochow University, San Xiang Road No. 1055, Suzhou 215004, China and 5The Institute for Chemical Oncology, The Second Affiliated Hospital of Soochow University, San Xiang Road No. 1055, Suzhou 215004, China

†These authors contributed equally to this work.

Interleukin-23 receptor (IL-23R) is a key element in the T-helper 17 cell-mediated inflammatory process, which plays an important role in the pathogenesis of cancer. In this study, we examined whether genetic polymorphisms in IL-23R are associated with cancer risk in 4936 cancer patients and 5664 control subjects from eastern and southern Chinese populations. We found that the C allele of the rs10889677A>C polymorphism in the 3'-untranslated region of IL-23R was inversely associated with risk of multiple types of cancer, including breast cancer, lung cancer and nasopharyngeal carcinoma. Healthy controls who harbored the rs10889677C allele had significantly decreased cancer risk (odds ratio = 0.74, 95% confidence interval = 0.71–0.78) compared with those who harbored the rs10889677A allele. Biochemical analysis demonstrated that the rs10889677A allele disrupted the binding site for the microRNA miR-let-7f, thereby increasing the transcription of the IL-23R in vitro and in vivo. Furthermore, cancer-free individuals carrying the rs10889677C homozygous genotype had a lower proportion of regulatory T cells (Tregs) and a higher T-cell proliferation rate upon stimulation with concanavalin A than individuals carrying the rs10889677A allele. Our findings indicate that the IL-23R rs10889677A>C polymorphism may influence T-cell proliferation, resulting in changes in the levels of Tregs in vivo and modifying cancer susceptibility.

Introduction

The interleukin-23 receptor (IL-23R) is composed of IL-23R and IL-12Rβ1 subunits, which is shared with IL-12R (1). IL-23/IL-23R is essential for the T-helper 17 (Th17) cell-mediated immune response (2,3). Th17 cells are a recently discovered proinflammatory CD4+ effector T-cell population that contributes to pathogen clearance and tissue inflammation by expressing high levels of the proinflammatory cytokine IL-17 in response to stimulation (4). Recently, the novel inflammatory pathway axis IL-23R→IL-12R→STAT3→Th17→IL-17/IL-17F (IL-23R→Th17 axis) has been shown to play a pivotal role in inflammatory and autoimmune diseases (5). IL-23R plays an important role in the initiating, maintaining and accelerating the IL-23/IL-17 inflammatory signal transduction pathway (6).

Studies on the roles of IL-17, IL-23, IL-23R and Th17 cells in cancer have produced largely contradictory or controversial results. However, IL-17 expression in tumors has been correlated with increased tumor microvascular density, tumor differentiation and a generally unfavorable prognosis (7). Moreover, previous studies have indicated that IL-23R can promote tumor growth and may decrease immunosurveillance by CD8+ T cells (8). IL-23R signaling in regulatory T cells (Tregs) may also promote the immunosuppressive function of Tregs in the tumor microenvironment, facilitating evasion of the immune system by the tumor (9–11). Interestingly, both Th17 cells and Tregs can promote tumor growth, and evidence suggests that there may be an association between these two T-cell subtypes (12). These findings suggest that IL-23R may play an important role in cancer development and progression.

IL-23R is encoded by the IL-23R gene, which maps within 151 kb of the IL1R2B gene on chromosome 1 (1p31.2–32.1). Several lines of evidence suggest that cellular immune surveillance play a pivotal role in the development of cancer, including breast cancer, lung cancer and nasopharyngeal carcinoma (13–16). Previous studies have reported that single nucleotide polymorphisms (SNPs) in IL-23R may be associated with the risk of gastric and esophageal cancer (17–19); however, the association between the SNPs in IL-23R and risk for breast cancer, lung cancer and nasopharyngeal carcinoma in Chinese population remains unclear, and biological functions of these SNPs have not yet been elucidated. We hypothesized that IL-23R SNPs enhance the IL-23/IL-17 pathway and may play a role in human cancer development. To test this hypothesis, we investigated the association between various IL-23R genotypes and the risk for the development of multiple types of cancer in two Chinese populations.

Materials and methods

Study subjects

Overall, all subjects in this study were unrelated Han Chinese from eastern or southern Chinese populations. There were no age, stage and histology restriction for cancer patients. Patients or controls who recently (in last 6 months) had blood transfusions were excluded. All healthy controls had no documented history of cancer and were frequency matched to each set of cancer patients based on their age (±5 years), sex and residential area (urban or rural). In the family history section of the interview, all subjects, self and surrogate respondents, were first asked the number of brothers and sisters they had. Subjects were then asked whether there was a history of all malignant tumors in the immediate family, which included fathers, mothers, brothers and sisters. The control subjects for breast cancer patients were all female. The tumor–node–metastasis classification and tumor staging were evaluated according to the 2002 American Joint Committee on Cancer staging system. Study subjects’ demographics are summarized in Supplementary Tables 1–3, available at Carcinogenesis Online. In the eastern Chinese population, patients with breast cancer (n = 1010), lung cancer (n = 503) and nasopharyngeal carcinoma (n = 684) were recruited from the First Affiliated Hospital of Soochow University (Suzhou) between March 2001 and May 2009 with a response rate of 89%. We selected these cancer sites because the samples were available to us from previous studies in our laboratory (20–22). The eastern Chinese healthy controls (n = 2701) were randomly selected from a database of 3500 individuals who underwent a hospital-based physical examination annually (regular check-up/preventive care) in the Suzhou city during the same time period, in which the cancer patients’ blood samples were collected, with a response rate of 90%. In the southern Chinese population, patients with breast cancer (n = 804), lung cancer (n = 1056) and nasopharyngeal carcinoma (n = 906) were recruited from the Tumor Hospitals affiliated with Guangzhou Medical University between 2002 and 2009 with a response rate of 91%. The southern Chinese healthy controls (n = 2963) were randomly selected from a pool of 3000 individuals who also participated in a hospital-based screening program for health check-ups conducted in the Guangdong province during the same time period, in which cancer patients’ blood samples were collected, with a response rate of 92%.

Our study included two independent case-control data sets (Supplementary Figure 1, available at Carcinogenesis Online) that were described previously (20,21). The discovery set included 1010 breast cancer patients and...
1014 sex, age and residential area (urban or rural) matched healthy controls from the eastern Chinese population (Supplementary Table 1, available at Carcinogenesis Online). The validation set included patients with lung cancer (n = 503), nasopharyngeal carcinoma (n = 684) and 1687 sex, age and residential area (urban or rural) matched healthy controls from the eastern Chinese population, as well as patients with breast cancer (n = 804), lung cancer (n = 1056), nasopharyngeal carcinoma (n = 906) and 2963 sex, age and residential area (urban or rural) matched healthy controls from the southern Chinese population (Supplementary Tables 1–3, available at Carcinogenesis Online).

For functional assays, sufficient blood samples were obtained from 37 healthy volunteers (20 males and 17 females) between the ages of 19 and 49 years, and 37 healthy controls were randomly selected from volunteers including 186 healthy individuals as determined by physical examination. At the time of recruitment for the study, informed consent was obtained from each subject, and this study was approved by both the medical ethics committee of Soochow University and the institutional review boards of Guangzhou Medical University.

SNP selection
Using the dbSNP database (http://www.ncbi.nlm.nih.gov/) and two bioinformatics analysis software programs (Miranda Java Interface v1.0 and http:// snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm), we found five functional SNPs either in the promoter region, coding exon or 3′UTR of the IL-23R gene with minor allele frequencies of >5% in the Chinese population (see Supplementary Materials and methods, available at Carcinogenesis Online). A bioinformatic-based analysis was performed on these polymorphisms using Haploview software 4.2 to analyze the haplotype block based on the Chinese Han Beijing (23) population data of HapMap (HapMap Data Release 27 Phase II + III, February 2009, on NCBI B36 assembly, dbSNP b126). There was one haplotype block in the Chinese population (Supplementary Figure 2A, available at Carcinogenesis Online). The haplotype-tagging SNPs were selected with the Haploview software 4.2 Tagger program; it was found that the rs6683039, rs6682925 and rs1884444 SNPs covered the haplotype in block 1 at a 100% frequency (rs6683039 and rs6682925: D′ = 1.0, r2 = 0.94; rs6683039 and rs11465754: D′ = 0.937, r2 = 0.877; rs6683039 and rs1884444: D′ = 1.0, r2 = 0.94; the SNP rs10889677 outside the block. Therefore, the SNPs rs6683039, rs6682925, rs1884444 and rs10889677 were chosen as four functional potential SNPs in the IL-23R gene to be analyzed for their associations with risk of various cancers.

Genomic analysis
Genomic DNA was isolated from peripheral blood lymphocytes of all the study subjects. All subjects were genotyped for the rs6682925, rs6683039, rs18844444 and rs10889677 SNPs by using the allele-specific matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (Sequenom, San Diego, CA) (21). A total of 80 samples were randomly selected for direct sequencing to confirm the genotyping results from the mass spectral analysis, and the results were in 100% agreement. Approximately, 10% of the samples were also randomly selected for a blinded repeat of the genotyping-based prior knowledge of the previous genotyping results or the status of being a case and control, and the results were in 100% agreement.

IL-23R 3′UTR luciferase constructs generation
The 3′UTR of Renilla luciferase in the vector pScheck2-2 (Promega, Madison, WI) was replaced with the full-length 3′UTR of the IL-23R gene containing either the rs10889677C or rs10889677A allele. The resulting constructs (pScheck2-2-IL-23R-3′UTR-A-allele and pScheck2-2-IL-23R-3′UTR-C-allele) were verified by sequencing (see Supplementary Materials and methods, available at Carcinogenesis Online).

Transient transfections and luciferase assays
The 293T or Jurkat cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. All analyses were performed at an identical passage and were suspended in RPMI 1640 supplemented with 1% Nutridoma SP (Boehringer Mannheim) and 1% fetal bovine serum. The samples were dispensed into culture wells, and the cells were stimulated with concanavalin A (ConA; Sigma–Aldrich) at a final concentration of 10 µg/ml.

CFDA-SE labeling of PBMCs
The diacetate form of CFDA-SE is a nontoxic, nonfluorescent molecule that can positively diffuse into cells and, once inside the cells, forms CFSE, which emits light with characteristic wavelengths, producing a homogeneous labeling of cell populations. CFDA-SE was used to assay lymphocyte proliferation as described previously (see Supplementary Materials and methods, available at Carcinogenesis Online). The samples were analyzed in triplicate in final volumes of 100 µl in 96-well plates. The samples were treated with ConA (0.3–40 µg/ml). To evaluate DNA synthesis, 0.5 µCi [3H]-thymidine (6.7 Ci/mmol; ICN; 1 Ci = 37 GBq) was added to each well, and after 8 h, the cells were harvested onto glass-fiber filters with a PHD cell harvester (Cambridge Technology, Watertown, MA). The filters were air dried, and radioactivity was measured by liquid scintillation spectroscopy on a Betaplate scintillation counter (LBK).

Enzyme-linked immunosorbent assay
A total of 41 serum samples were randomly selected from a serum database including 237 healthy individuals as determined by physical examination. The serum levels of IL-17 and IL-10 were measured with an enzyme-linked immunosorbent assay kit specifically designed (BioLegend, San Diego, CA) for the detection of IL-17 and IL-10 protein levels according to the manufacturer’s instructions. All analyses were performed in a blinded fashion in which the laboratory personnel were unaware of the genotyping data.

Flow cytometry analysis
A flow cytometer (FACSCalibur; BD Biosciences) was used to measure the cell surface expression of IL-23R, CD39, CD8 and CD4 as well as the intracellular expression of FOXP3 (see Supplementary Materials and methods, available at Carcinogenesis Online). Data were analyzed with CELLQUEST (BD Immunocytometry Systems) and FlowJo (Tree Star, Ashland, OR) software.

Peripheral blood mononuclear cell isolation and in vitro sensitization
Peripheral blood mononuclear cells (PBMCs) (1 × 10^6 cells/ml) were isolated by Ficoll–Paque (Amersham Pharmacia Biotech) density-gradient centrifugation and were suspended in RPMI-1640 supplemented with 1% Nutridoma SP (Boehringer Mannheim) and 1% fetal bovine serum. PBMCs were dispensed into culture wells, and the cells were stimulated with concanavalin A (ConA; Sigma–Aldrich) at a final concentration of 10 µg/ml.

Measurement of [3H]-thymidine incorporation
PBMCs (1 × 10^6 cells/ml) from 37 subjects were cultured in RPMI-1640 supplemented with 1% Nutridoma SP (Boehringer Mannheim) and 1% fetal bovine serum. The samples were analyzed in triplicate in final volumes of 100 µl in 96-well plates. The samples were treated with ConA (0.3–40 µg/ml). To evaluate DNA synthesis, 0.5 µCi [3H]-thymidine (6.7 Ci/mmol; ICN; 1 Ci = 37 GBq) was added to each well, and after 8 h, the cells were harvested onto glass-fiber filters with a PHD cell harvester (Cambridge Technology, Watertown, MA). The filters were air dried, and radioactivity was measured by liquid scintillation spectroscopy on a Betaplate scintillation counter (LBK).

Statistical analysis
Chi-squared tests were used to assess the differences in the age and sex distributions of the cases and controls as well as differences in the various polymorphisms and the presence of disease. The Hardy-Weinberg Equilibrium (HWE) was tested with a goodness-of-fit chi-squared test to compare the expected genotype frequencies with the observed genotype frequencies (p2 + 2pq + q2 = 1) in the cancer-free controls. Because we frequency matched the case and control on sex and age, only the unconditional logistic regression model was used to estimate the association between the SNPs and cancer risk. The cancer risk associated each SNP was measured by odds ratio (OR) and its corresponding 95% confidence interval (CI) by using an unconditional logistic regression model with adjustments for age, gender, smoking status, drinking status, body mass index (BMI) and family history of cancer (24). The recurrent tumors as well as the T-cell proliferation rates and Treg levels in 37 healthy control subjects. All tests were two sided and were performed with the SAS software (version 9.1, SAS Institute, Cary, NC, USA). A P value < 0.05 was considered statistically significant.
Results

Identification of IL-23R SNPs associated with cancer susceptibility

Four candidate SNPs of the IL-23R gene were genotyped in a discovery set consisting of 1010 breast cancer patients and 1014 cancer-free controls from the eastern Chinese population (Table I). A significant association with breast cancer risk was observed for the rs10889677 SNP, but the other SNPs, rs6682925, rs6683039 and rs1884444, were not significantly associated with the risk of breast cancer (Table I). Genotype distributions of all SNPs were found to be consistent with HWE. We also performed a linkage disequilibrium analysis of the control samples and found that the rs10889677 locus was not in high linkage disequilibrium with the other three SNPs (compared with rs1884444, \( r^2 = 0.023 \) and \( r^2' = 0.987 \); compared with rs1884444, \( r^2 = 0.026 \); compared with rs6682925, \( D' = 0.186 \) and \( r^2 = 0.023 \); however, the rs6683039 locus was in high linkage disequilibrium with the other two SNPs (compared with rs6682925, \( D' = 1 \) and \( r^2 = 0.987 \); compared with rs1884444, \( D' = 1 \) and \( r^2 = 0.726 \)).

From the analysis of the validation set, the rs10889677 SNP was selected for further analysis of the association with a decreased risk for breast, lung and nasopharyngeal cancer (Table II). The genotype distributions of all SNPs in these analyses also agreed with HWE (Table II). The ORs associated with breast, lung and nasopharyngeal cancer were adjusted for age, sex, smoking status, drinking status, BMI and family history of all malignant tumors (Table II).

Table I. Associations between candidate SNPs in IL-23R and risk of breast cancer in the discovery set of an eastern Chinese population, recruited between 2001 and 2009

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients (n = 1010)</th>
<th>Controls (n = 1014)</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
<th>( P_{\text{total}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6682925</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>385 (38.12)</td>
<td>361 (35.60)</td>
<td>1.00 (Reference)</td>
<td>1.00 (Reference)</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>479 (47.42)</td>
<td>487 (48.03)</td>
<td>0.91 (0.76–1.12)</td>
<td>0.92 (0.74–1.10)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>146 (14.46)</td>
<td>166 (16.37)</td>
<td>0.83 (0.63–1.08)</td>
<td>0.82 (0.64–1.06)</td>
<td>0.149</td>
</tr>
</tbody>
</table>

rs6683039

| TT       | 369 (36.53)         | 346 (34.12)         | 1.00 (Reference) | 1.00 (Reference)    |                  |
| TC       | 483 (47.83)         | 500 (49.31)         | 0.91 (0.74–1.10) | 0.92 (0.73–1.14)    |                  |
| CC       | 158 (15.64)         | 168 (16.57)         | 0.89 (0.67–1.16) | 0.88 (0.64–1.19)    | 0.277            |

rs1884444

| TT       | 528 (52.28)         | 510 (50.30)         | 1.00 (Reference) | 1.00 (Reference)    |                  |
| TC       | 379 (37.52)         | 406 (40.04)         | 0.90 (0.73–1.09) | 0.93 (0.75–1.08)    |                  |
| GG       | 103 (10.20)         | 98 (9.66)           | 1.02 (0.74–1.39) | 0.99 (0.72–1.32)    | 0.623            |

rs10889677

| AA       | 522 (51.68)         | 463 (45.66)         | 1.00 (Reference) | 1.00 (Reference)    |                  |
| AC       | 422 (41.78)         | 432 (42.60)         | 0.87 (0.72–1.05) | 0.86 (0.69–1.02)    |                  |
| CC       | 66 (6.54)           | 119 (11.74)         | 0.49 (0.35–0.69) | 0.51 (0.36–0.72)    | 0.0001           |

\[ ^a\text{Data were calculated by unconditional logistic regression; adjusted for age, BMI and family history of all malignant tumors.} \]
\[ ^b\text{Tests for trend of odds were two sided and based on likelihood ratio tests assuming a multiplicative model.} \]
(95% CI 0.52–0.95) to 0.85 (95% CI 0.69–1.01) or 0.44 (95% CI 0.31–0.63) to 0.64 (95% CI 0.44–0.93), respectively, when compared with the IL-23R rs10889677TA genotype. These associations were confirmed in the southern Chinese population, in which the ORs associated with the IL-23R rs10889677CC genotype for risk of breast cancer, lung cancer and nasopharyngeal carcinoma patient groups were 0.44 (95% CI 0.31–0.63), 0.49 (95% CI 0.34–0.69) and 0.55 (95% CI 0.38–0.79), respectively, when compared with the IL-23R rs10889677TA genotype. After adjusting for age, sex, smoking status, drinking status, BMI and family history of all malignant tumors, there were no significant changes in the respective ORs. FPRP analysis was also performed and revealed that, assuming a prior probability of 0.001 and a prior OR of 0.67, as suggested by Wacholder et al. (25), the FPRP for the observed association between the IL-23R rs10889677TA allele and the risk for all cancers yielded a value of 0.09, which was lower than the pre-set FPRP level of 0.20.

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry was used to genotype for rs6882925, rs6883039 and rs1884444 polymorphisms in breast, lung and nasopharyngeal cancer patients and healthy controls from eastern and southern Chinese populations. However, no significant association was observed between these polymorphisms and the risk of these cancers in either population or in their combination, as shown in Supplementary Table 4, available at Carcinogenesis Online.

IL-23R rs10889677TA>C genotypes affect IL-23R expression by inhibiting the binding of miR-let-7f in vitro

According to two bioinformatics analysis software programs (MIRanda Java Interface v1.0 and http://snpinfo.niehs.nih.gov/snpfunc.htm), the IL-23R rs10889677TA>C polymorphism lies within a predicted binding site for human miR-let-7f. Therefore, we hypothesized that miR-let-7f would bind tightly to IL-23R mRNA transcripts containing the rs10889677TA allele, resulting in the downregulation of IL-23R expression. Conversely, the binding of miR-let-7f to mRNA transcripts containing the rs10889677CA allele would be disrupted, resulting in the upregulation of IL-23R expression. To test this hypothesis, the human embryonic kidney cell line 293T was transiently co-transfected with miR-let-7f and either of the polymorphic reporter constructs and assessed for the luciferase activity. Compared with the construct containing the rs10889677TA allele, the construct containing the rs10889677CC allele exhibited significantly reduced luciferase activity in a concentration-dependent manner (Figure 1A). The same experiments were repeated using Jurkat cells, which generated similar results (Figure 1B).

Jurkat cells and 293T cells were also co-transfected with the psiCHECK-2-IL-23R-3′UTR vector, 40 pmol miR-let-7f and the miR-let-7f inhibitor. The miR-let-7f inhibitor is a single-stranded antisense RNA molecule that complements and knocks down endogenous miR-let-7f. In both cell lines, no significant differences in luciferase activity were observed in the presence of the micro RNA-let-7f inhibitor (Figure 1C). These results suggest that miR-let-7f negatively may regulate the transcription of IL-23R and that miR-let-7f regulation may be inhibited by the presence of the rs10889677CA allele.

Association of IL-23R rs10889677TA>C genotypes with IL-23R expression

To further explore the effects of IL-23R rs10889677TA>C on the transcription of the IL-23R gene, we used PBMCs from 37 cancer-free controls (6 rs10889677CC, 19 rs10889677AC and 12 rs10889677AA). The effect of the rs10889677TT SNP on the IL-23R mRNA expression was quantified by real-time PCR. Subjects with the rs10889677TT genotypes expressed significantly higher IL-23R mRNA levels (0.29 ± 0.07) than those with the rs10889677AC (0.10 ± 0.03) or rs10889677CC (0.08 ± 0.04; ANOVA test; P = 0.007; linear regression test: P = 0.004, Figure 1D). To determine whether miR-let-7f is constitutively expressed in PBMCs, quantitative real-time PCR was used to examine miR-let-7f expression. miR-let-7f was constitutively expressed in PBMCs; however, there was no significant association between the background expression of miR-let-7f and the IL-23R rs10889677TA>C genotypes in PBMCs collected from cancer-free individuals (0.346 ± 0.074 for AA; 0.322 ± 0.049 for AC and 0.298 ± 0.068 for CC; ANOVA test: P = 0.836; linear regression test: P = 0.562, Supplementary Figure 2B, available at Carcinogenesis Online).

Fig. 1. Representative graph of luciferase activity of variant allele on luciferase reporter genes bearing 3′UTR segments from human IL-23R in 293T (A) and Jurkat cells (B). Relative luciferase activity of the psiCHECK-2-IL-23R-3′UTR-A-allele and psiCHECK-2-IL-23R-3′UTR-C-allele constructs co-transfected with miR-let-7f and inhibitor in 293T and Jurkat cells (C). Renilla luciferase activity was measured and normalized to firefly luciferase. Six replicates were carried out for each group, and the experiment was repeated at least three times. Data are mean ± standard error of the mean. **P < 0.05; ***P < 0.01 compared with A allele. (D) IL-23R mRNA expression level in PBMCs from healthy individuals as a function of IL-23R genotype (6 rs10889677CC, 19 rs10889677AC and 12 rs10889677AA); data are mean ± standard error of the mean, normalized to β-actin, P = 0.007.
The expression level of IL-23R in PBMCs from healthy individuals was assessed by flow cytometry. The percentage of CD4+ T cells expressing IL-23R in PBMCs from healthy individuals with the rs10889677AA genotype (0.89±0.07%) was significantly higher than the percentage of CD4+ T cells from healthy individuals with the rs10889677AC (0.68±0.05%) or rs10889677CC genotypes (0.51±0.06%; ANOVA test: P = 0.0025; linear regression test: P = 0.0018). CD4+ T cells from subjects with the rs10889677AA genotype (708.43±37.19) also had a higher IL-23R mean fluorescence intensity than CD4+ T cells from subjects with the rs10889677AC (632.42±43.59) or rs10889677CC genotypes (513.91±40.56; ANOVA test: P = 0.0189; linear regression test: P = 0.0075; Figure 2A). Similarly, the percentage of CD8+ T cells expressing IL-23R in PBMCs from healthy individuals with the rs10889677AA genotype (1.08±0.14%) was higher than in PBMCs from healthy individuals with the rs10889677AC (0.78±0.07%) or rs10889677CC genotypes (0.58±0.07%; ANOVA test: P = 0.0034; linear regression test: P = 0.0003). CD8+ T cells from subjects with the rs10889677AA genotype also had a higher IL-23R mean fluorescence intensity (680.33±94.47) than those from healthy individuals with the rs10889677AC (610.72±30.40) or rs10889677CC genotypes (452.91±30.15; ANOVA test: P = 0.0027; linear regression test: P = 0.0043; Figure 2B).

Effects of the IL-23R rs10889677A>C genotypes on T-cell proliferation

We next sought to elucidate whether the different IL-23R rs10889677A>C genotypes affect the T-cell proliferation rate in vitro. The median percentage of CD4+ cells in PBMCs from healthy individuals expressing the IL-23R rs10889677AA genotype (19.61±1.59%) was significantly lower than that in PBMCs from healthy individuals expressing the rs10889677AC (26.87±1.26%) or rs10889677CC genotypes (33.03±2.14%; ANOVA test: P < 0.0001; linear regression test: P < 0.0001; Figure 3A). Similarly, the median percentage of CD8+ cells in PBMCs from healthy individuals expressing the IL-23R rs10889677AA genotype (15.19±1.64%) was also significantly lower than that in healthy individuals expressing the rs10889677AC (22.86±0.71%) or rs10889677CC genotypes (27.15±2.25%; ANOVA test: P < 0.0001; linear regression test: P < 0.0001; Figure 3B).

In addition, [3H]-thymidine incorporation assays were performed to detect T-cell proliferation after stimulation with ConA. PBMCs from healthy individuals expressing the IL-23R rs10889677AA genotype (1.34±0.10) exhibited a significantly lower proliferation rate than PBMCs from healthy individuals expressing the rs10889677AC (1.56±0.12) or rs10889677CC genotypes (3.31±0.58; ANOVA test: P = 0.0002; linear regression test: P = 0.0003; Figure 3C).

Serum levels of IL-17 and IL-10 in healthy controls with different IL-23R rs10889677A>C genotypes

The IL-23R signaling may trigger the development of the Th17 population and promote the immunosuppressive function of Tregs, thus leading to specific changes in the expression of the downstream cytokines IL-17 and IL-10 (26). Therefore, we assessed IL-17 and IL-10 protein levels in serum samples from 41 cancer-free controls according to the different rs10889677A>C genotypes (6 rs10889677CC, 17 rs10889677AC and 18 rs10889677AA). Our result revealed that subjects with the rs10889677AA genotype had significantly higher IL-17 levels (mean±standard error of the mean, 29.28±0.557 pg/ml) than those with the rs10889677AC (25.45±0.631 pg/ml) or rs10889677CC genotypes (22.11±1.007 pg/ml; ANOVA test: P < 0.001; linear regression test: P < 0.001), as shown in Figure 4B. Similarly, healthy individuals with the rs10889677AA (19.75±0.486 pg/ml) genotype exhibited higher IL-10 levels than those with the rs10889677AC (16.18±0.531 pg/ml) or rs10889677CC genotypes (13.96±0.802 pg/ml; ANOVA test: P < 0.001; linear regression test: P < 0.001; Figure 4C).
A stratified analysis assessing the associations between the IL-23R rs10889677A>C genotypes and the risk of various cancers was conducted. However, no significant association was found between the various rs10889677A>C genotypes and breast, lung and nasopharyngeal cancer risks (Supplementary Figure 3, available at Carcinogenesis Online).

**Discussion**

The IL-17/IL-23 inflammation pathway plays an important role in the regulation of cancer development and tumor immunity, but the mechanisms involved are poorly understood. Elucidating the role of IL-17/IL-23 and Th17 cells may aid in understanding the regulation of the inflammation response in tumor development and may provide novel ways of modulating the cancer microenvironment in patients. In this study, we have shown that the risks of breast, lung and nasopharyngeal cancer are inversely associated with the rs10889677C allele, which itself alters miR-let-7f-mediated IL-23R expression. In addition, the IL-23R rs10889677A allele was correlated with an increase in the proportion of Tregs in vivo and a lower T-cells proliferation rate in vitro relative to the IL-23R rs10889677C allele. The association between this polymorphism and several cancers with diverse etiologies raises the possibility that IL-23R variant might be a common susceptibility factor for human cancer.

The importance of IL-23R in tumor development and its influence on tumor immunity has been well recognized (7). Therefore, it is biologically reasonable that functional IL-23R polymorphisms may play a role in the development of cancer. In fact, studies have shown that IL-23R polymorphisms are associated with susceptibility to gastric cancer (19). In a previous study of gastric cancer, which included 941
cancer patients and 775 Chinese (Guangzhou) control subjects, Chen et al. (17) found that the rs10889677C allele was associated with a significantly reduced risk of gastric cancer, compared with the more common rs10889677A allele. Two other independent studies of Chinese populations have shown that the rs10889677C allele may increase the risk of oral cancer (27) and ovarian cancer (28), compared with the rs10889677A allele. Nevertheless, these two studies had a relatively small number of study subjects (240 oral cancer patients and 240 Taiwanese control subjects; 96 ovarian cancer patients and 115 Chinese control subjects), which did not have adequate statistical power to draw strong conclusions (20.2% and 14.4%, respectively). Our study achieved a >99.0% statistical power (two-sided test, \( \alpha = 0.05 \)) to detect an OR of 0.74 for the rs10889677C variant allele (which occurred at a frequency of 0.330 in the controls). The FPRP for the observed association between the \( IL-23R \) rs10889677C allele and the risk for all cancers yielded a value of 0.09, which was lower than the pre-set FPRP level of 0.20, suggesting that this finding is noteworthy. Recently, several genome-wide association studies (GWAS) have reported several novel SNPs (rs2046210, rs11249433, rs2981582, rs3817198, rs889312 and so on) that are associated with the development of breast cancer (29–35); however, most of the studies were based on Caucasians (29–33,35) and only one GWAS was based on Chinese (34). A GWAS of nasopharyngeal carcinoma also reported three susceptibility loci in Chinese population, including rs9510787, rs6774494 and rs1412829 (36). Interestingly, candidate genes of these loci may relate to the immune response (37–39). The \( IL-23R \) gene, which maps to chromosome 1 (1p31.2~32.1), GWAS in cancers, including breast, lung and nasopharyngeal cancers. We have also indicated that these loci may be associated with the development of cancers in Asian populations (40,41). Kiyotani et al. (40) identified loci at 1p31 associated with clinical outcomes of breast cancer patients with tamoxifen treatment in Japanese, and our previous GWAS data in Chinese populations have also indicated that these loci may be associated with lung cancer risk (42). Due to the limitation of SNP coverage in the Affymetrix Genome-Wide SNP Array 6.0 chip, previous GWAS regarding cancer in Chinese patients do not include these SNPs (34,42,43); therefore, the association between \( IL-23R \) rs10889677A>C polymorphism and the risk for cancer in Chinese population remains unclear, and case-control studies with large sample sizes and different cancer types are needed.

Evidence for the biological function of the \( IL-23R \) rs10889677A>C SNP has been limited in previous studies. Our results demonstrate that the \( IL-23R \) rs10889677A>C SNP may affect \( IL-23R \) expression by modifying miR-let-7i binding to the 3'UTR of the \( IL-23R \) gene. miR-let-7i binding affects the expression of \( IL-23R \) on the cell surface, thus influencing the \( IL-23R \)-mediated pathway and the immunosuppressive function of Tregs in the tumor environment. Li et al. (44) have shown that miR-let-7i inhibits \( IL-23R \) expression in human CD4+ memory T cells, and transfection of memory T cells with miR-let-7i mimic results in the downregulation of \( IL-23R \) and the downstream cytokine IL-17. Previous studies have reported that miR-let-7i may be a tumor suppressor gene that plays an important role in cancer development (45). IL-23R signaling in Tregs promotes an immunosuppressive phenotype in the tumor environment (46), which promotes carcinogenesis. Tumor-associated Tregs express the \( IL-23R \), which activates Stat3, resulting in the upregulation of the immunosuppressive cytokine IL-10. Our functional results are consistent with previous findings on the contributions of miR-let-7i and \( IL-23R \) to the immune response. Furthermore, T-cells proliferation experiments indicated that a subtle change in \( IL-23R \) function due to variations in the rs10889677A>C polymorphism may interfere with T-cell proliferation. Because T lymphocytes play an important role in the immune surveillance of cancer cells (47,48), individuals who carry the \( IL-23R \) rs10889677A allele and thus have lower rates of T-cell proliferation would be predicted to have a higher risk for developing breast, lung and nasopharyngeal cancers.

Our results regarding associations between the \( IL-23R \) rs10889677A>C polymorphism and susceptibility to cancer were obtained from multiple independent case-control analyses derived from eastern and southern Han Chinese populations, and genotyping of the samples was performed in two independent laboratories. The relatively large sample sizes decreased the size of ORs that can be detected statistically, due to increased power. The genotype frequencies of all the SNPs in the controls were aligned with HWE and were identical in the two populations, further supporting the random nature of our method for selecting controls. Moreover, the associations between the SNPs and cancer risks observed in our study are biologically plausible because they are consistent with our functional findings. Nevertheless, further studies of breast, lung and nasopharyngeal cancers as well as other cancers would be beneficial to confirm our results.

In conclusion, our study demonstrated an association between the \( IL-23R \) rs10889677C allele and reduced risk of breast, lung and nasopharyngeal cancers. The rs10889677C allele resides in the 3'UTR of the \( IL-23R \) gene and results in the inhibition of the interaction with Th17 and Treg cells, which consequentially increases the proliferation rate of T lymphocytes and may explain the observed decrease in cancer susceptibility. Together with previous studies (49,50), our results further support the hypothesis that genetic polymorphisms that influence the efficacy of the immune system may modify cancer susceptibility.

**Supplementary material**

Supplementary Tables 1–4 and Figures 1–3 can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

**Funding**

National Natural Science Foundation of China grants 81001278, 81171895, 30872178, 30872142 and 81072366; A project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, Jiangsu Provincial Natural Science Foundation (No. BK2011297); the Scientific Research Foundation for
the Returned Overseas Chinese Scholars, State Education Ministry; Jiangsu Province’s Key Medical Department in 2011.

Acknowledgements

The authors thank all the volunteers and the medical assistants from co-operating hospitals. The authors also appreciate the helpful comments by Prof Dongxin Lin of the Peking Union Medical College and Prof Qingyi Wei of The University of Texas: MD Anderson Cancer Center.

Conflicts of Interest Statement: None declared.

References


Received July 13, 2012; revised August 31, 2012; accepted September 23, 2012.