Functional variants in TNFAIP8 associated with cervical cancer susceptibility and clinical outcomes

Ting-Yan Shi1,2, Xi Cheng3, Ke-Da Yu4, Meng-Hong Sun2,4, Zhi-Ming Shao2,4, Meng-Yun Wang1,2, Mei-Ling Zhu1,2, Jing He1,2, Qiao-Xin Li1,2, Xiao-Jun Chen1,2, Xiao-Yan Zhou2,5, Xiao-hua Wu2,3,9 and Qingyi Wei1,6

1Cancer Institute, Fudan University Shanghai Cancer Center, Shanghai 200032, China; 2Department of Oncology, Shanghai Medical College, Fudan University, Shanghai 200032, China; 3Department of Gynecologic Oncology; 4Department of Breast Surgery and 5Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai 200032, China; and 6Department of Epidemiology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

Abstract

We investigated the associations between single nucleotide polymorphisms (SNPs) and messenger RNA (mRNA) expression levels of TNFAIP8 in head and neck squamous cell carcinoma (SCC) cell lines. The expression levels of TNFAIP8 were significantly associated with both resistance to cisplatin and nedaplatin, recurrence and death from cervical cancer. Taken together, the absence of information on human papillomavirus (HPV) infection, the TNFAIP8-rs11064 SNP may function by affecting the affinity of mir-22 binding to the 3′-untranslated region (UTR) of TNFAIP8 and regulating TNFAIP8 expression, thus contributing to cervical cancer risk. Additionally, the increased TNFAIP8 protein expression may predict platinum resistance and clinical outcomes in cervical cancer patients. Larger, prospective studies with detailed HPV infection data are warranted to validate our findings.

Introduction

The tumor necrosis factor-α-induced protein 8 (TNFAIP8) family is a newly identified and less well-characterized group of proteins that are important for maintaining immune homeostasis and inhibiting apoptosis. The mammalian TNFAIP8 family consists of TNFAIP8 (also named as SCC-S2, NDED), TNFAIP8-like 1 (TNFAIP8L1, TIPE1), TNFAIP8L2 (TIPE2) and TNFAIP8L3 (TIPE3) (1). These proteins share high degrees of sequence homology and are found to be involved in the process of proliferation, inflammation and cell death.

Several studies have found that TNFAIP8 and TNFAIP8L1 act as anti-apoptotic and pro-oncogenic signaling molecules and play important roles in immunity, oncogenesis and tumor progression. TNFAIP8L2 is an essential negative regulator of both innate and adaptive immunity and is involved in the development of inflammatory diseases, whereas little is known about TNFAIP8L3. Previous data have suggested that TNF stimulation and the activation of the nuclear factor-kappaB pathway can upregulate messenger RNA (mRNA) expression levels of TNFAIP8 in head and neck squamous cell carcinoma (SCC) cell lines. Moreover, the overexpression of TNFAIP8 is correlated with cancer progression and poor prognosis in several kinds of human cancers.

Cervical cancer is the second most commonly diagnosed cancer and the second leading cause of cancer death in women in developing countries. It is well known that persistent infection with an oncogenic high-risk human papillomavirus (HPV) type is the primary cause of cervical cancer. Because host immune response plays a role in eliminating the viral infection and preventing progression to cervical cancer, in which TNFAIP8 family genes may be activated and involved in the development and prognosis of cervical cancer.

A number of studies have demonstrated that deregulation of microRNAs (miRNAs) is involved in cell differentiation, proliferation, apoptosis and carcinogenesis. MicroRNAs are single-stranded 21–23 nucleotide long endogenous non-coding RNAs that may regulate thousands of target mRNAs by binding to their 3′-untranslated regions (UTRs). This process results in either the degradation of target mRNAs or repression of their translation, and these targets can be implicated in the regulation of almost all biological processes. Several recent studies have indicated that single nucleotide polymorphisms (SNPs) at miRNA-binding sites can remarkably alter the expression of target mRNAs or repression of their translation, and these targets share high degrees of sequence homology and are found to be involved in the process of proliferation, inflammation and cell death. Therefore, we performed a large case-control study to test the hypothesis that functional variants at the miRNA-binding sites of the TNFAIP8 family genes are associated with cervical cancer risk, platinum resistance and clinical outcomes. We searched and selected the following three common (minor allele frequency > 5%) potentially functional SNPs: TNFAIP8-rs11064, TNFAIP8-rs3813308 and TNFAIP8L1-rs1060555. To address the functional relevance of these selected SNPs, we also investigated the associations of their genotypes with expression levels of both mRNA and protein as well as the allelic functionality by the luciferase reporter assay.
TNFAIP8 variations and cervical carcinogenesis

Materials and methods

Study subjects

The study population consisted of 1584 cervical cancer patients and 1394 cancer-free female controls. The patients had been operated between February 2008 and March 2011 in Fudan University Shanghai Cancer Center. All tumors were histopathologically confirmed as primary cancer of the cervix independently by two gynecologic pathologists as routine diagnostic at Fudan University Shanghai Cancer Center. The controls were enrolled from women who had come to the Outpatient Department of Breast Surgery at Fudan University Shanghai Cancer Center for breast cancer screening and agreed to participate in this study. These female controls, with the selection criteria including no individual history of cancer, were frequency matched to the cases on age (± within 5 years) and residential regions. All subjects were unrelated ethnic Han Chinese and residents in the Eastern China. During an in-person survey, all potential subjects were interviewed to identify their willingness to participate in research studies and to collect their demographic and risk factor information. Appropriate response rates for the cases and controls was 95% and 95%, respectively. Because most Chinese women included in this study were non-smokers and non-drinkers, our study populations were restricted to women who did not smoke cigarettes or drink alcohol. For the cases, the detailed clinico-pathologic information was extracted from the patients’ electronic database, as described previously (19), including tumor histology (20), FIGO stage (International Federation of Gynecology and Obstetrics, 2009), tumor size (i.e. the size of the primary tumor was the largest tumor diameter), pelvic lymph node (LN) metastasis, lympho-vascular space invasion (LVSI) and depth of cervical stromal invasion (Supplementary Table II, available at Carcinogenesis Online). Each participant provided a one-time 10 ml of venous blood sample (after the diagnosis and before the initiation of treatment for the cases), which were kept frozen till DNA extraction for genotyping.

In the survival subset, we included 148 cervical cancer patients, who were histopathologically confirmed as SCC and underwent radical hysterectomy plus pelvic lymphadenectomy. The detailed treatment protocol has been described elsewhere (21). Briefly, 3 weeks after radical surgery, the patients with at least two high-risk factors, such as positive pelvic LN metastasis and/or large tumor size and/or positive LVSI and/or positive surgical margin and deep cervical stromal invasion, were treated with concurrent chemoradiation therapy that included the pelvic irradiation (45 Gy) with weekly cisplatin (30 mg/m² cisplatin and 45 mg/m² paclitaxel). We then followed up every 3 months for the first 2 years, every 6 months for the next 2 years and annually for the following years thereafter. Recurrence was defined as the outcome of disease after a disease-free interval of more than 3 months after primary treatment and grouped by different sites as follows: locally (i.e. vaginal apex), regionally (i.e. pelvic sidewall) or distantly (i.e. liver or lung) (Supplementary Table III, available at Carcinogenesis Online). Progression-free survival and overall survival times were calculated from the date of first surgery to the date of recurrence and death, respectively. Patients without progression or died from other causes were censored on their last date of record.

All samples were obtained from tissue bank of Fudan University Shanghai Cancer Center. The research was approved by the institutional review board of Fudan University Shanghai Cancer Center, and a written informed consent was obtained from all recruited individuals.

Identification and in silico analysis of potential functional variants

We evaluated all SNPs in the 3′-UTR of the four TNFAIP8 family genes with a minor allele frequency of at least 5% in Han Chinese populations, based on the dbSNP database of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/projects/SNP) and the International Map project database (http://mapscan.nci.nih.gov/). Supplementary Table I, available at Carcinogenesis Online, showed that there were three and seven SNPs in the TNFAIP8 and TNFAIP8L1 genes, respectively, but none was found in 3′-UTRs of TNFAIP8L2 and TNFAIP8L3. Of these 10 SNPs, two (i.e. rs1045244 and rs1045242) were in high linkage disequilibrium with another one (i.e. rs11064) by using an r² threshold of 0.8, and six were not predicted as potentially functional SNPs by SNP function prediction (FuncPred) software from National Institute of Environmental Health Sciences (http://snpinfo.nih.gov/). As a result, we selected 4 SNPs (one TNFAIP8 SNP [rs11064], one TNFAIP8L1 SNP [rs1045555] (3′-UTR 380) C>G). Additionally, we added one more potentially functional SNP in the 5′-flanking of the TNFAIP8 gene as a reference SNP (i.e. TNFAIP8-rs3813083 G>C). Therefore, this study included three SNPs of TNFAIP8 family genes, which capture 24 other untyped SNPs (Supplementary Figure 1A, B, available at Carcinogenesis Online).

We then performed miRBase (http://www.mirbase.org/), miRanda (http://www.microrna.org/microrna/releaseNotes.do) and TargetScanHuman 6.2 (http://www.targetscan.org/) to predict miRNA targets. The RNAfold online tool (http://rna.ibt.univie.ac.at/) was used to estimate the RNA secondary structure based on minimum free energy (MFE) values.

DNA extraction and genotyping

Genomic DNA was obtained from the whole blood, and the Taqman method was performed for genotyping, as described previously (22). Four negative controls without DNA template, duplicated positive and eight repeat samples were included in each 384-well plate for the quality control. As a result, the mean genotyping rate was 98.9%, and the discrepancy rate in all positive controls (i.e. duplicated samples, overlapping samples from previous studies and samples randomly selected to be sequenced) was <0.1%.

Cloning and site-directed mutagenesis

The 509 bp fragment of human TNFAIP8 3′-UTR containing the rs11064 A allele was amplified from a homozygous human genomic DNA sample (Supplementary Table IV, available at Carcinogenesis Online). After purification, the PCR product was cloned into the psiCHECK™-2 vector (Promega, Madison, WI) using the XhoI and NotI restriction sites located at the 3′ to the Renilla luciferase translational stop codon. The rs11064 G allele was generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. This method was used to avoid non-specific clamping during the construction of mutant libraries (23). All constructs used in this study were verified by direct sequencing, and the generated reporter vectors were named psiCHECK2:rs11064A and psiCHECK2:rs11064G, respectively (Figure 1B).

Quantitative PCR, transient transfection and luciferase reporter assay

Four cancer cell lines, the cervical adenocarcinoma HeLa and SCC SiHa cell lines, lung cancer cell line A549 and colorectal cancer cell line SW480, were used for the luciferase reporter assay. All these four cell lines were obtained from American Type Culture Collection. Type C virus infection in the cell line were recorded regularly to ensure the maintenance of phenotypes. Cells were used for no more than 3 months after resuscitation. They were cultured in Dulbecco’s modified Eagle’s medium ([Catt L0103; Biowest, Nuaille, France] for HeLa and SiHa), RPMI 1640 medium ([Cat# L0409; Biowest] for A549) and Leibovitz’s L-15 medium ([Cat#L4386, Sigma-Aldrich Co., St Louis, MO, for SW480), supplemented with 10% fetal bovine serum (Cat# S1810; Biowest) at 37°C, humidified incubator supplied with 5% CO₂. We first detected the baseline levels of the TNFAIP8 mRNA expression for the four cell lines. Total RNA was extracted from the cultured cells, and the SYBR Green fluorescent-based assay (TaKaRa Bio, Shiga, Japan) was performed for the quantitative PCR (Supplementary Table IV, available at Carcinogenesis Online), as described previously (22). We evaluated readout values by normalizing the copy number of TNFAIP8 to that of β-actin and calculated the relative expression of TNFAIP8 mRNA by using 2^{-ΔCT} (ΔCT = Avg. TNFAIP8 CT - Avg. β-actin CT) (24).

To conduct the luciferase reporter assay, a total of 2×10⁴ cells from each cell line were plated onto each well of six-well plates. After an overnight incubation, cells were co-transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA). Each co-transfection reaction contained 200 pmol miR-22 (GenePharma Co., Ltd, Shanghai, China) with 2 pg of the psiCHECK2 control, respectively. Cells were cultured for 72 hours after transfection, cells were washed and lysed with 500 µl passive lysis buffer (Promega), of which 20 µl lysed cells were used for the quantification of both Firefly and Renilla luciferase activities by the Dual-Luciferase Reporter Assay System (Cat#E1910; Promega) and Synergy™ M4 Multi-Mode Microplate Reader (BioTek, Winooski, VT) according to the manufacturers’ protocols. The relative luciferase activity = Renilla/Firefly luciferase activity. For the other lysed cells left, we evaluated mRNA expression levels of the Renilla/TNFAlpha 3′-UTR region by normalizing to that of β-actin using the quantitative PCR assay (Supplementary Table IV, available at Carcinogenesis Online).

Genotype and mRNA expression data of TNFAIP8 from the HapMap database

The data on TNFAIP8-rs11064 genotypes and TNFAIP8 mRNA expression levels were both available for 270 HapMap individuals, including 45 Chinese subjects, by the SNPexp online tool (http://app3.titan.uio.no/biotools/help. php?app=snpexp) (25). We used Student’s t-test and analysis of variance to test the differences in the relative mRNA expression levels among different genotype groups, as described previously (26).

TNFAIP8 protein expression levels by TNFAIP8 genotypes in the target tissues

The TNFAIP8 protein expression was performed by the immunohistochemistry (IHC) assay on 5 µm thick tissue sections prepared from formalin-fixed, paraffin-embedded tissue from the constructed 10×12 tissue microarray block using the antibody against TNFAIP8 (sc-80254, goat polyclonal IgG, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) and SABC (goat IgG) detection kit (Cat#SA1023; BOSTER, Wuhan, China). Two tissue cores were obtained for each case. A known positive sample was included as positive control. For the negative control, the primary antibody was replaced with...
Fig. 1. The TNFAIP8-rs11064 A>G SNP contributes to the binding affinity of miR-22 to the TNFAIP8 3′-UTR. (A) The rs11064 SNP is located at the TNFAIP8 3′-UTR, which is also at the miRNA-binding site with the A allele perfectly matching the corresponding U allele in miR-22. (B) Schematic drawing of the
TNFAIP8 variations and cervical carcinogenesis

Cell isolation and the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazo-
lium bromide assay

Histopathologically confirmed fresh tumor tissues obtained from patients by
surgery were cut into pieces smaller than 5×5 mm and suspended in the RPMI
1640 medium supplied with 15% fetal bovine serum at room temperature.
suspended tumor cells were poured over a 150 µm sterile steel mesh placed
in the dish. Percoll discontinuous gradient centrifugation with 1000 r.p.m. was
used to separate and purify tumor cells (28). Tumor cells were then collected and
resuspended at a concentration of 1×10^5 viable cells/ml and cultured in 96-well
plates at 37°C in a 5% CO2 incubator. On day 2, platinum drugs (cisplatin,
 carboplatin, nedaplatin and oxaliplatin with a final concentration of 100, 200,
100 and 120 ng/ml, respectively) were added into the culture system for 48h,
as described previously (29). The measurements were performed in duplicate.
For each patient, tumor cells without the drugs were cultured as negative assay
cells in the meantime. At the end of the cultivation, the medium was removed
and cells were incubated with 1 mg/ml thiazolyl blue tetrazolium bromide
for each patient, tumor cells without the drugs were cultured as negative assay
controls in the system for 48h, as described previously (29). The measurements were performed in duplicate.
For each patient, tumor cells without the drugs were cultured as negative assay
cells in the meantime. At the end of the cultivation, the medium was removed
and cells were incubated with 1 mg/ml thiazolyl blue tetrazolium bromide
Sigma-Aldrich) at 37°C for 4h. The formazan crystals were dissolved in solu-
tion buffer (10% sodium dodecyl sulfate, 5% isobutanol and 0.012 mol/l HCl)
overnight using a sonification. The absorbance at 490 nm was measured by both the positive and negative tumor
bio-Rad, Hercules, CA) and the inhibition rate = (1 - OD_{sample}/OD_{control}) × 100%.

Statistical analysis

Hardy-Weinberg Equilibrium was tested by χ^2 test for each SNP. The differ-
ences in selected variables between cervical cancer cases and controls, as well as
the distributions of TNFAIP8 protein expression levels among different
TNFAIP8 genotypes, were evaluated by the Pearson’s χ^2 test. Logistic regres-
sion models were used to evaluate associations of selected SNPs with cer-
vical cancer risk and with protein expression levels by computing the odds
ratios (ORs) and their 95% confidence intervals (CIs). These analyses were
performed with or without adjustment for age (in years), age of primiparity,
menopause status and body mass index (BMI) (30). These associations were also stratified by demographic and clinico-pathologic variables. We applied the PROC
HAPLOTYPE procedure in SAS software to infer haplotype frequen-
cy distributions for continuous variables. We performed Student’s t-test or Wilcoxon
rank test to compare continuous variables between two groups, and used analysis
of variance or Kruskal–Wallis test to compare continuous variables among
three or four groups. For survival analysis, Kaplan–Meier curve and log rank test,
as well as univariate and multivariate Cox proportional hazards regression
analyses, were conducted. All statistical analyses were performed with SAS
software (version 9.1, SAS Institute, Cary, NC), unless stated otherwise. All P
values were two-sided with a significance level of P < 0.05.

Results

Demographic characteristics of the study population

The final analysis included 1567 cases and 1380 controls. As shown in
Supplementary Table II, available at Carcinogenesis Online, there were no significant differences in the distributions of age between the
cases and the controls with similar mean ages of 45.8 (±9.8) and 46.2
(±8.8) years, respectively (P = 0.132). Among these 1567 cases, there
were 1240 cases with SCC, whose distribution of age was similar to that of the controls. However, there were significant differences in age of primiparity, menopausal status and BMI between cases and controls (all P < 0.001 for all cases and for cases with SCC only, respectively), with the cases more likely to be premenopausal, thin-
er and younger at primiparity than the controls. Thus, we further adjusted these variables for any residual confounding effect in later
multivariate logistic regression analyses.

Association between SNPs and cervical cancer risk

The genotype frequencies of the three selected SNPs and their associations
with cervical cancer risk are summarized in Table I. All the observed genotype distributions among the controls agreed
with Hardy-Weinberg Equilibrium (P = 0.057, 0.637 and 0.593 for
TNFAIP8-rs11064, TNFAIP8-rs3813308 and TNFAIP8L1-rs1060555,
respectively). Compared with the AA genotype and AA/AG genotypes,
the TNFAIP8-rs11064 variant GG genotype was found to be signifi-
cantly associated with an increased risk of cervical cancer (adjusted
OR = 2.15 and 2.16, 95% CI = 1.15–4.02 and 1.16–4.03, P = 0.016 and
0.015, respectively). However, this association was not observed for
the TNFAIP8-rs3813308 and TNFAIP8L1-rs1060555 SNPs or for
the haplotype analysis of the two TNFAIP8 SNPs (Supplementary Table V, available at Carcinogenesis Online). When combining these two
TNFAIP8 SNPs and assuming a recessive genetic model, we found that those women who carried two TNFAIP8 variant genotypes had a 4.73-fold increased risk (95% CI = 1.56–14.29, P = 0.006) of
cervical cancer, compared with those who carried one or less variant
 genotype (Table I).

In the stratified analysis, under a recessive genetic model, we found that the significantly increased risk of cervical cancer asso-
ciated with the TNFAIP8-rs11064 GG genotype was more evident in younger (≤24 years, P = 0.038) or thinner (BMI ≤ 25 kg/
 m², P = 0.012) women and in subgroups of FIGO stage I, tumor
size (<4 cm), negative pelvic LN, negative LVSI and depth of cer-
vical stromal invasion ≤ 1/2 (Supplementary Table VI, available at
Carcinogenesis Online). However, homogeneity tests suggested that there was no difference in risk estimates between subgroups
of the strata. Meanwhile, we observed a borderline-significant locus–
locus interaction between the two selected TNFAIP8 SNPs (i.e.
rs11064 and rs3813308; adjusted OR = 3.49, 95% CI = 0.94–13.04,
P = 0.063).

We then calculated FPRP values for all observed significant
associations. When the assumption of prior probability was 0.25,
the association with TNFAIP8-rs11064 GG genotypes was note-
worthy for the subgroups of tumor size ≤ 4 cm and negative LN
metastasis (FPRP = 0.143 and 0.189, respectively) as well as
TNFAIP8L1-rs1060555 GG genotypes for the women who were of
younger age at primiparity (≤24 years, FPRP = 0.138).

MIR-22 differentially regulates TNFAIP8 mRNA expression through allelic variants of rs11064

Because the mRNA secondary structure is critical for mRNAmiRNA
interactions (32), we further explored whether the TNFAIP8-rs11064
SNP in the 3′-UTR of TNFAIP8 could alter the local secondary
structure of the TNFAIP8 mRNA based on the MFE value. Using the
RNAfold online tool and inputting 162-nucleotide long DNA
sequence of the TNFAIP8 3′-UTR containing the rs11064 locus, we

Luciferase reporter vectors. The control pcisCHECK2 luciferase vector (top) and pcisCHECK2 vectors contain a partial TNFAIP8 3′-UTR sequence with either the A (middle) or G (bottom) allele at the rs11064 locus. The graphs on the right side show sequencing results for A and G alleles. (C) The predicted secondary structure of the TNFAIP8 mRNA. The secondary structures of the TNFAIP8 3′-UTR were predicted by inputting two 162 nucleotide long DNA sequences centering rs11064 into RNAfold, with either the A (left) or G (right) allele. The figures and the values of MFE were generated by RNAfold (http://rna.th.informatics. ike.ac.jp). (D) The TNFAIP8 mRNA was expressed in the four cancer cell lines with relatively higher levels in A549 and SiHa, and lower levels in SW480 and HeLa. (E) Luciferase reporter assay. For each transfected cell line, compared with the pcisCHECK2-rs11064G vector, the pcisCHECK2-rs11064A vector showed a significantly decreased level of the relative Renilla/Human luciferase activity (Student’s t-test, P < 0.001 for HeLa, SiHa and SW480; P = 0.009 for A549, respectively); (F) Quantitative PCR assay. For each transfected cell line, compared with the pcisCHECK2-rs11064G vector, the pcisCHECK2-rs11064A vector showed a decreased trend of Renilla/TNFACIP8 3′-UTR mRNA expression levels (Student’s t-test, P = 0.002, 0.035, 0.004 and 0.062 for HeLa, SiHa, A549 and
SW480, respectively).
Table I. Associations of TNFAIP8 and TNFAIP8L1 genotypes with the risk of cervical carcinoma

<table>
<thead>
<tr>
<th>Variant genotypes</th>
<th>Cases (N = 1567)</th>
<th>Controls (N = 1380)</th>
<th>P*</th>
<th>Crude OR (95% CI)</th>
<th>P</th>
<th>Adjusted OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFAIP8-rs11064</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1178 (75.2)</td>
<td>1039 (75.3)</td>
<td>0.015</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>350 (22.3)</td>
<td>326 (23.6)</td>
<td>0.95 (0.80–1.13)</td>
<td>0.535</td>
<td>0.98 (0.82–1.17)</td>
<td>0.820</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>39 (2.5)</td>
<td>15 (1.1)</td>
<td>2.29 (1.26–4.18)</td>
<td><strong>0.007</strong></td>
<td>2.15 (1.15–4.02)</td>
<td><strong>0.016</strong></td>
<td></td>
</tr>
<tr>
<td>Additive model</td>
<td></td>
<td></td>
<td>1.07 (0.92–1.24)</td>
<td>0.395</td>
<td>1.09 (0.93–1.27)</td>
<td>0.314</td>
<td></td>
</tr>
<tr>
<td>Dominant model</td>
<td>0.943</td>
<td>1.01 (0.85–1.19)</td>
<td>0.943</td>
<td>1.03 (0.87–1.23)</td>
<td>0.718</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recessive model</td>
<td><strong>0.005</strong></td>
<td>2.32 (1.27–4.23)</td>
<td><strong>0.006</strong></td>
<td>2.16 (1.16–4.03)</td>
<td><strong>0.015</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFAIP8-rs3813308</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>416 (26.6)</td>
<td>337 (24.4)</td>
<td>0.415</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>754 (48.1)</td>
<td>681 (49.4)</td>
<td>0.90 (0.75–1.07)</td>
<td>0.230</td>
<td>0.90 (0.74–1.08)</td>
<td>0.251</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>397 (25.3)</td>
<td>362 (26.2)</td>
<td>0.89 (0.73–1.09)</td>
<td>0.253</td>
<td>0.88 (0.71–1.09)</td>
<td>0.254</td>
<td></td>
</tr>
<tr>
<td>Additive model</td>
<td></td>
<td></td>
<td>0.94 (0.85–1.04)</td>
<td>0.253</td>
<td>0.94 (0.85–1.05)</td>
<td>0.255</td>
<td></td>
</tr>
<tr>
<td>Dominant model</td>
<td>0.87</td>
<td>0.89 (0.76–1.06)</td>
<td>0.188</td>
<td>0.89 (0.75–1.06)</td>
<td>0.200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recessive model</td>
<td>0.579</td>
<td>0.95 (0.81–1.13)</td>
<td>0.578</td>
<td>0.95 (0.80–1.13)</td>
<td>0.557</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Combined effects of two risk genotypes of TNFAIP8 by the recessive model

| 0  | 1152 (73.5) | 1007 (73.0) | 0.006 | 1.00 | 1.00 |
| 1  | 394 (25.1)  | 369 (26.7)  | 0.93 (0.79–1.10) | 0.413 | 0.92 (0.78–1.10) | 0.372 |
| 2  | 21 (1.3)    | 4 (0.3)     | 4.59 (1.57–14.31) | **0.005** | 4.63 (1.53–14.01) | **0.007** |
| P  | = 0.769     | = 0.877     |                  |      |      |
| 0–1| 1546 (98.7) | 1376 (99.7) | **0.002** | 1.00 | 1.00 |
| 2  | 21 (1.3)    | 4 (0.3)     | 4.67 (1.60–13.64) | **0.005** | 4.73 (1.56–14.29) | **0.006** |

The results are shown in bold, if P < 0.05.
χ² test for genotype distributions between cases and controls.
A Adjusted for age, age at primaparity, menopausal status and BMI in logistic regression models.

found that the MFE changed from -35.6 to -36.2 kcal/mol, when the rs11064 allele changed from A to G (Figure 1C).

Given that rs11064 A>G was located at the miRNA-binding site of TNFAIP8 with the A allele perfectly matching the corresponding U allele of mir-22 (Figure 1A), we then performed the luciferase reporter assay to determine whether mir-22 could differentially regulate the TNFAIP8 mRNA expression through different rs11064 alleles. Baseline levels of the TNFAIP8 mRNA expression in patients with cervical cancer were shown in Figure 1D, with relatively higher levels in A549, medium levels in HeLa and SiHa, and lower levels in SW480. For these cancer cell lines, the relative luciferase activities and mRNA expression levels of the two test groups (i.e. psiCHECK2:rs11064A and psiCHECK2:rs11064G) were normalized by those of control groups. We found a significantly decreased level of the relative luciferase activity in the psiCHECK2:rs11064A group compared with the psiCHECK2:rs11064G group (Student’s t-test, P = 0.001 for HeLa, SiHa and SW480, respectively; Figure 1E); likewise, a decreased trend of Renilla/TNF-α-3' UTR mRNA expression levels was observed in the psiCHECK2:rs11064A group, compared with the psiCHECK2:rs11064G group (Student’s t-test, P = 0.002, 0.035, 0.004 and 0.062 for HeLa, SiHa, A549 and SW480, respectively; Figure 1F).

Association between the TNFAIP8-rs11064 SNP and expression levels of the TNFAIP8 mRNA from the HapMap database

We evaluated 270 HapMap individuals for the association between variants and mRNA expression of TNFAIP8 based on the SNPs online database available to us. There were 167 AA, 80 AG and 17 GG carriers for the TNFAIP8-rs11064 SNP, of which 36 AA and 9 GG carriers were Chinese (no GG was observed in this study population). Because TNFAIP8 mRNA expression levels obtained from the HapMap database followed normal distributions in both all subjects and Chinese subjects (Shapiro–Wilk test, P = 0.084 and 0.842, respectively), the G allele was associated with significantly increased levels of TNFAIP8 mRNA expression compared with the A allele in both all 264 subjects (i.e. 528 alleles) and 45 Chinese subjects (i.e. 90 alleles) (Student’s t-test, P = 0.021 and 0.026, respectively; Figure 2A and B).
Fig. 2. Differential expression of TNFAIP8 mRNA and protein by different rs11064 variant alleles. The relative TNFAIP8 mRNA expression levels by rs11064 alleles were obtained from HapMap for (A) all 270 individuals and (B) the 45 Han Chinese in Beijing (CHB) individuals (http://app3.titan.uio.no/biotools/help.php?app=snpexp). Individuals who carry rs11064 G allele had a significantly increased level of TNFAIP8 mRNA expression than those who carry A allele in both all and Chinese subjects (Student's t-test, \( P = 0.021 \) and 0.026, respectively). (C) TNFAIP8 protein expression levels were also investigated in the corresponding tissue with IHC for 144 cervical cancer patients who had 288 alleles at Fudan University Shanghai Cancer Center. The rs11064 variant G allele (all three GG patients had high expression levels) was significantly associated with an increased expression level of TNFAIP8 protein compared with the A allele (\( \chi^2 \) test, \( P = 0.012 \)). The graphs on the right side show representative IHC results. Cervical cancer patients with high expression levels of TNFAIP8 showed poorer (D) progression-free survival and (E) overall survival than those with low TNFAIP8 expression levels (\( P = 0.066 \) and 0.342, respectively).
cisplatin and nedaplatin (Wilcoxon test, \( P = 0.043 \) and 0.009, respectively; Table III).

No significant association between TNFAIP8 SNPs and survival was observed (Supplementary Table VII, available at Carcinogenesis Online), mainly because there were only three homozygous variant GG carriers among the 144 patients. However, consistent with the results of platinum resistance, we found that the patient who had high expression levels of TNFAIP8 showed a decreased trend of progression-free survival and overall survival (log rank test, \( P = 0.066 \) and 0.342, respectively; Figure 2D). Further multivariate Cox proportional hazards regression models demonstrated that the TNFAIP8 protein expression was independently associated with recurrence and death (adjusted hazard ratio = 4.10 and 4.48, 95% CI = 1.38–12.15 and 1.02–19.64, \( P = 0.011 \) and 0.047, respectively; Supplementary Table VII, available at Carcinogenesis Online); in the same multivariate model, we found that highly advanced tumor stage (IIIB), large tumor size (≥4 cm) and positive LVSI were also independently associated with recurrence, and positive LVSI with death.

Discussion

To the best of our knowledge, this is the first study that has investigated associations of miRNA-binding site SNPs of the TNFAIP8 family genes with cervical cancer risk, platinum resistance and clinical outcomes in Eastern Chinese women. Although no significant association between genotypes of the TNFAIP8-like genes and cervical cancer risk was observed, we found that the TNFAIP8-rs11064 variant GG genotype was associated with an increased risk of cervical cancer. Further genotype–phenotype correlation analyses and additional experiments suggested that this SNP might function by affecting the affinity of miR-22 binding to the 3′-UTR of TNFAIP8 and regulating expression levels of the TNFAIP8 mRNA and TNFAIP8 protein, thus contributing to cervical cancer risk. Moreover, survival analyses of a subset showed a potential effect of TNFAIP8 expression on platinum resistance and clinical outcomes, indicating that functional variants in TNFAIP8 might be involved in tumor progression and prognosis of cervical cancer by affecting the response of tumor cells to platinum drugs, especially cisplatin and nedaplatin.

TNFAIP8 is one of the TNFAIP8 family genes that have been less well characterized. TNFAIP8 is located at chromosome 5q23.1 and encodes a 21 kDa cytosolic protein, which consists of 11 exons and 10 introns and spans ~13.5 kb of genomic DNA. It has been originally identified in a metastatic and radio-resistant primary human head and neck cancer cell line (PCI-06B) (33). Subsequently, several studies have showed that TNFAIP8 plays important roles in maintaining immune homeostasis and inducing the development and progression of tumors as an oncogene (2–4). Meanwhile, its overexpression could lead to cell proliferation (3). Others reported that TNFAIP8

### Table II. Logistic regression analysis of correlation between TNFAIP8 genotypes and TNFAIP8 protein expression in cervical cancer tissues from 144 patients

<table>
<thead>
<tr>
<th>TNFAIP8 variants</th>
<th>TNFAIP8 protein expression</th>
<th>( P^a )</th>
<th>Crude OR (95% CI)</th>
<th>( P^b )</th>
<th>Adjusted OR (95% CI)</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alleles</td>
<td>High N (%)</td>
<td>Low N (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cases (N = 288)</td>
<td>166 (57.6)</td>
<td>122 (42.4)</td>
<td>0.012</td>
<td>1.00</td>
<td>1.00</td>
<td>0.018</td>
</tr>
<tr>
<td>rs11064</td>
<td>A</td>
<td>147 (55.5)</td>
<td>118 (44.5)</td>
<td>0.82</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>19 (82.6)</td>
<td>4 (17.4)</td>
<td>3.81</td>
<td>0.018</td>
<td>0.017</td>
</tr>
<tr>
<td>rs3813308</td>
<td>C</td>
<td>86 (60.1)</td>
<td>57 (39.9)</td>
<td>0.39</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>80 (55.2)</td>
<td>65 (44.8)</td>
<td>0.39</td>
<td>1.00</td>
<td>0.315</td>
</tr>
</tbody>
</table>

The results are shown in bold, if \( P < 0.05 \).

\( \chi^2 \) test for genotype distributions between high and low levels of TNFAIP8 protein.

\( ^a \)Adjusted for age, age at primiparity, menopausal status and BMI in logistic regression models.

### Table III. Variants and TNFAIP8 protein expression as predictors of response to platinum agents in the survival subset

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases N (%)</th>
<th>Cisplatin</th>
<th>Carboplatin</th>
<th>Nedaplatin</th>
<th>Oxaliplatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median ± SE (%)</td>
<td>( P^a )</td>
<td>Median ± SE (%)</td>
<td>( P^a )</td>
<td>Median ± SE (%)</td>
</tr>
<tr>
<td>All patients(^b)</td>
<td>144 (100)</td>
<td>81.6 ± 2.1</td>
<td>35.7 ± 2.5</td>
<td>0.603</td>
<td>77.7 ± 2.1</td>
</tr>
<tr>
<td>TNFAIP8-rs11064(^c)</td>
<td>265 (92.2)</td>
<td>81.5 ± 1.6</td>
<td>35.0 ± 1.9</td>
<td>0.869</td>
<td>77.8 ± 1.6</td>
</tr>
<tr>
<td>A</td>
<td>23 (7.8)</td>
<td>85.1 ± 5.6</td>
<td>38.0 ± 5.7</td>
<td>0.869</td>
<td>71.1 ± 5.2</td>
</tr>
<tr>
<td>C</td>
<td>143 (50)</td>
<td>81.8 ± 2.2</td>
<td>36.4 ± 2.6</td>
<td>0.422</td>
<td>78.3 ± 2.0</td>
</tr>
<tr>
<td>G</td>
<td>145 (50)</td>
<td>79.6 ± 2.1</td>
<td>34.2 ± 2.5</td>
<td>0.422</td>
<td>74.5 ± 2.2</td>
</tr>
<tr>
<td>TNFAIP8L1-rs1060555(^d)</td>
<td>209 (74.8)</td>
<td>81.5 ± 1.7</td>
<td>35.0 ± 2.1</td>
<td>0.277</td>
<td>78.2 ± 1.7</td>
</tr>
<tr>
<td>C</td>
<td>71 (25.2)</td>
<td>84.9 ± 3.3</td>
<td>36.7 ± 3.4</td>
<td>0.277</td>
<td>77.6 ± 3.3</td>
</tr>
<tr>
<td>G</td>
<td>83 (57.1)</td>
<td>77.6 ± 2.6</td>
<td>44.9 ± 3.5</td>
<td>0.009</td>
<td>85.5 ± 3.2</td>
</tr>
</tbody>
</table>

The results are shown in bold, if \( P < 0.05 \).

\( ^a \)Wilcoxon test for platinum-inhibition rates between different groups.

\( ^b \)Of the 148 patients, three were lost to the follow-up, and one had a score of Not Applicable for the IHC staining of TNFAIP8.

\( ^c \)All 144 patients with 288 alleles.

\( ^d \)Four patients were unsuccessfully genotyped for rs1060555.
contains a death effector domain and thus may cause the inhibition of caspase-mediated apoptosis (34). For example, the downregulation of TNFAIP8 alone was sufficient to resist glucocorticoid-induced apoptosis (35). In cancers of the head and neck, breast, lung and esophagus, expression levels of TNFAIP8 in addition to a short term of follow-up. On the other hand, although genetic variations may contribute to cervical cancer risk by regulating gene expression, any single SNP may be insufficient for the prediction of cancer development and prognosis (44).

Several limitations in this study need to be addressed. First, there are selection bias and information bias by the study design, which may have been minimized by frequency-matching cases and controls as well as the adjustment for potential confounding factors in final multivariate analyses. Second, because of the retrospective nature of the study design, we did not have enough information on other risk factors, especially HPV infection that may be potential confounders. This was because the hospital did not perform HPV and related sub-type tests routinely for the diagnosis of all cases, let alone for the normal controls. A recent meta-analysis demonstrated that HPV16, 18 and 45 accounted for a greater or equal proportion of HPV infections in cervical cancer compared with normal cytology, but others, like high-risk HPV33, 51 and 58, were in a reverse direction (45). Therefore, the HPV infection status could be a confounder in estimating the risk associated with genetic factors.

In summary, in this case-control study of 1584 cases and 1394 controls, we found the TNFAIP8-rs11064 SNP to be associated with cervical cancer risk in Eastern Chinese women, and this SNP may function by affecting the affinity of mir-22 binding to the 3′-UTR of TNFAIP8 and regulating TNFAIP8 expression. Furthermore, TNFAIP8 might be a prognostic marker for cervical cancer, particularly for platinum resistance and clinical outcomes. However, well-designed larger, prospective studies with detailed information about HPV infection are warranted to validate our findings.

Supplementary material
Supplementary Tables I–VII and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

Funding
China’s Thousand Talents Program Recruitment at Fudan University and the Shanghai Committee of Science and Technology, China (grant no. 12DZ2260100).

Acknowledgements
We would like to thank Hong-Yu Gu, Yun-Hua Ling and Yu-Hu Xin from Fudan University Shanghai Cancer Center for their guidance on immunohistochemistry and MTT assay as well as for their technical support.

Conflict of Interest Statement: None declared.

References


Received August 13, 2012; revised November 27, 2012; accepted December 29, 2012.