A Sequence Polymorphism in miR-608 Predicts Recurrence after Radiotherapy for Nasopharyngeal Carcinoma

Jian Zheng1, Jieqiong Deng1, Mang Xiao4, Lei Yang5, Liyuan Zhang2, Yonghe You1, Min Hu2, Na Li1, Hongchun Wu1, Wei Li1, Jiachun Lu5, and Yifeng Zhou1

Abstract

Nasopharyngeal carcinoma is treated with radiotherapy and other modalities, but there is little information on individual genetic factors to help predict and improve patient outcomes. Single-nucleotide polymorphisms (SNP) in mature microRNA (miRNA) sequences have the potential to exert broad impact as miRNAs target many mRNAs. The aim of this study was to evaluate the effects of SNPs in mature miRNA sequences on clinical outcome in patients with nasopharyngeal carcinoma receiving radiotherapy. In particular, we analyzed associations between seven SNPs and nasopharyngeal carcinoma locoregional recurrence (LRR) in 837 patients from eastern China, validating the findings in an additional 828 patients from southern China. We found that miR-608 rs4919510C>G exhibited a consistent association with LRR in the discovery set [HR, 2.05; 95% confidence interval (CI), 1.35–3.21], the validation set (HR, 2.24; 95% CI, 1.45–3.38), and the combined dataset (HR, 2.08; 95% CI, 1.41–3.26). Biochemical investigations showed that rs4919510C>G affects expression of miR-608 target genes along with nasopharyngeal carcinoma cell growth after irradiation in vivo and in vitro. Notably, X-ray radiation induced more chromatid breaks in lymphocyte cells from rs4919510CC carriers than in those from subjects with other genotypes (P = 0.0024). Our findings reveal rs4919510C>G in miR-608 as a simple marker to predict LRR in patients with radiotherapy-treated nasopharyngeal carcinoma.

Introduction

Nasopharyngeal carcinoma is one of the most common head and neck malignancies in South China and South Asia, where the incidence is as high as 50 of 100,000, but it is rare in the Western world (1 of 100,000; refs. 1–3). Although significant progress has been made in the diagnosis and treatment of nasopharyngeal carcinoma in recent decades, it remains a highly frequent cause of cancer-related death in China. The worldwide 5-year overall survival (OS) rate ranges from 32% to 62% among a series of studies involving more than 9,500 patients in all stages of nasopharyngeal carcinoma (4). The main cause of death among patients with nasopharyngeal carcinoma is recurrence, and 80% of all recurrences occur during the first 3 years after pathogenesis (4). Currently, radiotherapy is the main treatment modality for this malignancy; however, differences in individual sensitivity to radiotherapy have a great impact on the recurrence rate of nasopharyngeal carcinoma (5).

microRNAs (miRNA) comprise a group of endogenous, single-stranded, small, noncoding RNAs that have emerged as key regulators of fundamental biologic processes via their control over the expression of more than 30% of human genes (6, 7). miRNAs are initially transcribed as primary miRNAs (pri-miRNA) with several hundred nucleotides that are further processed into hairpin-structured precursor miRNAs (pre-miRNA) and then into mature miRNAs (8–10). Mature miRNAs consist of approximately 22 to 25 nucleotides. To date, more than 1,000 miRNAs have been detected in humans (11, 12). Physiologically, miRNAs act as negative gene regulators that fine-tune translational output through targeted mRNA binding. A variety of pathologic associations have been attributed to altered miRNA networks, particularly in cancer, because miRNAs can function as both oncogenic and tumor suppressor factors (13–15).

Genome-wide association studies (GWAS) have identified several single-nucleotide polymorphisms (SNP) related to nasopharyngeal carcinoma susceptibility (1, 16). To date, candidate gene approaches remain the primary strategy used in association studies of clinical outcomes. Genetic variants, such as SNPs in miRNAs, can affect their biogenesis, processing, and target site binding in a variety of ways (10). A SNP in
the mature sequence can alter target site interactions by either strengthening or weakening hybridization kinetics, and SNPs can significantly transform the target library of the miRNA itself. Numerous studies have linked genetic variation in mature miRNA sequences to cancer risk and prognosis (17–19). Nonetheless, to the best of our knowledge, the importance of mature miRNA sequence SNPs in the locoregional recurrence (LRR; ref. 20) of nasopharyngeal carcinoma after radiotherapy remains unknown. In this study, we evaluated the frequencies of mature miRNA sequence SNPs in patients with nasopharyngeal carcinoma and assessed their impact on LRR after radiotherapy.

Materials and Methods

Study population

A total of 1,665 patients with histologically confirmed nasopharyngeal carcinoma [International Classification of Disease (ICD) 9:147, ICD10:C11] were recruited from Jiangsu Province in eastern China [The First Affiliated Hospital of Soochow University (Suzhou, China), The Second Affiliated Hospital of Soochow University (Suzhou, China), The Third Hospital Affiliated to Nantong University (Wuxi, China), and Huai'an No.1 Hospital (Huai'an, China)] as well as Guangzhou City in southern China (The Tumor Hospitals affiliated to Guangzhou Medical College, Guangzhou, China) between 2000 and 2009 and were followed up until 2012 (Table 1). All patients were treated with definitive radiotherapy at urban hospitals, and no patients underwent surgery. In our eastern Chinese population, 837 newly diagnosed patients were analyzed as a discovery set in this study. In the southern Chinese population, 828 newly diagnosed patients were used as a validation set (21, 22).

A self-administered questionnaire was used for all patients to collect epidemiologic data including demographical characteristics, tobacco and alcohol use, family history of cancer, and medical history. All LRRs were diagnosed by endoscopy and biopsy and/or computed tomography (CT) scan of the

| Table 1. Baseline demographic and clinical characteristics of study populations |
|-------------------------|-------------------------|-------------------------|
| Variables               | Discovery set (eastern Chinese, N = 837) | Validation set (southern Chinese, N = 828) |
|                         | Recurrence (N = 176) | No recurrence (N = 661) | P | Recurrence (N = 218) | No recurrence (N = 610) | P |
| Age, mean (SEM)         | 53.91 (0.879)        | 52.85 (0.411)          | 0.856 | 50.81 (0.821)        | 51.54 (0.409)          | 0.413 |
| Gender, N (%)           | Male 116 (65.91)      | 466 (70.50)            | 0.239 | 162 (74.32)          | 438 (71.81)            | 0.476 |
|                         | Female 60 (34.09)     | 195 (29.50)            |      | 56 (25.68)           | 172 (28.19)            |      |
| Family history of cancer, N (%) | 0.859 | | | 0.466 | |
| No                      | 156 (90.64)          | 589 (89.11)            |      | 197 (90.37)          | 561 (91.96)            |      |
| Yes                     | 20 (11.36)           | 72 (10.89)             |      | 21 (8.63)            | 49 (8.04)              |      |
| Smoking status, N (%)   | Never 82 (46.59)      | 291 (44.02)            | 0.542 | 106 (48.62)          | 278 (45.57)            | 0.438 |
|                         | Ever 94 (53.41)       | 370 (55.98)            |      | 112 (51.38)          | 332 (54.43)            |      |
| Drinking status, N (%)  | Never 87 (49.43)      | 352 (53.25)            | 0.367 | 107 (49.08)          | 319 (52.30)            | 0.415 |
|                         | Ever 89 (50.57)       | 309 (46.75)            |      | 111 (50.92)          | 291 (47.70)            |      |
| BMI, N (%)              | <20 28 (15.91)        | 116 (17.55)            | 0.911 | 62 (28.44)           | 205 (33.61)            | 0.151 |
|                         | 20–28 133 (75.57)     | 481 (72.77)            |      | 148 (67.89)          | 387 (63.44)            |      |
|                         | ≥28 15 (8.52)         | 64 (9.68)              |      | 8 (3.67)             | 18 (2.95)              |      |
| EBV infection status, N (%) | Positive 159 (90.34) | 552 (83.51)           | 0.024 | 202 (92.66)          | 522 (85.58)            | 0.007 |
|                         | Negative 17 (9.66)    | 109 (16.49)            |      | 16 (7.34)            | 88 (14.42)             |      |
| Stage, N (%)            | I 8 (4.55)            | 28 (4.23)              | 0.285 | 9 (4.13)             | 26 (4.26)              | 0.194 |
|                         | II 42 (23.86)         | 155 (23.45)            |      | 47 (21.55)           | 163 (26.72)            |      |
|                         | III 68 (38.64)        | 316 (47.81)            |      | 92 (42.21)           | 245 (40.16)            |      |
|                         | IV 58 (32.95)         | 162 (24.51)            |      | 70 (32.11)           | 176 (28.86)            |      |
| Histologic grade, N (%) | Undifferentiated 139 (78.97) | 484 (73.22) | 0.119 | 166 (76.15) | 448 (73.44) | 0.434 |
|                         | Differentiated 37 (21.02) | 177 (26.78) | | 52 (23.85) | 162 (25.56) | |
| Chemotherapy, N (%)     | Yes 143 (81.25)       | 593 (89.71)            | 0.003 | 175 (80.28)          | 533 (87.38)            | 0.011 |
|                         | No 33 (18.75)         | 68 (10.29)             |      | 43 (19.72)           | 77 (12.62)             |      |
nasopharynx and the skull base showing progressive bone erosion and/or soft tissue swelling (23). Diagnosis of a second primary tumor was based on a modification of the criteria of Warren and Gates (24), and we did not find any second primary tumor cases in our present study. Each patient saw his or her doctor during the follow-up period for assessment of recurrence once a month in the first year, every 2 months in years 2 and 3, and every 6 months thereafter. We reviewed patients’ medical records during the follow-up period to collect clinical information including the date of diagnosis, recurrence status, Epstein–Barr virus (EBV) infection status, histologic grade, pathologic stage, and treatment. Immunoglobulin-A antibodies to EBV capsid antigen (EBV/IgA/VCA) and immunoglobulin-A antibodies to EBV early antigen were confirmed by serologic testing at the time of study enrollment. The tumor, node, metastasis classification and tumor staging were evaluated according to the 2002 American Joint Committee on Cancer staging system. At recruitment, informed consent was obtained from each patient, and the study was approved by the Medical Ethics Committee of Soochow University (SZUM2009061002) and the Institutional Review Board of Guangzhou Medical College (GZMC2009060426).

Radiotherapy technique
Radiotherapy was conducted largely using standard procedures (5). For details, see Supplementary Materials and Methods. The patients received a month of radiotherapy, and treatment was delivered once daily (5 fractions/week). The lag time between date of diagnosis and date of first treatment is within 2 weeks.

Follow-up and endpoint selection
All patients were evaluated weekly during the treatment period, and after the completion of treatment, they were followed up every 3 months by telephone for the first 3 years, every 6 months in years 4 and 5, and annually thereafter. Overall, we recruited 2,073 patients with nasopharyngeal carcinoma (1,075 from the eastern Chinese and 998 from the southern Chinese). A total of 1,665 patients (837 from the eastern Chinese and 828 from the southern Chinese) had complete follow-ups and clinical information. Among the remaining 408 patients (238 from the eastern Chinese and 170 from the southern Chinese) with incomplete follow-up or clinical information or both, 125 cases (6.03%) lacked stage and/or histology information, 132 cases (6.37%) had incorrect telephone numbers, 49 cases (2.36%) refused to participate, 66 cases (3.18%) had ambiguous death date and/or indirect death because of nasopharyngeal carcinoma, and 36 cases (1.74%) moved or were unavailable for unknown reasons. However, there was no significant difference in the distributions of demographic characters (e.g., age and gender), smoking status, drinking status, body mass index (BMI), EBV infection status, stage, histologic grade, and chemotherapy between the patients with nasopharyngeal carcinoma with and without follow-up/clinical information (P = 0.642 for age; P = 0.365 for gender; P = 0.516 for smoking status; P = 0.287 for drinking status; P = 0.258 for BMI; P = 0.539 for EBV infection status; P = 0.225 for stage; P = 0.169 for histologic grade; and P = 0.258 for chemotherapy). The endpoints of the current study included the time to recurrence (TTR) and OS. The TTR was calculated as the time from the date of diagnosis of nasopharyngeal carcinoma to the date of the first observation of LRR, or until the last follow-up if the patient was recurrence-free at that time. TTR was censored at the time of death or at the last follow-up if the patient remained recurrence-free at that time. The OS was defined as the time from pathologic diagnosis to death from any cause, or the last contact if the patient was alive.

Tissue samples
To determine the expression levels of selected genes, we collected 35 nasopharyngeal carcinoma tissues from patients who had undergone resection from The Second Affiliated Hospital of Soochow University. All cases were histopathologically diagnosed as nasopharyngeal carcinoma by biopsy and without radio- or chemotherapy.

SNP selection and genotyping
According to the bioinformatics analysis, SNPs located in mature miRNA sequences with allelic frequencies in Chinese populations were selected. First, based on the miRBase database (http://mirbase.org; up to January 1, 2012), 205 polymorphic loci in mature miRNA sequences (Supplementary Table S1) were screened. Second, based on the HapMap public database [HapMap Data Rel 28 phase II+III, August 10, on National Center for Biotechnology Information (NCBI) B36 assembly; dbSNP b126; up to January 1, 2012], we found that there were only seven SNPs in 205 polymorphic loci that had allelic frequencies in Chinese populations (Supplementary Table S1). Finally, we selected these seven SNPs located in mature miRNA sequences with the allelic frequencies in the Chinese population for genotyping: rs49-499 (rs3746444C>T), miR-608 (rs4919510C>G), miR-3152 (rs13299349A>G), miR-4513 (rs2168518C>T), miR-4520a (rs8078913C>T), miR-4741 (rs7227168C>T), and miR-4762 (rs41524547C>G).

Genomic DNA was isolated from the peripheral blood lymphocytes of all the study subjects. All subjects were genotyped for the seven SNPs by using allele-specific matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry (Sequenom; refs. 25, 26). The DNA isolation and genotyping were conducted in Suzhou Center (Suzhou, China; for the eastern Chinese population) and Guangzhou Center (Guangzhou, China; for the southern Chinese population), respectively. The cross-trained laboratory personnel conducting the genotyping were blinded to patient information. Approximately, 10% of the samples were also randomly selected for a blinded repeat of the genotyping without prior knowledge of the previous genotyping result or the patient information, and the results were in 100% agreement.

Cell culture
Human nasopharyngeal carcinoma cell lines (CNE-1 and CNE-2) and 293T cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Biochemistry and Cell Biology.
The primers used for PCR amplification were quantified with the assistance of Qiagen (Qiagen) according to the manufacturer’s instructions. Gene expression profiling was conducted using the Human OneArray microarray (Phalanx Biotech; refs. 29, 30). Details are given in Supplementary Materials and Methods. The array data have been deposited in Gene Expression Omnibus (GEO; accession number: GSE46372).

RNA extraction and microarray analysis

Total RNA was isolated from 35 nasopharyngeal carcinoma tissue samples with TRizol reagent (Molecular Research Center). The relative gene expression for the selected genes was calculated using the Human OneArray microarray (Phalanx Biotech; refs. 29, 30). Details are given in Supplementary Materials and Methods. The array data have been deposited in Gene Expression Omnibus (GEO; accession number: GSE46372).

Quantitative real-time PCR analysis

Total RNA was isolated from 35 nasopharyngeal carcinoma tissue samples with TRizol reagent (Molecular Research Center). The relative gene expression for the selected genes was quantified using the ABI Prism 7000 sequence detection system (Applied Biosystems) based on the SYBR Green method. The primers used for PCR amplification of the candidate genes are listed in Supplementary Table S2. The expression of miR-608 in nasopharyngeal carcinoma cells was calculated relative to the U6 small nuclear RNA (Supplementary Materials and Methods).

Construction of FBXO32 3′-UTR luciferase reporter plasmid

The reporter vector psiCHECK-2 (Promega) was prepared by amplifying a 683-bp FBXO32 3′-untranslated region (3′-UTR) region from a human genomic DNA sample, including the artificial XhoI and NotI enzyme restriction sites with the forward primer 5′-TGTATATGCTGAGCCATAGTTCTC-3′ and reverse primer 5′-CGCTCTAGTCTAAAGCGGCCGCGC-3′. Construction of the FBXO32 3′-UTR luciferase reporter plasmid was conducted according to a previously described method (21). The resulting construct (psiCHECK-2-FBXO32-3′-UTR) was verified by sequencing.

Transient transfections and luciferase assays

The CNE-1 and CNE-2 cells were seeded at 1 × 10^5 cells per well in 24-well plates (BD Biosciences). Sixteen hours after plating, the cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions (Supplementary Materials and Methods).

Mutagen sensitivity assay

To further explore the differences in DNA repair ability among individuals with different miR-608 rs4919510C>G genotypes, we evaluated X-ray radiation sensitivities in 135 additional control subjects according to published protocols (Supplementary Materials and Methods; refs. 31, 32). The values of chromatic breaks per cell (b/c) were used to indicate the DNA repair capacity of the individual (31, 33).

Cell growth analysis in response to irradiation

CNE-1-empty vector, CNE-1-miR-608-C, CNE-1-miR-608-G, CNE-2-empty vector, CNE-2-miR-608-C, and CNE-2-miR-608-G cells were plated in a 24-well culture plates (2.5 × 10^4/well). After incubation for 24 hours, the cells received 2 Gy of irradiation. Cell growth was monitored by counting cell numbers at various time intervals. Three independent experiments were carried out in triplicate.

Animal model

The animal experiments were carried out in accordance with National guidelines and approved by the Laboratory Animal Center of Soochow University. CNE-1-empty vector, CNE-1-miR-608-C, CNE-1-miR-608-G, CNE-2-empty vector, CNE-2-miR-608-C, and CNE-2-miR-608-G cells were diluted to a concentration of 5 × 10^6/mL in physiologic saline. Nude mice were injected subcutaneously with 0.1 mL of the suspension into the back flank (6 mice/group). For details, see Supplementary Materials and Methods.

Radiation delivery

Local irradiation of the implanted tumor was conducted using a customized mouse jig with other parts of the body shielded with lead. Each mouse was confined to a customized mouse jig with a circular window, through which the tumor bed was exposed to the radiation, and was irradiated locally. Mice were exposed to X-rays with 5-mm thick lead shields when the tumor bed was gently extended into the radiation field. Tumors were locally irradiated at a dose of 2, 10, or 20 Gy using a RS2000 X-ray Biological Irradiator (Rad Source Technologies) at a dose rate of 1 Gy/min through a 0.2-mm copper filter beginning 1 week after transplantation.

Statistical analysis

The χ^2 test or Fisher exact test was applied separately to compare the distribution of selected demographic and clinical variables according to recurrence status. The Cox proportional hazards model was used to estimate HRs and their 95% confidence intervals (CIs) for multivariate analyses in the discovery set, validation set, and combined dataset. The analyses were adjusted for age, gender, BMI, smoking status, drinking status, family history of cancer, EBV infection status, stage, histologic grade, and chemotherapy, as appropriate. Associations between genotypes and TTR and OS were estimated using the Kaplan–Meier method, and statistical significance was determined using the log-rank test (34). For the functional analyses, data were presented by using mean ± SEM; the comparison of mean between two groups was conducted by using Student t test; statistical comparisons of more
than two groups were conducted using the one-way ANOVA (35), and then least-significant difference (LSD) for multiple comparisons. The statistical analyses were conducted using STATA software (version 10; STATA Corporation). All P values were two-sided and \( P < 0.05 \) was considered statistically significant.

Results

**Patient characteristics**

The clinical information and demographic characteristics of the 1,665 patients with nasopharyngeal carcinoma included in this study are shown in Table 1. For the eastern Chinese population, the median patient age was 54 years (range, 21–82 years) and the median follow-up time was 3.1 years (range, 0.4–10.8 years); 176 patients (21.03%) showed tumor LRR after radiotherapy, resulting in a 3-year recurrence probability of 0.21 ± 0.04, and the median TTR was 1.9 years (95% CI, 1.6–2.3 years); 180 patients (21.51%) have died (including 150 LRRs, 21 distant metastases, and 9 other reasons), but the median OS had not been reached during the follow-up time. No significant differences were noted with regard to sex, age, family history of cancer, smoking status, drinking status, BMI, stage, or histologic grade according to recurrence status; however, significant differences were found in EBV infection status and the number of patients who received chemotherapy (Table 1).

For the southern Chinese population, the median patient age was 52 years (range, 19–87 years) and the median follow-up time was 2.9 years (range, 0.5–10.2 years); 218 patients (26.33%) exhibited tumor recurrence after radiotherapy, resulting in a 3-year recurrence probability of 0.28 ± 0.04, and the median TTR was 2.2 years (95% CI, 2.1–2.5 years); 223 patients (26.93%) have died (including 182 LRRs, 29 distant metastases, and 12 other reasons), but median OS had not been reached during the follow-up time.

**Associations between SNPs and recurrence risk**

Genotype distributions of all SNPs were found to be consistent with Hardy–Weinberg equilibrium (HWE), with the exception of rs7227168C>T and rs41524547C>G. No significant associations were noted in the SNPs and baseline demographic, clinical, or pathologic characteristics according to recurrence status. The selected seven candidate SNPs were genotyped in a discovery set consisting of 837 patients with nasopharyngeal carcinoma from the eastern Chinese population. In the univariate analysis, patients carrying the miR-608 rs4910510GG genotype had a median TTR of 6.2 years, compared with a median TTR of 8.9 years for patients with rs4910510CC genotype (HR, 2.02; 95% CI, 1.29–3.16; \( P = 0.0008 \), Fig. 1A; Table 2). However, the other tested SNPs did not show any statistically significant associations with nasopharyngeal carcinoma LRR in the univariate analyses. In the multivariate analysis, a Cox proportional hazards model was adjusted for age, gender, BMI, smoking status, drinking status, family history of cancer, EBV infection status, stage, histologic grade, and chemotherapy, and the miR-608 rs4910510GG genotype remained significantly associated with nasopharyngeal carcinoma LRR (HR, 2.05; 95% CI, 1.35–3.21; \( P = 0.0012 \); Table 2). These results were also confirmed in the southern Chinese population, in which rs4910510CC genotype had a consistent association with recurrence in the validation (HR, 2.24; 95% CI, 1.45–3.38; \( P = 0.0006 \); Fig. 1B; Table 2) and combined (HR, 2.08; 95% CI, 1.41–3.26; \( P < 0.0001 \)) datasets (Fig. 1C; Table 3).

About 85% of patients for both populations received the chemotherapy during the treatment; however, there were no significant differences in the associations with miR-608 rs4919510C>G polymorphism by chemotherapy (\( P > 0.05 \)), as shown in Supplementary Fig. S1.

**Associations between miR-608 rs4919510C>G genotypes and OS**

As shown in Supplementary Fig. S2A–S2C, we found that miR-608 rs4919510C>G exhibited a consistent association with the OS in the discovery set (HR, 2.13; 95% CI, 1.39–3.28; \( P = 0.0006 \)), the validation set (HR, 1.89; 95% CI, 1.23–3.07; \( P = 0.0023 \)), and the combined dataset (HR, 1.95; 95% CI, 1.31–3.15; \( P < 0.0001 \)). However, we also analyzed the time from
### Table 2. Univariate and multivariate analysis of mature miRNAs sequence polymorphisms and nasopharyngeal carcinoma recurrence in discovery set and validation set

<table>
<thead>
<tr>
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<th>Discovery set (eastern Chinese, N = 837)</th>
<th>Validation set (southern Chinese, N = 828)</th>
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<tr>
<td></td>
<td>Univariate analysis</td>
<td>Multivariate analysis</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>MAF</td>
</tr>
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<td>miR-608 rs4919510</td>
<td>0.509</td>
<td>0.0008</td>
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<tr>
<td>CC</td>
<td>201</td>
<td>0.17 ± 0.03</td>
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<tr>
<td>GC</td>
<td>420</td>
<td>0.19 ± 0.03</td>
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<tr>
<td>GG</td>
<td>216</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>GG+GG</td>
<td>636</td>
<td>0.22 ± 0.03</td>
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<td>miR-4513 rs2168518</td>
<td>0.194</td>
<td>0.120</td>
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<td>CC</td>
<td>552</td>
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<tr>
<td>TC</td>
<td>246</td>
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<tr>
<td>TT</td>
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<tr>
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<td>368</td>
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<tr>
<td>CT</td>
<td>357</td>
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<tr>
<td>CC</td>
<td>112</td>
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<tr>
<td>TC</td>
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<td>GG</td>
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<tr>
<td>AG</td>
<td>167</td>
<td>0.21 ± 0.05</td>
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<tr>
<td>AA</td>
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<tr>
<td>AG+AA</td>
<td>184</td>
<td>0.24 ± 0.04</td>
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</table>

*aProbability ± SE of 3-year recurrence.

*bOn the basis of log-rank test.

*cOn the basis of Wald test within Cox proportional hazards model with adjusted for age, gender, BMI, smoking status, drinking status, family history of cancer, EBV infection status, stage, histologic grade, and chemotherapy.

dNumber of patients with the given genotype.

eMinor allele frequency.
Polymorphism in microRNA and Recurrence of Nasopharyngeal Carcinoma

Table 3. Univariate and multivariate analysis of mature miRNAs sequence polymorphisms and nasopharyngeal carcinoma recurrence in combined dataset

<table>
<thead>
<tr>
<th></th>
<th>Combined dataset (eastern and southern Chinese, (N = 1,665))</th>
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<td></td>
<td><strong>Univariate analysis</strong></td>
<td><strong>Multivariate analysis</strong></td>
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<tr>
<td></td>
<td>(N^a)</td>
<td>MAF(^b)</td>
<td>Probability(^a)</td>
<td>HR (95% CI)</td>
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<tr>
<td>mIR-608 rs4919510</td>
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<td>0.18 ± 0.03</td>
<td>1.00 (Reference)</td>
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<td>CC</td>
<td>410</td>
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<td>0.22 ± 0.02</td>
<td>1.48 (1.01–2.08)</td>
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<td>GC</td>
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<td>2.18 (1.59–2.99)</td>
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<tr>
<td>GC+GG</td>
<td>1,255</td>
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<td>2.08</td>
<td>1.53 (1.07–2.18)</td>
</tr>
</tbody>
</table>

\(^a\)Probability ± SE of 3-year recurrence.

\(^b\)On the basis of log-rank test.

\(^c\)On the basis of Wald test within Cox proportional hazards model with adjusted for age, gender, BMI, smoking status, drinking status, family history of cancer, EBV infection status, stage, histologic grade, and chemotherapy.

\(^d\)Number of patients with the given genotype.

\(^e\)Minor allele frequency.

Effects of the rs4919510C>G genotypes on the mIR-608 target genes expression

The nasopharyngeal carcinoma cell line CNE-1 was infected with mIR-608-C-allele lentivirus, mIR-608-G-allele lentivirus, or control lentivirus, and the infection efficiency exceeded 90% for all lentiviruses, as shown in Supplementary Fig. S3. Next, the expression levels of mIR-608 target genes were detected by microarray analysis (Fig. 2A). We compared RNA transcription levels between the CNE-1-mIR-608-C and CNE-1-mIR-608-G groups. Overall, 2,242 genes were differentially expressed with a \(P < 0.01\). Of these genes, 801 were upregulated and 1,441 genes were downregulated (GEO; accession number: GSE46372); the genes with altered expression after infection induced immunity and defense genes, DNA repair genes, cell growth–related genes, tumor invasion and metastasis-related genes, cancer stem cell–related genes, and cell death–related genes. We also compared gene expression levels between the CNE-1-mIR-608-C and CNE-1-mIR-608-G groups and the control group (CNE-1-empty vector). We identified 108 genes that were downregulated with a \(P < 0.01\) (GEO; accession number: GSE46372). Interestingly, four differentially expressed genes were present in both comparisons: FBXO32, RHOD, TRIM31, and TSC22D3 (Fig. 2A). Differentially expressed genes from the microarray experiments were analyzed using STRING, a database of known and predicted protein–protein interactions. Figure 2B summarizes the network of predicted associations for differentially expressed gene-encoded proteins. The results indicated that FBXO32 was the key gene of this protein interaction net. This gene was linked to TNF and KITLG, and these genes were linked to many downstream genes. All of these genes were interrelated, thereby forming a large network. However, the other 3 genes (RHOD, TRIM31, and TSC22D3) were not linked to other genes (outside the network).

The rs4919510C>G genotypes affect FBXO32 expression by inhibiting the binding of mIR-608 in vitro

According to a bioinformatics analysis software program (MIRanda Java Interface v1.0), the FBXO32 3’-UTR was predicted to bear a mIR-608–binding site. CNE-1 cells were transiently cotransfected with two mIR-608 mimics (containing different rs4919510C>G alleles) and the reporter constructs and then assessed for luciferase activity. Compared with the rs4919510C allele, the rs4919510G allele was associated with significantly reduced luciferase activity in a concentration-dependent manner (Fig. 3B). The same experiments were repeated using CNE-2 cells with similar results (Fig. 3C).

Effects of the mIR-608 rs4919510C>G genotypes on X-ray radiation-induced chromatid breaks in lymphocytes

We investigated the phenotype of X-ray radiation-induced chromatid breaks in lymphocyte cells from 125 control subjects and the rs4919510C>G genotype–phenotype association in these individuals. The exact b/c value was defined as the b/c value of the treatment group minus the

recurrence to death among patients with nasopharyngeal carcinoma (Supplementary Fig. S2D) and found that there was no significant difference among patients carrying the different mIR-608 rs4919510C>G genotypes (ANOVA test, \(P > 0.05\)).
spontaneous b/c value of the untreated group. As shown in Fig. 3D, we found that the mean ± SEM b/c value in the 28 rs4919510CC carriers was 0.225 ± 0.008, which was significantly higher than those of 39 individuals with rs4919510GG genotype (0.182 ± 0.007) and 58 individuals with the rs4919510GC genotype (0.204 ± 0.007; ANOVA test, $P = 0.0024$).

Effects of the miR-608 rs4919510C–G genotypes on the cell growth in response to irradiation

CNE-1-empty vector, CNE-1-miR-608-C, and CNE-1-miR-608-G cells were subjected to 2 Gy of radiation to examine the effect on cell growth. As shown in Fig. 4A, the cell growth delay after irradiation was shorter for CNE-1-miR-608-G cells than for CNE-1-miR-608-C cells. The same experiments were
Effects of the miR-608 rs4919510C>G genotypes on tumor growth

We evaluated the effects of radiation on tumor growth in different animal models. During a 1-week period, all mice were injected with CNE-1-miR-608-C, CNE-1-miR-608-G, or CNE-1-empty vector cells developed tumors, after which mice were assigned to receive 2, 10, or 20 Gy of local radiation. As shown in Fig. 4B, no significant tumor growth inhibition was observed in CNE-1-empty vector, CNE-1-miR-608-C, or CNE-1-miR-608-G xenografts that were locally irradiated with 2Gy of radiation ($P = 0.625$). In contrast, it was showed that the CNE-1-miR-608-G xenografts grew faster than the CNE-1-miR-608-C xenografts after 10-Gy irradiation (average volumes ±SEM: 593 ± 59.49 mm$^3$ vs. 891 ± 93.44 mm$^3$; $P = 0.009$). Unfortunately, approximately 70% of the mice died after being locally irradiated at a dose of 20 Gy (Fig. 4B). The same experiments were repeated using the CNE-2-empty vector, CNE-2-miR-608-C, and CNE-2-miR-608-G cells with similar results (Fig. 4A).

Discussion

In the present study, we investigated the effects of SNPs in mature miRNA sequences on LRR in patients with nasopharyngeal carcinoma after radiotherapy. We found that carrying at least one G allele (GC, GG) of the miR-608 rs4919510C>G SNP significantly increased the risk of LRR compared with that in patients carrying a homozygous C allele. Importantly, these results remained significant after adjustment for other potential predictors of patient outcome in this patient cohort study, and our functional results were also consistent with these findings. This study represents the first finding that the miR-608 rs4919510C>G SNP may serve as a predictive marker for the LRR of nasopharyngeal carcinoma in patients who were treated with radiotherapy.

Nasopharyngeal carcinoma is highly sensitive to radiation that alone for early nasopharyngeal carcinoma can achieve a relatively high cure rate; however, its efficacy is disappointing for locally advanced disease. It is well known that recurrence in the nasopharynx is one of the important causes of treatment failure (36, 37); therefore, assessments of recurrence are crucial for the selection of appropriate treatment. Previous reports indicated that miRNAs were aberrantly expressed in nasopharyngeal carcinoma compared with that in normal epithelial tissue, and this aberrant expression promoted an aggressive tumor phenotype by changing the expression of mRNA targets (38–40). Therefore, miRNA-related SNPs may be used individually and jointly to predict the risk of recurrence of early-stage head and neck cancer (41). Liu and colleagues suggested that some important miRNAs had a significant value for determining the survival prognosis in addition to nasopharyngeal carcinoma development and progression (42). As the important roles of miRNAs in cancer are gradually being revealed, their potential applications as predictive markers and treatment targets have generated great interest for cancer diagnosis, classification, prognosis, risk factor evaluation, and therapy strategies.
A series of epidemiologic studies revealed that the miR-608 rs4919510C>G SNP has a key role in cancer progression (17), and evidence of its influence on prognosis has also accumulated recently (18, 34, 43). However, the importance of the miR-608 rs4919510C>G SNP in the LRR of nasopharyngeal carcinoma after radiotherapy remains unclear, and the biologic functions of this SNP have not yet been elucidated. The rs4919510C>G SNP is located within the mature sequence of miR-608 and at the joint of the stem with the canonical hairpin loop (18). Because this rigid secondary structure is a requisite for recognition, and thus processing, of pre-miRNA by the RNase Drosha, structural disruptions at this critical point may affect recognition or subsequent processing. Each miRNA has hundreds of targets, and thus, a singular change in a mature miRNA sequence could have an exponentially large effect on protein output, perhaps an effect sufficient to skew, even slightly, the clinical outcome of cancer. Our results indicated that the rs4919510C>G SNP might influence the expression of miR-608 target genes, which include immunity and defense genes, DNA repair genes, cell growth–related genes, tumor invasion and metastasis-related genes, cancer stem cell–related genes, and cell death–related genes. These genes could have consequences directly related to cancer cell survival and tumor growth, thereby influencing the LRR of nasopharyngeal carcinoma after radiotherapy. Moreover, the X-ray radiation induced more chromatid breaks in lymphocyte cells from rs4919510CC carriers than in those from subjects with other genotypes. As we know, medical radiation can cause DNA...
double-strand breaks in cancer cells and suppress cancer cell growth (44), which is consistent with the rs4919510C variant genotype having low DNA repair genes expression and deficient DNA repair capacity, and the association between rs4919510C variant genotype and medical radiation on decreasing LRR risk of nasopharyngeal carcinoma is biologically plausible. Our results also indicated that miR-608 rs4919510C>G exhibited an association with the OS; however, there was no significant difference in time from recurrence to death among patients carrying different miR-608 rs4919510C>G genotypes. All these results suggested that miR-608 rs4919510C>G was associated with higher risk of LRR and then the TTR in advance, which finally may lead to the higher risk of all-cause mortality. Therefore, our present study detected the importance of mature miRNA sequence SNPs in the LRR of nasopharyngeal carcinoma after radiotherapy.

FBXO32 (also known as atrogin-1) is a member of the F-box protein family and constitutes 1 of 4 subunits of the ubiquitin protein ligase complex (45, 46). FBXO32 has been reported to play a role in muscle atrophy (47), and recent findings have suggested that FBXO32 is a novel apoptosis regulator that is transcriptionally silenced by epigenetic mechanisms in cancer cells (48). Furthermore, Chou and colleagues found that FBXO32 might be a novel tumor suppressor gene that was associated with poor prognosis in human ovarian cancer (50). Our reporter gene assays suggested that the rs4919510C allele affected FBXO32 expression by inhibiting the binding of miR-608 in nasopharyngeal carcinoma cell lines, and these results are consistent with previous findings on the contributions of FBXO32 to tumor suppression.

In summary, our preliminary study provides the first evidence that miR-608 rs4919510C>G may be a predictive marker to identify patients with a high risk of nasopharyngeal carcinoma recurrence, and these data may help to predict response in a subgroup of patients treated with radiotherapy. In addition, this may help to select subgroups of patients with nasopharyngeal carcinoma who may benefit from newly developed gene-therapy strategies. Nevertheless, a median follow-up time of approximately 3 years for both populations included in the present studies may not be sufficient to identify all of the recurrences that would occur, and further validation of our hypothesis-generating findings in prospective biomarker-embedded clinical trials is needed. Therefore, large patient cohort studies are warranted to further confirm our results.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Zheng, J. Lu, Y. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Zheng, J. Deng, M. Xiao, L. Yang, L. Zhang, Y. You, W. Li, J. Lu, Y. Zhou
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Zheng, J. Deng, Y. You, N. Li, J. Lu, Y. Zhou
Writing, review, and/or revision of the manuscript: J. Zheng, J. Deng, H. Wu, J. Lu, Y. Zhou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Zheng, J. Deng, L. Zhang, M. Hu, N. Li, J. Lu, Y. Zhou
Study supervision: J. Lu, Y. Zhou

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