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 Hu3, 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. Cancer Res; 73(16); 5151–62. ©2013 AACR.
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 Huaiian No.1 Hospital (Huaiian, 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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Discovery set (eastern Chinese, N = 837)</th>
<th>Validation set (southern Chinese, N = 828)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recurrence (N = 176)</td>
<td>No recurrence (N = 661)</td>
</tr>
<tr>
<td>Age, mean (SEM)</td>
<td>53.91 (0.879)</td>
<td>52.85 (0.411)</td>
</tr>
<tr>
<td>Gender, N (%)</td>
<td>Male 116 (65.91)</td>
<td>466 (70.50)</td>
</tr>
<tr>
<td></td>
<td>Female 60 (34.09)</td>
<td>195 (29.50)</td>
</tr>
<tr>
<td>Family history of cancer, N (%)</td>
<td>No 156 (88.64)</td>
<td>589 (89.11)</td>
</tr>
<tr>
<td></td>
<td>Yes 20 (11.36)</td>
<td>72 (10.89)</td>
</tr>
<tr>
<td>Smoking status, N (%)</td>
<td>Never 82 (46.59)</td>
<td>291 (44.02)</td>
</tr>
<tr>
<td></td>
<td>Ever 94 (53.41)</td>
<td>370 (55.98)</td>
</tr>
<tr>
<td>Drinking status, N (%)</td>
<td>Never 87 (49.43)</td>
<td>352 (53.25)</td>
</tr>
<tr>
<td></td>
<td>Ever 89 (50.57)</td>
<td>309 (46.75)</td>
</tr>
<tr>
<td>BMI, N (%)</td>
<td>≤20 28 (15.91)</td>
<td>116 (17.55)</td>
</tr>
<tr>
<td></td>
<td>20–28 133 (75.57)</td>
<td>481 (72.77)</td>
</tr>
<tr>
<td></td>
<td>≥28 15 (8.52)</td>
<td>64 (9.68)</td>
</tr>
<tr>
<td>EBV infection status, N (%)</td>
<td>Positive 159 (90.34)</td>
<td>552 (83.51)</td>
</tr>
<tr>
<td></td>
<td>Negative 17 (9.66)</td>
<td>109 (16.49)</td>
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<td>Stage, N (%)</td>
<td>I 8 (4.55)</td>
<td>28 (4.23)</td>
</tr>
<tr>
<td></td>
<td>II 42 (23.86)</td>
<td>155 (23.45)</td>
</tr>
<tr>
<td></td>
<td>III 68 (38.64)</td>
<td>316 (47.81)</td>
</tr>
<tr>
<td></td>
<td>IV 58 (32.95)</td>
<td>162 (24.51)</td>
</tr>
<tr>
<td>Histologic grade, N (%)</td>
<td>Undifferentiated 139 (78.97)</td>
<td>484 (73.22)</td>
</tr>
<tr>
<td></td>
<td>Differentiated 37 (21.02)</td>
<td>177 (26.78)</td>
</tr>
<tr>
<td>Chemotherapy, N (%)</td>
<td>Yes 143 (81.25)</td>
<td>593 (89.71)</td>
</tr>
<tr>
<td></td>
<td>No 33 (18.75)</td>
<td>68 (10.29)</td>
</tr>
</tbody>
</table>
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 about 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 clinical information or both, 125 cases (6.03%) lacked stage 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).

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(Shanghai, China) and passaged for fewer than 6 months. The cell lines were characterized by DNA fingerprinting analysis using short-tandem repeat (STR) markers. Cells were maintained according to the Cell Bank’s protocols.

Plasmids, lentiviral production, and transduction
Lentiviral expression plasmids construction, lentiviral production, and transduction were conducted by following the established and previously published procedures (27, 28). For details, see Supplementary Materials and Methods. Finally, 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 stably selected with G418 at 500 μg/mL (Gibco), and the drug-resistant cell populations were used for subsequent studies.

RNA extraction and microarray analysis
Total RNA was extracted from the cultured cells in both experimental (CNE-1-miR-608-C and CNE-1-miR-608-G) and control (CNE-1-empty vector) groups using RNeasy Mini Kits (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).

Quantitative real-time PCR analysis
Total RNA was isolated from 35 nasopharyngeal carcinoma tissue samples with TRIzol reagent (Molecular Research Center, Inc.). The relative gene expression for the selected genes was quantified using SYBR Green methodology (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’-TGTATTATGCTGAGCGGCTATCTC-3’ and reverse primer 5’-CGCTCTAAGTCGTAAAAGCGGCCTGCG-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 chromatid 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-G displayed 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 rs4910510C>G polymorphism by chemotherapy (P > 0.05), as shown in Supplementary Fig. S1.

Associations between miR-608 rs4910510C>G genotypes and OS

As shown in Supplementary Fig. S2A–S2C, we found that miR-608 rs4910510C>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>
<th>miRNA</th>
<th>N&lt;sup&gt;d&lt;/sup&gt;</th>
<th>MAF&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Probability&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HR (95% CI)</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HR (95% CI)</th>
<th>P&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-608</td>
<td>368</td>
<td>0.049</td>
<td>0.0008</td>
<td>0.0012</td>
<td>0.0482</td>
<td>0.001</td>
<td>0.0006</td>
</tr>
<tr>
<td>CC</td>
<td>201</td>
<td>0.17 ± 0.03</td>
<td>1.00 (Reference)</td>
<td>1.00</td>
<td>209</td>
<td>0.21 ± 0.04</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>GC</td>
<td>420</td>
<td>0.19 ± 0.03</td>
<td>1.45 (0.92–2.18)</td>
<td>0.493</td>
<td>439</td>
<td>0.27 ± 0.03</td>
<td>1.55 (1.03–2.15)</td>
</tr>
<tr>
<td>GG</td>
<td>216</td>
<td>0.26 ± 0.05</td>
<td>2.05 (1.35–3.21)</td>
<td>0.180</td>
<td>180</td>
<td>0.37 ± 0.04</td>
<td>2.21 (1.49–3.24)</td>
</tr>
<tr>
<td>GC+GG</td>
<td>636</td>
<td>0.22 ± 0.03</td>
<td>1.68 (1.18–2.61)</td>
<td>0.178</td>
<td>619</td>
<td>0.29 ± 0.03</td>
<td>1.65 (1.17–2.39)</td>
</tr>
<tr>
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<td>357</td>
<td>0.019 ± 0.03</td>
<td>1.00 (Reference)</td>
<td>1.00</td>
<td>378</td>
<td>0.28 ± 0.03</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>CC</td>
<td>552</td>
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<td>1.00 (Reference)</td>
<td>1.00</td>
<td>560</td>
<td>0.26 ± 0.03</td>
<td>1.00 (Reference)</td>
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<tr>
<td>GC</td>
<td>246</td>
<td>0.23 ± 0.03</td>
<td>1.21 (0.87–1.67)</td>
<td>0.234</td>
<td>234</td>
<td>0.28 ± 0.04</td>
<td>1.11 (0.71–1.53)</td>
</tr>
<tr>
<td>GT</td>
<td>39</td>
<td>0.073 ± 0.03</td>
<td>1.18 (0.84–1.71)</td>
<td>0.705</td>
<td>268</td>
<td>0.29 ± 0.03</td>
<td>1.17 (0.78–1.61)</td>
</tr>
<tr>
<td>GC+GT</td>
<td>285</td>
<td>0.22 ± 0.05</td>
<td>1.14 (0.82–1.68)</td>
<td>0.335</td>
<td>286</td>
<td>0.29 ± 0.03</td>
<td>1.17 (0.78–1.61)</td>
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<tr>
<td>miR-4520a</td>
<td>385</td>
<td>0.0347</td>
<td>0.737</td>
<td>0.705</td>
<td>1.00</td>
<td>0.29 ± 0.03</td>
<td>1.17 (0.78–1.61)</td>
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<tr>
<td>GT</td>
<td>368</td>
<td>0.18 ± 0.02</td>
<td>1.00 (Reference)</td>
<td>1.00</td>
<td>378</td>
<td>0.28 ± 0.03</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>CT</td>
<td>112</td>
<td>0.22 ± 0.04</td>
<td>0.91 (0.54–1.51)</td>
<td>0.104</td>
<td>104</td>
<td>0.25 ± 0.05</td>
<td>0.92 (0.73–1.14)</td>
</tr>
<tr>
<td>CT+CC</td>
<td>469</td>
<td>0.22 ± 0.04</td>
<td>1.09 (0.81–1.52)</td>
<td>0.450</td>
<td>450</td>
<td>0.26 ± 0.04</td>
<td>0.96 (0.78–1.17)</td>
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<tr>
<td>miR-499</td>
<td>3746444</td>
<td>0.148</td>
<td>0.647</td>
<td>0.523</td>
<td>0.150</td>
<td>0.28 ± 0.02</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>CT</td>
<td>611</td>
<td>0.19 ± 0.02</td>
<td>1.00 (Reference)</td>
<td>1.00</td>
<td>605</td>
<td>0.28 ± 0.02</td>
<td>1.00 (Reference)</td>
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<tr>
<td>CC</td>
<td>204</td>
<td>0.20 ± 0.05</td>
<td>1.13 (0.79–1.60)</td>
<td>0.199</td>
<td>199</td>
<td>0.25 ± 0.03</td>
<td>0.87 (0.47–1.64)</td>
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<tr>
<td>CC+GC</td>
<td>226</td>
<td>0.21 ± 0.03</td>
<td>1.06 (0.73–1.54)</td>
<td>0.234</td>
<td>223</td>
<td>0.27 ± 0.02</td>
<td>0.86 (0.57–1.17)</td>
</tr>
<tr>
<td>miR-3152</td>
<td>313299349</td>
<td>0.120</td>
<td>0.339</td>
<td>0.256</td>
<td>0.122</td>
<td>0.27 ± 0.02</td>
<td>0.86 (0.57–1.17)</td>
</tr>
<tr>
<td>GG</td>
<td>653</td>
<td>0.18 ± 0.02</td>
<td>1.00 (Reference)</td>
<td>1.00</td>
<td>639</td>
<td>0.27 ± 0.03</td>
<td>1.00 (Reference)</td>
</tr>
<tr>
<td>AG</td>
<td>167</td>
<td>0.21 ± 0.05</td>
<td>1.13 (0.70–1.67)</td>
<td>0.177</td>
<td>177</td>
<td>0.29 ± 0.05</td>
<td>1.15 (0.80–1.61)</td>
</tr>
<tr>
<td>AA</td>
<td>17</td>
<td>0.073 ± 0.03</td>
<td>1.18 (0.82–1.68)</td>
<td>0.705</td>
<td>1.00</td>
<td>0.29 ± 0.03</td>
<td>1.17 (0.78–1.61)</td>
</tr>
<tr>
<td>AG+AA</td>
<td>184</td>
<td>0.24 ± 0.04</td>
<td>1.19 (0.78–1.93)</td>
<td>0.198</td>
<td>189</td>
<td>0.30 ± 0.04</td>
<td>1.07 (0.57–1.69)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Probability ± SE of 3-year recurrence.<br>On the basis of log-rank test.<br>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.<br>Number of patients with the given genotype.<br>Minor allele frequency.
Four differentially expressed TRIM31, FBXO32, TSC22D3, and RHOD genes were present in both comparisons: associations for differentially expressed gene-encoded protein interactions. Figure 2B summarizes the network of predicted genes from the microarray experiments were analyzed using CaneCNE-1-miR-608-also compared gene expression levels between the CNE-1-stem cell genes, tumor invasion and metastasis-related genes, cancer-infinity and defense genes, DNA repair genes, cell growth–related 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. The results for the 20 selected genes had shown that the direction of expression changes were consistent with those found by microarray analysis (Fig. 3A).

**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).

On the basis of the microarray array results, the expression levels of 20 selected genes (9 upregulated and 11 downregulated) were evaluated using the quantitative PCR (qPCR) analysis, and all these selected 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. The results for the 20 selected genes had shown that the direction of expression changes were consistent with those found by microarray analysis (Fig. 3A).

**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

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**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>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate analysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UniGene ID</td>
<td>MAF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-608 rs4919510</td>
<td>0.496</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>CC</td>
<td>410</td>
<td>0.22 ± 0.02</td>
</tr>
<tr>
<td>GC</td>
<td>859</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>GG</td>
<td>396</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>GC+GG</td>
<td>1,255</td>
<td></td>
</tr>
</tbody>
</table>

*Probability ± SE of 3-year recurrence.

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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
repeated using CNE-2-empty vector, CNE-2-miR-608-C, and CNE-2-miR-608-G cells with similar results (Fig. 4A).

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 2 Gy 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 mm³ vs. 891 ± 93.44 mm³; 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 cell xenografts with similar results (Fig. 4B).

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

Figure 4. A, 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 subjected to 2 Gy of radiation to examine the effect on cell growth. Cells were seeded in 24-well culture plate 24 hours before irradiation. Cell numbers were counted at different times after irradiation. Cell multiplication of CNE-1-empty vector and CNE-2-empty vector cells were used as controls. Three experiments were carried out; points, mean; bars, SEM. B, subcutaneously implanted 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 xenografted tumors were established. During a 1-week period, all mice developed tumors, after which mice were assigned to receive 2, 10, or 20 Gy of local radiation. Each point represents the mean tumor volume. Bars, SEM. Mean tumor volume from 6 nude mice of each group are shown.
double-strand breaks in cancer cells and suppress cancer cell growth (44), which is consistent with the rs4919510CC variant genotype having low DNA repair gene expression and deficient DNA repair capacity, and the association between rs4919510CC 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 associated with poor prognosis in human ovarian cancer (50).

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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References
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