Radiotherapy technique

All patients were immobilized in the supine position with thermoplastic masks. Contrast-enhanced planning computed tomography (25) scans with a 3-mm slide thickness were then obtained, with coverage from the skull vertex to 2 cm below the clavicles. Magnetic resonance imaging/computed tomography was performed for all patients to accurately delineate of tumor volumes and critical structures. The primary tumor and upper neck above the bottom of the hyoid bone were treated with intensity-modulated radiotherapy (IMRT) techniques using 7 coplanar beams with an inverse treatment planning system and simulated annealing. IMRT was delivered using a simultaneous integrated boost technique (5).

The gross tumor volume (GTV) included the primary nasopharyngeal tumor (GTVnx) and the involved lymph nodes (GTVnd) as demonstrated by imaging and physical examinations. The high-risk clinical tumor volume (CTV-1) included the GTV plus a 5-mm margin and encompassed the entire nasopharyngeal mucosa plus a 5-mm submucosal volume. CTV2 covered the CTV1 and areas at risk, including the posterior third of the nasal cavity and maxillary sinus, pterygopalatine fossa, posterior ethmoid sinus, parapharyngeal space, skull base, and clivus, or conversely, it was based on tumor invasion. CTV3 covered lower risk lymphatic levels. The planning target volume (PTV) was created on the basis of each CTV with an additional 3-5 mm margin, accounting for organ motion/daily treatment set-up uncertainties. In areas where the GTV or the CTV was adjacent to critical normal structures (i.e., brainstem), a smaller margin was delineated. The prescribed dose was 68-74 Gy to the gross
disease PTV for the nasopharynx and 66-72 Gy for positive lymph nodes in 30-33 fractions. The prescribed doses to high- and low-risk region PTVs were 60-63 Gy and 50.4-56 Gy in 30-33 and 28 fractions, respectively.

**Plasmids, lentiviral production, and transduction**

The precursor sequence of miRNA-608 containing rs4919510C>G was amplified with the forward primer 5′-GACTCGAGCTCTAATAAAAAATAAT-3′ and reverse primer 5′-TCGCGGCGCCAGAATAAAAAACCCAAAC-3′ from a homozygous human genomic DNA sample. The amplified fragment was digested with XhoI and NotI (Fermentas, Hanover, MD, USA) and then cloned into the lentiviral expression vector pLVX-IRES-neo (Clontech Laboratories Inc., San Francisco, CA) (28-29). The Quick Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to mutate the rs4919510C nucleotide to guanine. The resulting constructs (pLVX-IRES-neo-miRNA-608-C-allele and pLVX-IRES-neo-miRNA-608-G-allele) were verified by sequencing. Replication-defective VSV-G pseudotyped viral particles were packaged in LentiX 293T human embryonic kidney cells (Clontech) by using a 3-plasmid transient cotransfection method (Lenti-T HT packaging mix, Clontech). Viruses were harvested and concentrated. For transduction, CNE-1 and CNE-2 cells were infected with control lentivirus (empty vector, only lentivirus without the miR-608 fragment was inserted), miRNA-608-C-allele lentivirus, and miRNA-608-G-allele lentivirus. After 48 hr of transduction, the cells were stably selected with G418 at 500μg/ml (Gibco, Lyon, France), 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-miRNA-608-C and CNE-1-miRNA-608-G) and control (CNE-1-empty vector) groups using RNeasy Mini Kits (Qiagen, CA, USA) according to the manufacturer’s instructions. RNA quantity and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). Gene expression profiling was conducted using the Human OneArray™ microarray (Phalanx Biotech, Palo Alto, CA) containing over 30,000 60-mer polynucleotide probes, with each probe mapped to the latest draft of the human genome (GoldenPath) and printed on standard 1-in. × 3-in. glass slides(30-31). Analysis was performed according to the manufacturer’s recommendations. Two technical repetitions were performed. After hybridization, arrays were scanned using GenePix 4000B (Axon Instruments, Union City, CA) and analyzed with GenePix Pro6.0 (Axon Instruments, Union City, CA) to obtain gene expression levels(31). A linear regression model was used to detect differentially expressed changes among the study groups.

Quantitative real-time PCR analysis

On the basis of the microarray array results, the expression levels of 20 selected genes were evaluated using qPCR analysis, 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. Total RNA was isolated from 35 NPC tissue samples with TRIzol reagent (Molecular Research Center, Inc). The
concentration and purity of RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, MA, USA). cDNA was generated from mRNA using the appropriate oligo primers and Superscript II (Invitrogen, CA) according to the manufacturer’s instructions. 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. \( \beta \text{-actin} \) was used as an internal reference gene in each reaction. The primers used for PCR amplification of the candidate genes are listed in Supplemental Table 2. The PCR reaction mixture consisted of 0.1\( \mu \)M of each primer, 1\( \times \)SYBR Premix EX Taq (Perfect Real Time) premix reagent (TaKaRa, Dalian, China), and 50ng of cDNA in a final reaction volume of 20\( \mu \)l. The cycling conditions were 95\(^\circ\)C for 2min, followed by 40 cycles at 95\(^\circ\)C for 15s and 60\(^\circ\)C for 1min. The relative gene expression for the precursor \( \text{miRNA-608} \) was also quantified using the ABI Prism 7000 sequence detection system (Applied Biosystems) based on the SYBR-Green method. The expression of \( \text{miRNA-608} \) in NPC cells was calculated relative to the U6 small nuclear RNA. The primers used for PCR amplification of the precursor \( \text{miRNA-608} \) cDNA were 5\(^{\prime}\)-GCTGCGTTTTAAAAAGGCATCTCC-3\(^{\prime}\) and 5\(^{\prime}\)-GTTCCATCTGCTCCCAGCCCCCAT-3\(^{\prime}\). For U6, the primers used were 5\(^{\prime}\)-CTCGCTTCGCGCAGACACA-3\(^{\prime}\) and 5\(^{\prime}\)-AACGCTTCACGAATTTGCGT-3\(^{\prime}\).

**Transient transfections and luciferase assays**

The CNE-1 and CNE-2 cells were seeded at 1\( \times \)10\(^5\) cells per well in 24-well plates (BD Biosciences, Bedford, MA). Sixteen hours after plating, the cells were
transfected with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions. In each well, 800ng of the psiCHECK-2-\textit{FBXO32}-3’UTR vector were cotransfected with 0, 1 or 40pmol of \textit{miR-608} miRNA mimics (containing different rs4910510C>G alleles, Ambion, Austin, TX). In addition, 100pmol of nonspecific miRNA purchased from Ambion were used as a negative control in every miRNA transfection experiment. Six replicates were included for each group, and the experiment was repeated at least 3 times. After transfection for 24h, the cells were assayed by adding 100μl of luciferase assay reagent. \textit{Renilla} luciferase activity in the cell lysate was measured using the Dual-Luciferase Reporter assay system (Promega, Madison, WI) with a TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) and normalized to the activity of firefly luciferase.

\textbf{Mutagen sensitivity assay}

One milliliter of whole blood from each subject and 9ml of RPMI 1640 medium (GIBCO; Invitrogen, China) supplemented with 20% fetal bovine serum (GIBCO; Invitrogen, China) were equally inoculated into 6-well plates as treatment and control groups respectively, to stimulate lymphocyte growth. A final concentration of 50μg/ml phytohemagglutinin was also added into the cultures. After 67h of culture, the treatment group cultures were irradiated with 1.5Gy of incident X-ray radiation at a dose rate of 0.289Gy/min for 5min at room temperature using a 200-kV (constant potential) X-ray machine (PW2184; Philips); the control group cultures were left untreated. Next, both groups of cultures were incubated for another 4 h and treated with 0.3μg of colcemid (GIBCO BRL, Carlsbad, CA) to induce mitotic arrest. One
hour later, the cells were harvested as follows: after 15min of treatment with 60mM KCl hypotonic solution, the cells were fixed for another 15min with freshly prepared methanol: acetic acid (3:1 vol:vol) and air-dried slides were stained with 4% Giemsa (Biomedical Specialties, Santa Monica, CA) for 7min. Chromosomal aberrations in each slide were examined using a Labphoto-2 photomicroscope (Nikon Instrument Group, Melville, NY). The number of simple chromatid breaks was scored from 50 well-spread metaphases for both treated and untreated samples from each subject. A chromatid break was scored as 1 break, and each isochromatid break set and each exchange figure (or interstitial deletion) were scored as 2 breaks. Gaps were not included in the analyses. The value of chromatid breaks per cell (b/c) were used to indicate the DNA repair capacity of the individual.

Animal model

Female BALB/c nude mice, aged 4-5 weeks, were purchased from the Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). Mice were allowed to acclimate to local conditions for at least 1 week and maintained under a 12-h dark/12-h light cycle with food and water provided ad libitum. All of the experiments were conducted in accordance with guidelines approved by the Laboratory Animal Center of Soochow University. CNE-1-empty vector, CNE-1-miRNA-608-C, CNE-1-miRNA-608-G, CNE-2-empty vector, CNE-2-miRNA-608-C, and CNE-2-miRNA-608-G cells were diluted to a concentration of 5×10⁷/ml in physiological saline. Nude mice were injected subcutaneously with 0.1ml of the suspension into the back flank (6 mice/group). For
palpable tumors, the tumor volume was measured using a caliper every other day and calculated according to the following formula: $V = L \times W^2 \times 0.5$ (L, length; W, width).

**Bioinformatics analysis**

TargetScan Human 5.2 (http://www.targetscan.org/) and MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) were employed to predict the targets of *miRNA-608*. Differentially expressed genes from the microarray analysis were analyzed using STRING (http://string-db.org/), a program that predicts associations between proteins.