Genome-Wide Identification of a Methylation Gene Panel as a Prognostic Biomarker in Nasopharyngeal Carcinoma

Wei Jiang1, Na Liu1, Xiao-Zhong Chen2, Ying Sun1, Bin Li2, Xian-Yue Ren1, Wei-Feng Qin2, Ning Jiang1, Ya-Fei Xu1, Ying-Qin Li1, Jian Ren3, William CS Cho4, Jing-Ping Yun1, Jing Zeng1, Li-Zhi Liu1, Li Li1, Ying Guo1, Hai-Qiang Mai1, Mu-Sheng Zeng1, Wei-Feng Qin2, Ning Jiang1, Ya-Fei Xu1, Ying-Qin Li1, Jian Ren3, William CS Cho4, Tie-Bang Kang1, Wei-Hua Jia1, Jian-Yong Shao1, and Jun Ma1

Abstract

DNA methylation, the best known epigenetic marker, can be used as a prognostic biomarker in many cancers. We examined DNA methylation status and survival in nasopharyngeal carcinoma (NPC) patients. Aberrant DNA-methylated genes in 24 NPC tissues and 24 noncancer nasopharyngitis biopsy tissues (NCNBT) were identified using Illumina 450K BeadChip. Correlations between DNA methylation and clinical outcomes were evaluated using bisulfite pyrosequencing in 454 NPC patients. Genome-wide methylation analysis demonstrated that NPC tissues had distinct DNA methylation patterns compared with NCNBT. Among all significant CpG sites, 2,173 CpG sites with β change ≥ 0.2 (1,880 hypermethylated, 293 hypomethylated) were identified (P < 0.05). A methylation gene panel comprising six hypermethylated genes was constructed with the average Z-score method. Patients in the training cohort with high methylation had poorer disease-free survival (DFS, HR, 2.26; 95% confidence interval [CI], 1.28–4.01; P, 0.005) and overall survival (OS, HR, 2.47; 95% CI, 1.30–4.71; P, 0.006) than those with low methylation. There were similar results in the validation (DFS, HR, 2.07; 95% CI, 1.17–3.67; P, 0.013; OS, HR, 1.83; 95% CI, 1.01–3.31; P, 0.046) and independent cohorts (DFS, HR, 1.94; 95% CI, 1.08–3.47; P, 0.026; OS, HR, 2.09; 95% CI, 1.10–3.98; P, 0.022). Analysis indicated that the methylation gene panel was an independent prognostic factor. Furthermore, patients with low methylation had a favorable response to concurrent chemotherapy with an improved DFS (P = 0.045) and OS (P = 0.031), whereas patients with high methylation did not benefit from concurrent chemotherapy. The six-hypermethylated gene panel was associated with poor survival in patients with NPC, demonstrating its potential usefulness as a prognostic biomarker to clinicians in NPC management.

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Introduction

Nasopharyngeal carcinoma (NPC) is a common malignancy in Southern China and Southeast Asia, whereas it is rare in most of the other parts around the world; the age-standardized incidence per 100,000 males ranges 20 to 50 in Southern China as compared with 0.5 in Western countries (1). Annually, approximately 80,000 new NPC cases and 50,000 deaths are reported worldwide (2).

Radiotherapy is the mainstay of treatment for NPC. Radiotherapy with concomitant chemotherapy increases survival for patients with locoregionally advanced NPC. Currently, the tumor-node-metastasis (TNM) staging system is the most important clinical parameter that is providing prognostic information and guiding treatment decisions for patients with NPC. However, TNM stage-based prognostic model is not successful for determining the best personalized treatment. Unpredictably, approximately 20% to 30% of NPC patients with the same stages and who received similar treatment regimens have local recurrence or distant metastasis (3, 4). This suggests that molecular subclassification may be clinically relevant, and there is a need for molecular tools that can stratify patients with respect to prognosis and response to therapy. Nevertheless, there is little available on individual prognostic biomarkers to help predict and improve the patients’ outcome. The development of new effective prognostic biomarkers for NPC is clearly imperative.

To date, emerging evidence has convincingly demonstrated that aberrant epigenetic silencing of many functional genes is a common and early event in human cancers (5–8). Recently, genome-wide gene methylation profiling was used as a strategy for classifying tumor subtypes or predicting clinical outcomes in...
multiple malignancies (9–14). Recent advances also indicate that many genes are predominantly or even exclusively silenced by DNA methylation in epithelial cells during the carcinogenesis of NPC (15, 16). The correlation between DNA methylation and early diagnosis has been explored in NPC in single genes or combinations of multiple genes (17–19). However, the causal relationship between DNA methylation status and clinical outcomes in NPC is still not well understood.

To investigate this important area, we conducted a genome-wide DNA methylation profiling study to identify and validate methylated genes as biomarkers for prognosis in a training cohort and two validation cohorts involving 454 NPC patients in total, providing insight into the development of personalized therapy for NPC patients.

**Materials and Methods**

**Clinical specimens**

For the genome-wide methylation study, the discovery set consisted of 24 fresh-frozen NPC tissues, and 24 noncancer nasopharyngitis biopsy tissues (NCNBT) were obtained from the primary site from the Sun Yat-Sen University Cancer Center (Guangzhou, China). The patients’ characteristics are listed in Supplementary Table S1.

The study included 454 biopsy-proven formalin-fixed paraffin-embedded (FFPE) NPC samples from the primary site. All samples were obtained before any anticancer treatment; 301 samples were collected at Zhejiang Cancer Hospital (Hangzhou, China) based on the 7th edition AJCC/UICC Cancer Staging Manual criteria. All patients (mean age, 47.08 years; range, 18–70 years) were treated with definitive-intent radiotherapy, the cumulative doses were >66 gray (Gy) to the gross tumor, 60 to 66 Gy to the involved areas of the neck, and >50 Gy to uninvolved areas, a 2 Gy fraction 5 times weekly; 147 of 337 (43.6%) patients with stages III to IV also received concurrent platinum-based chemotherapy with weekly cisplatin (40 mg/m² on day 1) intravenously for 7 weeks during radiotherapy. All patients underwent regular postradiotherapy clinical assessment; the median follow-up period was 82 months (range, 2–115 months). The basic descriptive information of the cases is listed in Supplementary Table S2.

**Study design**

We employed genome-wide methylation microarray to identify a hypermethylated methylation gene panel in a discovery set of 24 age- and sex-matched NPC tissues and NCNBT. The associations between the hypermethylated methylation gene panel and survival were analyzed using bisulfite pyrosequencing. The 301 samples from Sun Yat-Sen University were randomly divided into training (n = 151) and validation cohorts (n = 150). The prognostic value of the methylation gene panel was evaluated in the former and validated internally in the latter. The 153 samples from Zhejiang Cancer Hospital were used as an independent cohort to assess whether the panel possessed the same or similar prognostic value in different populations.

**DNA preparation and bisulfite modification**

DNA was extracted from fresh tissues using an AllPrep RNA/DNA Mini kit (Qiagen) according to the manufacturer’s instructions. Briefly, tissues were placed in a container of suitable size with 600 µL RNeasy lysis buffer (RLT) for homogenization using the TissueRuptor. After centrifugation, the supernatant was transferred to an AllPrep column to capture the DNA on the membrane. After the membrane was washed, the DNA was eluted in 100 µL elution buffer (EB).

DNA from FFPE tissues was isolated with a QIAamp DNA FFPE Tissue kit (Qiagen) according to the manufacturer's protocol. Briefly, eight FFPE tissue sections, each 10-µm thick, were placed in 1.5-mL tubes and deparaffinized with xylene. After deparaffinization, the pellet was resuspended in 180 µL buffer ATL and 20 µL proteinase K, and incubated at 56°C for 1 hour and at 90°C for 1 hour. After adding 200 µL buffer Al and 200 µL ethanol (96%–100%), the lysate was transferred to a QIAamp MinElute column (Qiagen) to capture the DNA on the membrane. After the membrane was washed, the DNA was eluted in 50 µL EB buffer.

DNA quality and quantity were tested using Agilent 2100 Bioanalyzer (Agilent Technologies) and NanoDrop ND 1000 units (Thermo Fisher).

The DNA was then bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s recommendations. Briefly, 500 ng DNA from fresh-frozen samples and 1 µg DNA from FFPE samples were denatured by adding M-Dilution buffer and incubating at 37°C for 15 minutes. CT conversion reagent containing sodium bisulfite was added to the denatured DNA, and the samples were incubated for 16 cycles of 95°C for 30 seconds and 50°C for 60 minutes. Subsequently, the bisulfite-converted DNA was added to a Zymo-Spin IC Column, washed with M-desulfonation buffer and M-wash buffer after desulphonation, and then eluted in 10 µL M-elution buffer.

**Genome-wide methylation profiling**

We used an Infinium Human Methylation 450K BeadChip (Illumina) according to the manufacturer’s standard protocol to analyze DNA methylation. Briefly, 4 µL bisulfite-converted DNA was used for whole-genome amplification, fragmentation, precipitation, and resuspension, and then hybridized on the BeadChip at 48°C for 16 hours. After washing away unhybridized and nonspecifically hybridized DNA, the hybridized bisulfite-converted DNA was used as a template for a single-nucleotide extension. Lastly, the BeadChip was stained and scanned with an illumina HiScan SQ scanner.

We used Illumina GenomeStudio Software (version 2011.1) to determine DNA methylation intensities from the scanned arrays and to perform background adjustment. Beta (β) value is calculated to estimate the methylation level for each CpG using the formula as follows: $\beta = M/(M + U + 100)$, where $M$ and $U$ are the signals of the methylated and unmethylated probes, respectively. $\beta$ values range from 0 (unmethylated) to 1 (fully methylated). The DNA methylation data were then imported into R version 2.14.1, and quantile was normalized using the IMA package in R (20).
All of the samples met the quality control criteria (CpG coverage > 95%). We removed probes with detection \( P \) value > 0.05, all single-nucleotide polymorphism–associated probes, and non-specific probes. We retained an eventual 377,621 sites from the original 485,577 sites.

We identified differential DNA methylation sites between NPC tissues and NCNBT using a nonparametric Wilcoxon rank-sum test with Bonferroni correction for multiple testing (\( P < 0.05 \)) in R. A significant difference in \( \beta \) value was defined as sites with a Bonferroni-corrected \( P \) value ≤ 0.05 and \( \beta \) change ≥ 0.2 between tumor and normal tissues. We conducted K-means hierarchical clustering analysis to cluster tissue status. The microarray data are available at www.ncbi.nlm.nih.gov/geo/ (GSE52068).

**RNA isolation and real-time PCR**

Total RNA was extracted using the AllPrep RNA/DNA Mini Kit (Qiagen); complementary DNA (cDNA) was synthesized from 1 \( \mu \)g RNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer’s instructions. Quantitative PCR was performed using a Bio-Rad CFX96 sequence detection system (Bio-Rad Laboratories) in 10 \( \mu \)L reactions containing 50 ng cDNA, 400 \( \mu \)mol/L each primer, deionized water, and 5 \( \mu \)L Platinum SYBR Green qPCR SuperMix-UDG reagents (Life Technologies). All reactions were incubated at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 7 minutes. Supplementary Table S3 lists the PCR primers used. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference, and relative expression was determined using the comparative threshold cycle \( 2^{-\Delta\Delta CT} \) method.
Bisulfite pyrosequencing
Quantitative DNA methylation analysis was performed by bisulfite pyrosequencing as previously described (21). The region selected for interrogation included CpG sites identified as differentially methylated based on the array data. The pyrosequencing primers were designed with PyroMark Assay Design Software 2.0 (Qiagen); the primer sequences and PCR conditions are outlined in Supplementary Table S4. Pyrosequencing was performed using a Pyro Gold SQA Reagent kit (Qiagen) in a PyroMark Q96 ID System (Qiagen) according to the manufacturer’s instructions. CpG site quantification was performed using PyroMark Q96 ID System software (Qiagen).

Figure 2.
Flowchart of hypermethylated gene selection.
methylation levels <15% were considered unmethylated CpG sites (22).

Statistical analysis
Quantitative methylation data by pyrosequencing were normalized by the Z-score method as described previously (23). Z-score of methylation for each gene in each sample was derived as follows: (methylation level of each gene in each sample – mean methylation level of each gene among all samples)/SD of methylation level for each gene. Categorical variables were compared using the \( \chi^2 \) test or Fisher exact test. Differences in continuous variables were evaluated using the Student t test. Correlation
among genes from bisulfite pyrosequencing data was analyzed using Spearman correlation.

The primary and secondary end points were disease-free survival (DFS) and overall survival (OS). DFS was calculated from treatment to the date of the first relapse at any site or death from any cause, whichever occurred first. OS to the death from any cause. DFS and OS were estimated with the Kaplan–Meier method and the log-rank test. Independent prognostic factors were determined with multivariate Cox regression analysis with the backward stepwise method. The methylation gene panel, age, sex, TNM staging, pathology type, and concurrent chemotherapy were used as covariates. Statistical analyses were performed using Stata 10 (StataCorp LP). Statistical significance was defined as $P < 0.05$ with a two-tailed test.

**Results**

**Differential methylation profiles between NPC tumor and NCNBT**

To avoid potential confounding factors, an age and sex-matched case set was used to identify DNA methylation marker with different methylation levels between NPC samples and NCNBT. There were 21,902 CpG sites (10,102 hypermethylated
and 11,800 hypomethylated) that significantly differed in methylation level between tumor and nontumor tissues after Benferroni adjustment. Among all significant CpG sites, 2,173 CpG sites with \( \beta \) change \( \geq 0.2 \) (1,880 hypermethylated, 293 hypomethylated) were identified (\( P < 0.05 \)), which discriminated NPC tissues from NCNBt with 93.8% accuracy (Fig. 1; Supplementary Table S5). Both hyper- and hypomethylation alterations are common events in NPC tumor tissues.

**Selection of candidate hypermethylated genes for methylation analysis**

In order to identify biologically meaningful methylation changes, 780 hypermethylated CpG sites (covering 496 genes) were selected for further analysis from 2,173 CpG sites based on the following exclusion criteria: (i) hypomethylated CpG sites; (ii) mean NCNBt methylation level \( > 25\% \); (iii) CpG sites not located in proximal promoter regions [including transcription start site (TSS) 1,500 and TSS200, 5'UTR, and first exon]; (iv) all imprinted and X chromosome genes (Fig. 2).

Among 496 hypermethylated genes, we prioritized 33 genes reported as having promoter DNA hypermethylation in PubMeth (http://matrix.ugent.be/pubmeth/; ref. 24), among which only 28 exhibited a prognostic value in cancer in the NCBI PubMed literature (Supplementary Table S6). We examined the mRNA expression of these 28 genes in the 24 NPC tissues and NCNBt: only eight genes had \( > 2\)-fold downregulation in the NPC tissues (Fig. 3A). There was restored expression (\( > 5\)-fold) of six genes (\( WIF1, UCHL1, RASSF1A, CCNA1, TP73, \) and \( SFRP1 \)) in NPC cell lines (CNE2, SUNE1, C666-1, HK1, and HONE1) following 5-aza-2-deoxycytidine treatment (Figs. 2 and 3B), and bisulfite pyrosequencing also validated their hypermethylation in NPC tissues (Figs. 2 and 3C; Supplementary Fig. S1). Finally, we identified six genes for methylation and survival analysis.

**Identification of a methylation gene panel associated with survival in training cohort**

In the training cohort (\( n = 151 \)), the frequencies of methylation (\( > 15\% \)) were 84% for \( WIF1 \), 61% for \( UCHL1 \), 49% for \( RASSF1A \), 52% for \( CCNA1 \), 95% for \( TP73 \), and 85% for \( SFRP1 \). Spearman correlation analysis revealed that the methylation levels of these genes were significantly positively associated with each other within the same patient cohort (Supplementary Table S7). In light of their correlation, we constructed a prognostic model by combining the six methylated genes using the average Z-score method (23, 25), calculating for each patient an average Z-score based on averaging the Z-scores of the six methylated genes.

Patients in the training cohort were separated into high and low methylation groups according to the model using the median average Z-score as the cutoff point (\( < 0.085 \)). Significant associations were found between high methylation and shorter DFS [HR, 2.26; 95% CI, 1.28–4.01; \( P = 0.005 \)] and worse OS [HR, 2.47; 95% CI, 1.30–4.71; \( P = 0.006 \)] as compared with low methylation (Fig. 4A and B). In addition, the distribution of clinical characteristics did not vary significantly between the groups (Supplementary Table S2).

**Validation of methylation gene panel in the validation and independent cohorts**

To validate the survival prognostic accuracy of the proposed model with methylation gene panel, patients in the validation cohort were classified into high and low methylation groups using the same cutoff point as classified in the training cohort. Similar to the training cohort, patients with high methylation had shorter DFS (HR, 2.07; 95% CI, 1.17–3.67; \( P = 0.013 \)) and shorter OS (HR, 1.83; 95% CI, 1.01–3.31; \( P = 0.046 \)) than patients with low methylation (Fig. 4C and D). Likewise, there were no significant differences in the distribution of clinical characteristics between the two groups (Supplementary Table S2).

To determine whether the prognostic model had the same power to define outcome in a different population of NPC patients, we measured the methylation levels of the six genes in an independent cohort (\( n = 153 \)), dividing patients into high and low methylation groups with the same cutoff point used in the training cohort. Patients with high methylation had shorter DFS (HR, 1.94; 95% CI, 1.08–3.47; \( P = 0.026 \)) and shorter OS (HR, 2.09; 95% CI, 1.10–3.98; \( P = 0.022 \)) than patients with low methylation (Fig. 4E and F). Clinical characteristics were similar between the two groups (Supplementary Table S2).

**The methylation gene panel was associated with survival independent of TNM stage**

We performed multivariate Cox regression analysis to determine whether the methylation gene panel was an independent prognostic factor. In the training cohort, the panel (HR, 2.26; 95% CI, 1.28–4.01; \( P = 0.005 \); HR, 2.47; 95% CI, 1.30–4.71; \( P = 0.006 \)) and TNM stage (HR, 2.05; 95% CI, 1.37–3.07; \( P < 0.001 \); HR, 2.66; 95% CI, 1.63–4.34; \( P < 0.001 \)) were independent prognostic factors for DFS and OS, respectively. The results for the validation and independent cohorts were similar (Table 1).

To investigate whether the methylation gene panel was associated with survival within TNM staging, we performed stratified analyses of patients with stage I to II or stage III to IV
disease from the combined cohort (n = 454). Overall, stage I to II patients with high or low methylation had similar DFS (HR, 1.66; 95% CI, 0.75–3.66; P, 0.290) and OS (HR, 1.07; 95% CI, 0.45–2.59; P, 0.884; Fig. 5A and B); however, stage III to IV patients with high methylation had shorter DFS (HR, 2.19; 95% CI, 1.52–3.16; P < 0.001) and OS (HR, 2.35; 95% CI, 1.58–3.49; P < 0.001) than patients with low methylation (Supplementary Fig. S2A and S2B).

The methylation gene panel was associated with the efficacy of concurrent chemotherapy

We further analyzed whether the methylation gene panel could predict the efficacy of concurrent chemotherapy in locoregionally advanced NPC patients (stage III–IV) from the three cohorts. A total of 147 of 337 (43.6%) patients received concurrent platinum-based chemotherapy in addition to radiotherapy. Patients with low methylation had a better response to concurrent chemotherapy with improved DFS (HR, 0.58; 95% CI, 0.28–0.96; P, 0.045) and OS (HR, 0.45; 95% CI, 0.22–0.83; P, 0.031; Fig. 5C and D), whereas patients with high methylation did not benefit from concurrent chemotherapy (DFS, HR, 0.81; 95% CI, 0.52–1.25; P, 0.342; OS, HR, 0.78; 95% CI, 0.49–1.24; P, 0.293, respectively; Supplementary Fig. S2C and S2D).

Discussion

In NPC therapy, it has become increasingly important to stratify patients who would or would not benefit from current treatment, and the TNM staging system is the key prognostic determinant. However, there are large variations in clinical outcome in patients with the same TNM stage (3, 4). This suggests that prognostic models based on clinical variables are not alone sufficient for determining the best personalized treatment. Following the development of prognostic molecular biomarkers, numerous studies have been reported that the molecular characteristics of tumors added predictive power. Among them, inclusion of DNA methylation profiles was shown with abilities to better define the prognoses of numerous cancers (9–14). However, NPC-related DNA methylation studies focus on examining the epigenetic inactivation of single or multiple tumor suppressor genes, such as RASSF2A, THY1, and 14-3-3sigma, in small cohorts (26–28). Clearly, the identification of new effective prognosis biomarkers for NPC will likely contribute to improved patient-tailored treatment. As expected, large-scale epigenetic alterations were detected between NPC tissues and NCNBT using a whole-genome methylation platform, which strongly suggests that aberrant DNA methylation within CpG islands plays a critical role and is one

Figure 5. Kaplan–Meier curves estimation of DFS and OS for patients with high or low methylation levels stratified by tumor stage or the receipt of concurrent chemotherapy. A and B, stage III–IV patient DFS and OS. C and D, stage III–IV patients with low methylation DFS and OS. HR and P values were calculated with adjusted multivariate Cox proportional hazard models. The following parameters were included in the model as the covariates for each analysis: methylation gene panel (high methylation vs. low methylation), sex, age (<45 years vs. >45 years), World Health Organization (WHO) pathology type (undifferentiated nonkeratinizing vs. differentiated nonkeratinizing), and concurrent chemotherapy (yes vs. no). CT, chemotherapy.
of the most common alterations in NPC pathogenesis; and importantly, genome-wide methylation profiling also allowed us to discover widespread hypermethylation in the NPC genome and we identified it to be a subset of patients with concordant methylation at multiple genes, suggesting the existence of a CpG island methylator phenotype (CIMP) in NPC.

Based on these promising findings, we developed and validated a panel of six hypermethylated genes significantly associated with poor prognosis in NPC patients. A key finding was the results of the confirmatory retrospective clinical studies we conducted involving larger cohorts of consecutive patients who had undergone radiotherapy to provide reasonable assurance that the six-hypermethylated gene panel was an independent prognostic factor. This demonstrated the strong reproducibility and robustness of our panel in all cohorts, a finding that is consistent with results of similar studies in other cancers (9–14) and that indicates that methylation status is significantly associated with tumor progression. These results support the premise that this novel methylation gene panel enabled better assessment of the prognosis of NPC, and it may be a more specific and rational method by more accurately identifying a subgroup of patients at high risk of failure, which may help to develop new patient-tailored treatment strategies for NPC patients.

Currently, treatment decisions for NPC patients are made according to the National Comprehensive Cancer Network guidelines. Early-stage patients receive radiotherapy alone, whereas patients with advanced disease receive chemoradiotherapy. With current treatment protocols, 20% to 30% of patients will suffer recurrence or distant metastasis after primary treatment (3, 4). There is evidence that a subgroup of patients do not benefit from the current treatment strategies. Thus, the accurate identification of patient subgroups will improve the prognostic system and lead to more personalized therapy. In the present study, we studied the association of methylation status and response to concurrent platinum-based chemotherapy and found that patients with low methylation had a favorable response to concurrent chemotherapy, which could be due to the fact that genes were activated by epigenetic alterations. It has been shown that WIF1 and SFRP1 are involved in Wnt/b-catenin signaling, which induces apoptosis and inhibits carcinoma cell growth, invasion, and angiogenesis (29, 30), that UCHL1 inhibits carcinoma cell growth and induces apoptosis through activating the p14ARF-p53 tumor-suppressive pathway (31), that RASSF1A involved in the RAS-mediated signaling pathways promotes apoptosis, cell-cycle arrest, and inhibits cell migration (32), that CCNA1 mediates apoptosis, G2-M arrest, and mitotic catastrophe in multiple cells (33), and that activation of TP73 promotes E2F-1–induced apoptosis (34). Transcriptional silencing of six genes by inappropriate promoter methylation involved in biologic pathways that confer aggressive behavior and facilitate tumor development or dissemination has been observed in our study, which may therefore reflect different biologic characteristics of NPC tumors and serve as predictors of response to anticancer agents. One exciting prospect is that this observation may suggest new potential targets for therapeutic intervention in the clinical management of NPC.

As far as we are aware, this is the first study with such large sample size to reveal the genome-wide methylation profiles of NPC tumors and show that NPC with high methylation level is associated with poorer survival. Notably, the prognostic model with the six–methylated gene panel is robust and easily applicable to routinely obtained diagnostic paraffin-embedded specimens, demonstrates its potential usefulness to clinicians, and may increase the therapeutic options for patients diagnosed with NPC. Prospective large-scale multicenter studies are necessary to replicate these findings.

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

Authors’ Contributions
Conception and design: W. Jiang, N. Liu, J. Ma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Liu, X.-Z. Chen, Y. Sun, B. Li, X.-Y. Ren, W.-F. Qin, J. Zeng, L.-Z. Liu, H.-Q. Mai, W.-H. Jia
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Jiang, N. Liu, X.-Z. Chen, Y.-F. Xu, J. Ren, Y. Guo, H.-Q. Mai, J. Ma
Writing, review, and/or revision of the manuscript: W. Jiang, N. Liu, Y.-Q. Li, W.C.S. Cho, H.-Q. Mai, M.-S. Zeng, J. Ma
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Jiang, N. Liu, B. Li, W.-F. Qin, N. Jiang, Y.-Q. Li, H.-Q. Mai, T.-B. Kang, J.-Y. Shao
Study supervision: N. Liu, L. Li, J. Ma

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References
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