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Original Article

Up-regulation of the *IRX2* gene predicts poor prognosis in nasopharyngeal carcinoma

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Abstract: Aberrant expression of the *IRX2* gene contributes to the oncogenesis and progression of various cancers. In this study, we analyzed the clinical significance and the prognostic value of mRNA expression level of the *IRX2* gene in nasopharyngeal carcinoma (NPC) patients, with the goal to find a novel prognostic biomarker for NPC. Tissue samples were collected prior to treatment from 71 NPC patients for the detection of mRNA expression level of a total of 31503 genes, with high throughput screening of the mRNA expression profile. The Kaplan-Meier curves and log-rank test were used for univariate analyses to determine if the mRNA expression level of *IRX2* and other 31502 genes, as well as clinical characteristics were of prognostic value for overall survival (OS), distant metastasis-free survival (DMFS) and disease-free survival (DFS). Regularized Cox regression was performed to test the contribution of prognostic factors to OS, DMFS, and DFS of NPC patients. The Cox proportional hazard model was used to test the independence of prognostic effect of *IRX2* and other clinical features. The receiver operator characteristic curve was drawn and the area under the curve (AUC) was calculated to evaluate the predictive power of *IRX2* gene. Univariate analyses showed a higher mRNA expression level of the *IRX2* gene correlated with shorter OS (P = 0.001), DMFS (P = 0.003), and DFS (P = 0.007). Regularized Cox regression and Cox proportional hazard model analyses further showed that ahigher mRNA expression level of the *IRX2* gene in the primary NPC was an independent prognostic factor for OS (Coxnet beta = 0.03, Cox proportion hazard model P = 0.038), DMFS (Coxnet beta = 0.018, Cox proportion hazard model P = 0.01) and DFS (Coxnet beta = 0.008, Cox proportion hazard model P = 0.029). The AUC showed that the mRNA expression level of the *IRX2* gene is a significant predictor for predicting the OS (AUC value = 0.7105) and DMFS (AUC value = 0.7027) of NPC patients. Our results demonstrated that the *IRX2* gene may be a novel independent unfavorable prognostic factor for NPC patients.

Keywords: Nasopharyngeal carcinoma, *IRX2* gene, prognostic factor

Introduction

Nasopharyngeal carcinoma (NPC) is a common head and neck cancer having its highest incidence rate in southern China, especially in Guangxi Zhuang Autonomous region [1]. Although NPC is different from the other types of head and neck cancers with anundifferentiated histology, that are radiosensitive and chemosensitive, and irradiation techniques and chemoradiotherapy have greatly improved in the few past decades [2, 3], local-regional failure and distant metastasis are still the major causes of treatment failure and poor prognosis of NPC patients. The oncogenesis and progression of NPC are complicated and multistep, andcomprise acquisition of multiple malignant potentials, such as sustaining proliferative signaling, evading growth suppressors, resisting cell death, activating invasion, and metastasis. Underlying the acquisition of these malignant potentials are genome instability caused by Epstein-Barr virus infection [4-6], gene amplification and mutation, epigenetic dysregulation, and modification. Because the mRNA expression profile may be reflective of all the combined factors above, we performed high throughput screening of the mRNA expression profile in 71 tissue samples of NPC with the goal to find the mRNA expression differences

among the 31503 genes from the expression profiles. We further performed univariate and multivariate analyses to identify genes whose mRNA expression levels are correlated with the OS, DMFS and DFS of NPC patients. According to the results, 301,342 and 280 genes were independently correlated with OS, DMFS and DFS of NPC patients respectively; and among these genes, *IRX2* drew our attention for its high correlation with the poorer OS, DMFS, and DFS of NPC patients.

Iroquois homeobox 2 (*IRX2*) is a member of the Iroquois homeobox gene family and located on chromosomal region 5p15.33 [7]. *IRX2* geneencodes a transcription factor and appears to play multiple roles during pattern formation of vertebrate embryos [7-9]. The relevance of *IRX2* gene expression in the progression of multiple malignant tumors has been reported in the past few years. Amplification of *IRX2* gene locus on chromosome 5p15.33 has been identified in breast cancer and soft tissue sarcomas [10-12]. mRNA expression of *IRX2* is upregulated in osteosarcoma, and *IRX2* promotes osteosarcoma cell growth and invasion through the PI3K/Akt signaling-mediated activation of MMP-9 and VEGF [12, 13]. Anelevated expression level of *IRX2* gene predicts a worse outcome ininfants with acute lymphoblastic leukemia [14]. In summary, all these studies suggest a possible function of the *IRX2* gene in promoting oncogenesis and progression of malignant tumors. In contrast, some other studies also showed that hypermethylation of the promoter and other CpG islands in *IRX2* gene frequently occurs in lung cancer and luminal A breast cancer [15-17], and the *IRX2* protein has been found to inhibit the cellular motility of breast cancer cells, suggesting a metastasis suppression function of *IRX2* protein [18]. Therefore, *IRX2* might play different functions depending on the type of malignant tumor or the stage of tumor development [18].

However, it remains unknown whether mRNA expression level of the *IRX2* gene is associated with the prognosis of NPC patents. To address this question, we examined the mRNA expression level of *IRX2* gene in 71 cases of NPC tissue and analyzed its correlation with the overall survival, distant metastasis-free survival and disease-free survival of NPC patients. We also drew the Receiver Operator Curve (ROC) and calculated the area under the curve (AUC value) to further evaluate the predictive power of *IRX2* gene.

Materials and methods

Patient selection and tissue sample collection

The Clinical Ethics Review Board of People's Hospital of Guangxi Zhuang Autonomous region approved this study. All of the patients signed informed consent documents prior to participating in this study.

A total of 71 patients from the Otorhinolaryngology Head and Neck Cancer Department of People's Hospital of Guangxi Zhuang Autonomous region were newly diagnosed between September 2007 and September 2012. Eligibility criteria of patients for inclusion in the study were as follows: (1) pathological confirmation of undifferentiated non-keratinized carcinoma of the nasopharynx, (2) a Union for International Cancer Control (UICC) staging system 2010 clinical classification of I to IVb, without a history of other malignant tumor or anticancer therapy, (3) Karnofsky performance score $\geq$ 70. Exclusion criteria included a history of severe systemic disease, pregnancy or lactation, and the presence of contradiction for receiving chemotherapy, radiotherapy, or surgery. Fresh NPC tissue samples of sixty-nine nasopharyngeal carcinoma patients were obtained by nasopharynx biopsy under narrowband imaging (NBI) endoscopy prior to anticancer therapy and then frozen in liquid nitrogen until analysis.

Pretreatment evaluation of patients

All of the patients underwent a pretreatment evaluation that included a precise clinical examination of the head and neck region, fiber optic nasopharyngoscopy, head and neck MRI, chest X-ray, ultrasonography of the abdominal region, bone scan, and a complete blood count and biochemical profile.

Patient treatment

All patients were treated with IMRT once a day 5 times a week. The target area was delineated based on the tumor boundary shown byMRI and CT. The prescribed doses were 69.76-76.3 Gy for the PGTVnx, 64-70 Gy for PGTVnd, 59.4-64.0 Gy for PTV1, and 50.0-54.0 Gy for PTV2.

Additional treatment with concurrent chemotherapy with platinum and fluorouracil was given to all patients.

Patient follow-up

All patients who had completed the NPC treatment attended follow-up visits at 3-month intervals for the first year, every 6 months for the second to the fourth years, and annually thereafter. The follow-up methods were as follows: (1) All patients received the EB virus serological examination and nasopharyngeal endoscopic examination,and the endoscopic positive patients were given anasopharyngeal biopsy. (2) All patients were confirmed by the nasopharyngeal skull base magnetic resonance imaging, chest X-ray, cervical lymph nodal, and abdominal ultrasoundexaminations. (3) Patients who had been diagnosed with abnormal bone metabolism by ECT scan received aPET/CT scan.

The survival time was calculated from the date of first treatment completion to either the date of event of interest (death caused by NPC, local-regional recurrence ordistant metastasis) or last follow-up. Among the 71 patients, 2 patients were excluded from OS analysis who died of non-neoplastic diseases, 1 patient was excluded from DMFS analysis because of distant metastasis occurred during the first treatment period, and 4 patients were excluded from DFS analysis because a local-regional (nasopharynx and cervical lymph nodes) residual or distant metastasis occurred during the first treatment period, respectively. Therefore, 69, 70, and 67 of 71 patients were selected to analyze the relationship between *IRX2* gene expression and OS, DMFS, and DFS, respectively.

RNA extraction

Tissue RNA was isolated from fresh frozen tissue samples using QIAGEN AllPrep DNA/RNA Mini Kit (Qiagen, Cat# 80204) according to the manufacturer's protocol.

RNA quantification

RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) or Nanodrop 2000 (Thermo Scientific). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using RNA Nano 6000 Assay Kit on an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Library preparation

For high-quantity samples, a total of 1.5 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5×). First strand cDNA was synthesized using random hexamer primer and M-MuLVReverse Transcriptase (RNaseH-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3' ends of DNA fragments, NEBNext Adaptors with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of correct length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. Finally, products were purified (AMPure XP system) and library quality was assessed on the Agilent BioAnalyzer 2100 system (Agilent Technologies, CA, USA). For low-quantity RNA samples (typically less than 100 ng total RNA), NEB Next rRNA Depletion Kit (Human/Mouse/Rat) (NEB E6310, USA) was used to deplete rRNA. Next, cDNA was synthesized from rRNA-depleted total RNA with NEBNext RNA First Strand Synthesis Module (NEB E7525S, USA) and double-stranded cDNA was generated from first-strand cDNA usingNEB Next mRNA Second Strand Synthesis Module (NEB E6111S, USA) according to the manufacturer's instruction.

Table 1. Clinical characteristics of the 71 NPC patients and their association with *IRX2* expression

	All patients	IRX2 expression level		P
	n = 71 (%)	High	Low	
		n = 26 (%)	n = 45 (%)	
Age (years)				
≤ 45	31 (44)	12 (46)	19 (42)	0.941
> 45	40 (56)	14 (54)	26 (58)	
Gender				
Male	53 (75)	21 (81)	32 (71)	0.537
Female	18 (25)	5 (19)	13 (29)	
T classification				
T1-2	26 (37)	10 (38)	16 (36)	1.000
T3-4	45 (63)	16 (62)	29 (64)	
N classification				
N0-1	43 (61)	15 (58)	28 (62)	0.901
N2-3	28 (39)	11 (42)	17 (38)	
Clinical classification				
I-II	16 (23)	6 (23)	10 (22)	1.000
III-IVb	55 (77)	20 (77)	35 (78)	

Abbreviation: NPC, nasopharyngeal carcinoma.

DNA fragmentation and adapter ligation werecarried out using TruePrep TM DNA Library Prep Kit V2 for Illumina (Vazyme, China). The concentration of each library was quantified by Qubit® RNA Assay Kit on a Qubit®2.0 Flurometer (Life Technologies, CA, USA) according to the manufacturer's instruction. The size distribution was evaluated using an Agilent high-sensitivity chip on an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

mRNA sequencing

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq 4000 PE Cluster Kit (Illumina) or NextSeq 500/550 High Output Kit V2 (Illumina) according to the manufacturer's instruction. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 4000 or NextSeq500 platform and 150 bp paired-end reads were generated.

Sequencing data analyses

Raw reads were trimmed to remove adapters and low-quality bases using Cutadapt (version 1.10) [19] with the following parameters: --quality-base = 33 --quality-cutoff = 20,20 --format = fastq -a CTGTCTCTTATACACATCT-ACTGTCTCTTATACACATCT -a AAAAAAAA$ -A AAAAAAAA$ -g ^TTTTTTTT -G ^TTTTTTTT -g AGATGTGTATAAGAGACAG-GAGATGTGTATAAGAGACAG --times = 6 --minimum-length = 20 --max-n = 0.1 --trim-n. To minimize the impact of rRNA on mRNA quantification, reads mapped to 5S rRNA, 5.8S rRNA, 18S rRNA, 28S rRNA, 16S mitochondrial rRNA, and 12S mitochondrial rRNA were filtered out using Bowtie2 (version 2.2.5) [20]. After cleaning and filtering, the remaining high-quality reads were mapped to human reference genome GRCh38 using STAR (version 2.5.2b) [21] with the following parameters: --outSAMunmapped Within --outFiterTypeBySJout --outSAMattributes NH HI AS NM MD --outFilter-MultimapNmax 20 –outFilterMismatchNmax 999 --outFilterMismatch-NoverLmax 0.04 --alignIntronMin 20 --alignIntronMax 1000000 --alignMatesGapMax 1000000 --alignSJoverhangMin 8 --alignSJDBoverhangMin 1 --sjdbScore 1 --runThreadN 2 --genomeLoadNoSharedMemory --outSAMtype BAM Unsorted --quantModeTranscriptomeSAM --outSAMheaderHD/@HD VN: 1.4 SO: unsorted --readFilesCommandzcat. Finally, gene expression was quantified using RSEM (version 1.3.0) [22]. Using the following parameters: rsem-calculate-expression -p 4 --seed-length 20 --paired-end --bam --no-bam-output --estimate-rspd--append-names. In addition, the batch difference between high-quantity and low-quantity samples was removed together with the gender imbalance by the Bioconductor package SVA (version 3.26.0) [23].

Statistical analyses

The average *IRX2* expression level among the 71 NPC samples wasused as a cut-off value to divide the patients into a low *IRX2* expression group and a high *IRX2* expression group. The chi-square test was employed to compare data between the two groups. The Kaplan-Meier curve and log-rank test were used for univariate analyses to determine whether gene

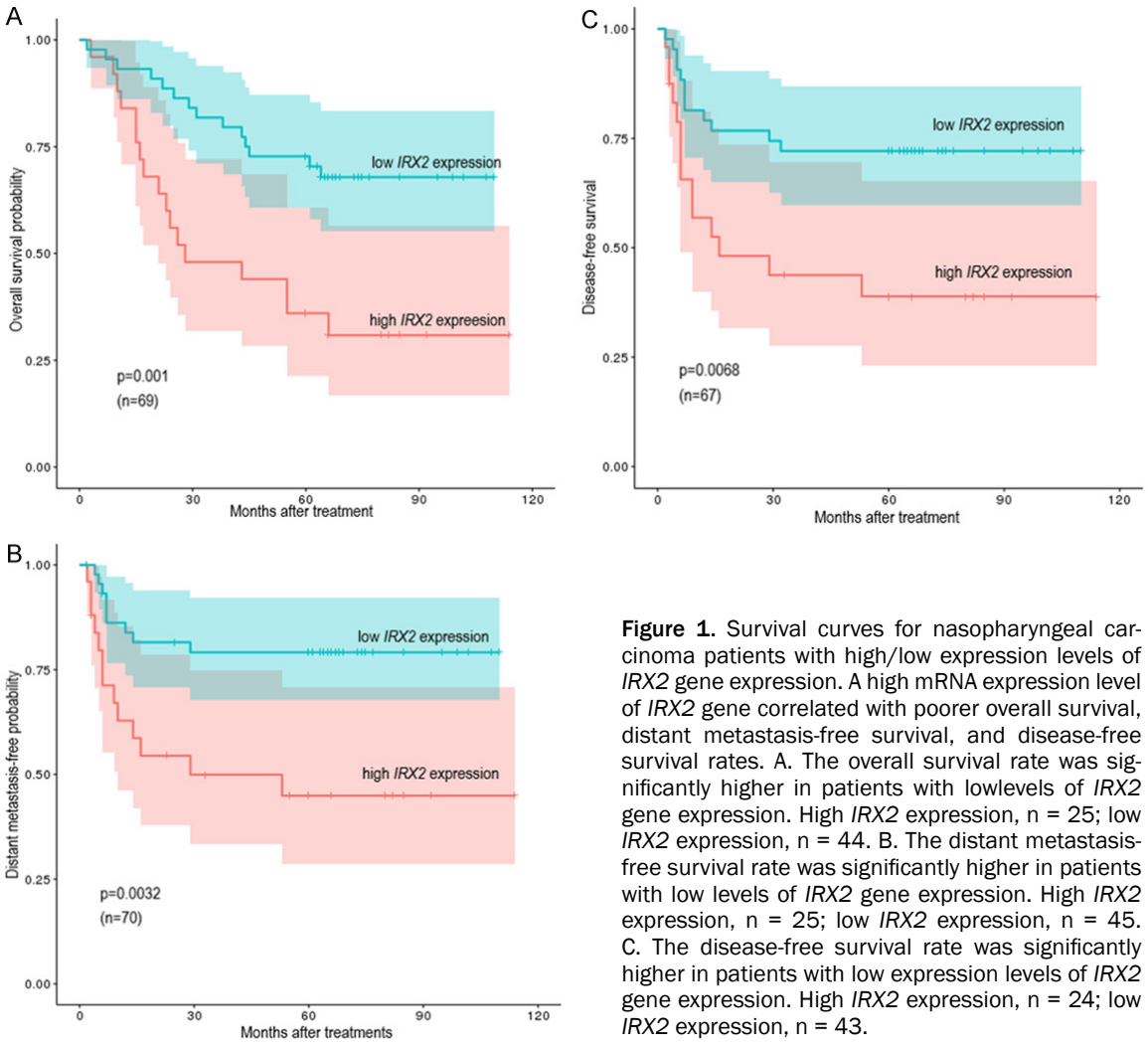

Figure 1. Survival curves for nasopharyngeal carcinoma patients with high/low expression levels of *IRX2* gene expression. A high mRNA expression level of *IRX2* gene correlated with poorer overall survival, distant metastasis-free survival, and disease-free survival rates. A. The overall survival rate was significantly higher in patients with lowlevels of *IRX2* gene expression. High *IRX2* expression, n = 25; low *IRX2* expression, n = 44. B. The distant metastasis-free survival rate was significantly higher in patients with low levels of *IRX2* gene expression. High *IRX2* expression, n = 25; low *IRX2* expression, n = 45. C. The disease-free survival rate was significantly higher in patients with low expression levels of *IRX2* gene expression. High *IRX2* expression, n = 24; low *IRX2* expression, n = 43.

expression and other clinical characteristics (age, sex, clinical classification, etc.) were significantly associated with overall survival (OS), distant metastasis-free survival (DMFS), and disease-free survival (DFS). Regularized Cox regression [24] was used for multivariate analyses to estimate the contribution of prognostic factors to OS, DMFS and DFS. The Cox proportional hazard model was usedto test the independence of prognostic value of *IRX2* and other clinical features. The receiver operator characteristic curve (ROC) wasdrawn and the area under the curve (AUC value) wascalculated to evaluate the predictive power of the *IRX2* gene for predicting the OS, DMFS, and DFS of NPC patients. All statistical tests were 2-sided, and *P*-values < 0.05 were considered statistically significant. All analyses were performed using statistical software R (version 3.3.3).

Results

Patients' clinical characteristics and follow-up outcome

The latest patient follow-up visit occurred in July 2017. The follow-up time ranged from 2 to 114 months, with a median of 64 months. Among the 71 patients enrolled in this study, 67 patients achieved clinical cure after completion of the first treatment and 4 patients experienced tumor residual (3 of 4 patients experienced local-regional residual and 1 of 4 patient developed distant metastasis during the first treatment period). Among the 67 patients who achieved clinical cure after completion of first

Table 2. Univariate analysis with Kaplan-Meier survival curves and log-rank test for the OS, DMFS, and DFS of NPC patients

Prognosis	Chisq	P	Exp (B)	95% CI for Exp (B)	
				Lower	Upper
OS					
Age (years) > 45 vs. ≤ 45	0.633	0.426	0.747	0.369	1.510
Gender Female vs. Male	4.155	0.042	2.830	1.308	6.121
Clinical classification I-II vs. III-IVb	2.681	0.102	2.335	1.044	5.226
T classification T2-4 vs. T1	0.257	0.612	1.662	0.343	8.056
N classification N1-3 vs. N0	5.592	0.018	2.986	1.437	6.205
IRX2 expression level High vs. Low	10.486	0.001	0.332	0.153	0.723
DMFS					
Age (years) > 45 vs. ≤ 45	0.038	0.846	1.086	0.467	2.524
Gender Female vs. Male	1.159	0.282	1.788	0.708	4.515
Clinical classification I-II vs. III-IVb	1.643	0.200	2.159	0.827	5.637
T classification T2-4 vs. T1	0.004	0.947	0.935	0.118	7.399
N classification N1-3 vs. N0	2.625	0.105	2.364	0.981	5.695
IRX2 expression level High vs. Low	8.682	0.003	0.304	0.122	0.753
DFS					
Age (years) > 45 vs. ≤ 45	0.369	0.543	0.786	0.362	1.703
Gender Female vs. Male	2.801	0.094	2.386	1.033	5.509
Clinical classification I-II vs. III-IVb	1.801	0.180	2.023	0.850	4.815
T classification T2-4 vs. T1	0.033	0.855	1.201	0.191	7.547
N classification N1-3 vs. N0	3.088	0.079	2.311	1.035	5.161
IRX2 expression level High vs. Low	7.313	0.007	0.366	0.158	0.848

Abbreviations: NPC, nasopharyngeal carcinoma; OS, overall survival; DMFS, distant metastasis-free survival; DFS, disease-free survival.

treatment, 19 patients developed distant metastasis, 5 patients developed local-regional recurrence, and 2 patients developed both distant metastasis and local-regional recurrence. The clinical characteristics of the 71 patients are summarized in **Table 1**.

Relationship between mRNA expression level of IRX2 and clinical characteristics of NPC patients

The mRNA expression levels of *IRX2* gene in the 71 cases of patients ranged from 0 to 447, with an average value of 58. We used the average value to divide the patients into a low *IRX2* expression group and a high *IRX2* expression group. The relationship between *IRX2* expression and clinical characteristics issummarized in **Table 1**. The sex, age, clinical classification, T and N classification had no association with mRNA expression level of the *IRX2* gene.

The mRNA expression levels of IRX2 gene in predicting survival of NPC patients

The Kaplan-Meier curve survival analyses and the log-rank test were used for univariate analyses to determine if age, gender, T classification, N classification, clinical classification, and mRNA expression levels are prognostic factors for OS, DMFS, and DFS in the NPC patients. The results demonstrated that among the 31503 genes from the expression profiles, there were 1260, 1021 and 620 genes significantly correlated with OS, DMFS and DFS of NPC patients, respectively (p value < 0.05). The patients with higher mRNA expression level of *IRX2* had a significant poorer OS (P = 0.001), DMFS (P = 0.003) and DFS (p value = 0.007) compared to those with lower mRNA expression level of *IRX2* (**Figure 1A-C**). Moreover, the gender (P = 0.042) and N classification (P = 0.018) were significantly associated with OS of NPC patients. The univariate analysis results are summarized in **Table 2**.

Table 3. Cox proportion hazard model analysis for the OS, DMFS, and DFS of NPC patients

Prognosis	Chisq	p	Exp (B)	95% CI for Exp (B)	
				Lower	Upper
OS					
Age (years) > 45 vs. ≤ 45	0.016	0.899	1.055	0.459	2.425
Gender Female vs. Male	6.465	0.011	4.307	1.390	13.345
Clinical classification I-II vs. III-IVb	1.138	0.286	1.903	0.584	6.204
T classification T2-4 vs. T1	0.074	0.785	0.731	0.077	6.951
N classification N1-3 vs. N0	3.483	0.062	2.766	0.952	8.037
IRX2 expression level High vs. Low	4.305	0.038	0.423	0.188	0.952
DMFS					
Age (years) > 45 vs. ≤ 45	0.628	0.428	1.476	0.563	3.868
Gender Female vs. Male	2.704	0.100	2.724	0.825	8.991
Clinical classification I-II vs. III-IVb	1.469	0.226	2.246	0.607	8.317
T classification T2-4 vs. T1	0.410	0.522	0.466	0.045	4.833
N classification N1-3 vs. N0	0.831	0.362	1.732	0.531	5.646
IRX2 expression level High vs. Low	6.609	0.010	0.296	0.117	0.749
DFS					
Age (years) > 45 vs. ≤ 45	0.165	0.685	1.211	0.480	3.059
Gender Female vs. Male	5.235	0.022	3.857	1.214	12.256
Clinical classification I-II vs. III-IVb	2.016	0.156	2.309	0.727	7.328
T classification T2-4 vs. T1	0.232	0.630	0.570	0.058	5.624
N classification N1-3 vs. N0	1.618	0.203	1.998	0.690	5.731
IRX2 expression level High vs. Low	4.776	0.029	0.387	0.165	0.907

Abbreviation: NPC, nasopharyngeal carcinoma; OS, overall survival; DMFS, distant metastasis-free survival; DFS, disease-free survival.

Regularized Cox regression and Cox proportion hazard model analyses

Regularized Cox regression wasperformed to select the factors that are associated with OS, DMFS, and DFS of NPC patients from all the significant factors identified by the univariate analyses. A total of 301, 342 and 380 genes were identified to be significantly associated with OS, DMFS and DFS of NPC patients, respectively (Coxnet beta > 0). The mRNA expression level of *IRX2* gene was determined-to contribute to the prognostic effects on OS (Coxnet beta = 0.03), DMFS (Coxnet beta = 0.018) and DFS (Coxnet beta = 0.008) of NPC patients. Moreover, gender was a contributor to OS of NPC patients (Coxnet beta = 0.106).

The Cox proportional hazard model was used to verify the independence of prognostic effect of mRNA expression level of *IRX2* and other clinical features. As summarized in **Table 3**, the mRNA expression level of *IRX2* gene was an independent predictor of OS (*p* value = 0.038), DMFS (*p* value = 0.01), and DFS (*p* value = 0.029). Moreover, gender was an independent predictor of OS (*p* value = 0.011).

The predictive power of mRNA expression level of IRX2 gene for prognosis of NPC patients

The receiver operator characteristic curve (ROC) wasdrawn and the area under the curve (AUC value) wascalculated to evaluate the predictive power of the *IRX2* gene for predicting the OS, DMFS, and DFS of NPC patients. The results showed that the mRNA expression level of *IRX2* gene is of certain accuracy in predicting the OS (AUC value = 0.7105, **Figure 2A**) and DMFS (AUC value = 0.7027, **Figure 2B**), but less accurate in predicting the DFS (AUC value = 0.6731, **Figure 2C**).

Discussion

A common phenomenon found during the therapeutic process of NPC is that, although the treatments given to the patients and other clini-

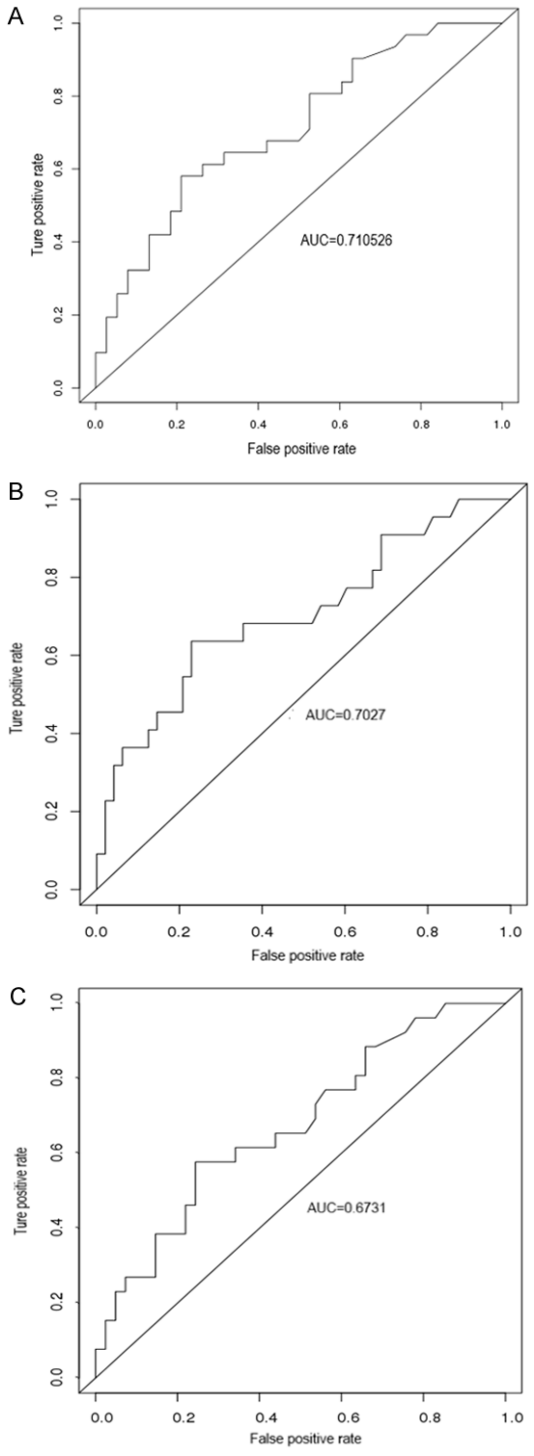

Figure 2. Receiver operating characteristic curves of mRNA expression level of *IRX2* in predicting the OS, DMFS, and DFS of NPC patients. A. Receiver operating characteristic curves of mRNA expression level of *IRX2* in predicting the OS of NPC patients. B. Receiver operating characteristic curves of mRNA expression level of *IRX2* in predicting the DMFS of NPC patients. C. Receiver operating characteristic curves of mRNA expression level of *IRX2* in predicting the DFS of NPC patients.

cal characteristics of patients are identical, the treatment responses and outcomes of patients are far more varied. Therefore, it is important to explore the genomic alternations underlying the oncogenesis and progression of NPC. In a previous study, the gene expression profiling and methylation profiling difference between the non-cancerous nasopharyngeal epithelium and NPC or between different NPC subtypes have been reported [25, 26]. In this study, we performed high throughput screening of the mRNA expression profile in 71 fresh tissue samples from NPC patients and investigated the relationship between the gene expression level and prognosis of NPC patients for the first time and eventually screened out 301, 342 and 380 genes whose mRNA expression level contributed to prognostic effects on OS, DMFS, and DFS of NPC patients, respectively. Among these genes, the mRNA expression level of the *IRX2* gene was identified to contribute to the prognostic effects on OS, DMFS, and DFS of NPC patients.

Although the direct transcriptional targets oftranscription factor *IRX2* and the exact mechanism of how the transcription factor *IRX2* exerts its function during oncogenesis and progression of malignant tumors are still unclear, it was reported that some oncogenes were correlated with the *IRX2* gene. For example, *TERT*, another oncogene located in the same chromosomal region 5p15.33 as *IRX2* gene, may be affected by abnormal *IRX2* expression [27]. The *TERT* gene encodes the telomerase subunit TERT and the maintaining of *TERT* gene expression may be a mechanism of supporting self-renewal in leukemic cells [28]. Amplification of the *IRX2* locus was found to coexist with an activating mutation of the *PIK3CA* gene in breast cancer, suggesting the possible function of promoting cell proliferation in breast cancer [10]. Moreover, the *IRX2* protein was reported to mediate the upregulation of MMP-9 and VEGF inosteosarcoma cells [12]. VEGF isconsidered to play an important role in mediating the vascularization of various cancers [29, 30] andMMP-9 proved to be a marker of highly metastaticcancers [31, 32]. Taken together, all these findings imply a possible oncogenic function of *IRX2* in promoting the proliferation and progression of various malignant tumors.

In thepresent study, we further confirmed the independence of the prognostic effect of *IRX2* gene by conducting Cox proportion hazard model and the predictive power of *IRX2* gene

in predicting OS, DMFS and DFS by evaluation using ROC analyses. The results showed that the mRNA expression level of *IRX2* accurately for predictedthe OS and DMFS of NPC patients. Taken together, these results suggest that the mRNA expression level of *IRX2* gene could be a prognostic factor for predicting the outcome and treatment response of NPC patients, or even facilitate the individualized treatment of NPC patients in the future. However, the impact of *IRX2* gene on the malignant biological behaviors of NPC and the exact mechanism of how the transcription factor *IRX2* exerts its functions remain unclear. Thus, we acknowledge that much more needs to be verified before the *IRX2* gene is eventually applied as a prognosis predictor or a therapeutic target of NPC in clinical practice. For example, the protein expression level of *IRX2* in NPC tissue and the *IRX2* expression difference between NPC tissue and non-cancerous tissues should be investigated. Moreover, it will be meaningful to explore the oncogenic functions of *IRX2* gene and the direct transcriptional targets oftranscription factor *IRX2* in NPC.

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Disclosure of conflict of interest

None.

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References

[1] Wei KR, Zheng RS, Zhang SW, Liang ZH, Li ZM and Chen WQ. Nasopharyngeal carcinoma incidence and mortality in China, 2013. Chin J Cancer 2017; 36: 90.

[2] Lee AW, Lau WH, Tung SY, Chua DT, Chappell R, Xu L, Siu L, Sze WM, Leung TW, Sham JS, Ngan RK, Law SC, Yau TK, Au JS, O'Sullivan B, Pang ES, O SK, Au GK, Lau JT; Hong Kong Nasopharyngeal Cancer Study Group. Preliminary results of a randomized study on therapeutic gain by concurrent chemotherapy for regionally-advanced nasopharyngeal carcinoma: NPC-9901 Trial by the Hong Kong Nasopharyngeal Cancer Study Group. J Clin Oncol 2005; 23: 6966-6975.

[3] Lai SZ, Li WF, Chen L, Luo W, Chen YY, Liu LZ, Sun Y, Lin AH, Liu MZ, Ma J. How does intensity modulated radiotherapy versus conventional two-dimensional radiotherapy influence the treatment results in nasopharyngeal carcinoma patients? Int J Radiat Oncol Biol Phys 2011; 80: 661-668.

[4] Dong JQ, Li MZ, Liu ZG, Zhong Q, Xiong D, Xu LH, Du Y, Xia YF and Zeng MS. Establishment and characterization of a novel nasopharyngeal carcinoma cell line (SUNE2) from a Cantonese patient. Chin J Cancer 2012; 31: 36-44.

[5] Ji MF, Yu YL, Cheng WM, Zong YS, Ng PS, Chua DT, Ng MH. Detection of Stage I nasopharyngeal carcinoma by serologic screening and clinical examination. Chin J Cancer 2011; 30: 120-123.

[6] Liang FY, Sun W, Han P, Lu X, Lian YN, Huang XM. Detecting plasma Epstein-Barr virus DNA to diagnose postradiation nasopharyngeal skull base lesions in nasopharyngeal carcinoma patients: a prospective study. Chin J Cancer 2012; 31: 142-149.

[7] Gómez-Skarmeta JL, Modolell J. Iroquois genes: genomic organization and function in vertebrate neural development. Curr Opin Genet Dev 2002; 12: 403-408.

[8] Cavodeassi F, Modolell J, Gomez-Skarmeta JL. The Iroquois family of genes: from body building to neural patterning. Development 2001; 128: 2847-55.

[9] Kim KH, Rosen A, Bruneau BG, Hui CC, Backx PH. Iroquois homeodomain transcription factors in heart development and function. Circ Res 2012; 110: 1513-24.

[10] Kadota M, Sato M, Duncan B, Ooshima A, Yang HH, Diaz-Meyer N, Gere S, Kageyama S, Fukuoka J, Nagata T, Tsukada K, Dunn BK, Wakefield LM, Lee MP. Identification of novel gene amplifications in breast cancer and coexistence of gene amplification with an activating mutation of PIK3CA. Cancer Res 2009; 69: 7357-65.

[11] Adamowicz M, Radlwimmer B, Rieker RJ, Mertens D, Schwarzbach M, Schraml P, Benner A, Lichter P, Mechtersheimer G and Joos S. Frequent amplifications and abundant expres-

sion of TRIO, NKD2, and *IRX2* in soft tissue sarcomas. Genes Chromosomes Cancer 2006; 45: 829-38.
[12] Liu TL, Zhou WW, Zhang F, Shi GD, Teng HL, Xiao JR and Wang Y. Knockdown of *IRX2* inhibits osteosarcoma cell proliferation and invasion by the AKT/MMP9 signaling pathway. Mol Med Rep 2014; 10: 169-74.
[13] Liu TL, Zhou WW, Cai B, Chu JJ, Shi GD, Teng HL Xu J, Xiao JR and Wang Y. *IRX2*-mediated upregulation of MMP-9 and VEGF in a PI3K/AKT-dependent manner. Mol Med Rep 2015; 12: 4346-4351.
[14] Kang H, Wilson CS, Harvey RC, Chen IM, Murphy MH, Atlas SR, Bedrick EJ, Devidas M, Carroll AJ, Robinson BW, Stam RW, Valsecchi MG, Pieters R, Heerema NA, Hilden JM, Felix CA, Reaman GH, Camitta B, Winick N, Carroll WL, Dreyer ZE, Hunger SP and Willman CL. Gene expression profiles predictive of outcome and age in infant acute lymphoblastic leukemia: a Children's Oncology Group study. Blood 2012; 119: 1872-81.
[15] Rauch TA, Wang Z, Wu X, Kernstine KH, Riggs AD and Pfeifer GP. DNA methylation biomarkers for lung cancer. Tumour Biol 2012; 33: 287-96.
[16] Sato T, Arai E, Kohno T, Takahashi Y, Miyata S, Tsuta K, Watanabe S, Soejima K, Betsuyaku T and Kanai Y. Epigenetic clustering of lung adenocarcinomas based on DNA methylation profiles in adjacent lung tissue: Its correlation with smoking history and chronic obstructive pulmonary disease. Int J Cancer 2014; 135: 319-34.
[17] Kamalakaran S, Varadan V, Giercksky Russnes HE, Levy D, Kendall J, Janevski A, Riggs M, Banerjee N, Synnestvedt M, Schlichting E, Kåresen R, Shama Prasada K, Rotti H, Rao R, Rao L, Eric Tang MH, Satyamoorthy K, Lucito R, Wigler M, Dimitrova N, Naume B, Borresen-Dale AL, Hicks JB. DNA methylation patterns in luminal breast cancers differ from non-luminal subtypes and can identify relapse risk independent of other clinical variables. Mol Oncol 2011; 5: 77-92.
[18] Werner S, Stamm H, Pandjaitan M, Kemming D, Brors B, Pantel K and Wikman H. Iroquois homeobox 2 suppresses cellular motility and chemokine expression in breast cancer cells. BMC Cancer 2015; 15: 896.
[19] Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMB Net Journal 2011; 17: 10-12.
[20] Langmead B, Salzberg LS. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012; 9: 357-359.
[21] Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013; 29: 15-21.
[22] Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 2011; 12: 323.
[23] Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The SVA package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics 2012; 28: 882-3.
[24] Simon N, Friedman J, Hastie T, Tibshirani R. Regularization paths for cox's proportional hazards model via coordinate descent. J Stat Softw 2011; 39: 1-13.
[25] Ali SM, Yao M, Yao J, Wang J, Cheng Y, Schrock AB, Chirn GW, Chen H, Mu S, Gay L, Elvin JA, Suh J, Miller VA, Stephens PJ, Ross JS, Wang K. Comprehensive genomic profiling of different subtypes of nasopharyngeal carcinoma reveals similarities and differences to guide targeted therapy. Cancer 2017; 123: 3628-3637.
[26] Hui L, Zhang J, Ding X, Guo X, Jang X. Identification of potentially critical differentially methylatedgenes in nasopharyngeal carcinoma: a comprehensive analysis of methylation profiling and gene expression profiling. Oncol Lett 2017; 14: 7171-7178.
[27] Gessner A, Thomas M, Castro PG, Büchler L, Scholz A, Brümmendorf TH, Soria NM, Vormoor J, Greil J, Heidenreich O. Leukemic fusion genes MLL/AF4 andAML1/MTG8 support leukemic self-renewal by controlling expression of the telomerase subunit TERT. Leukemia 2010; 24: 1751-1759.
[28] Greaves MF, Maia AT, Wiemels JL, Ford AM. Leukemia in twins: lessons in natural history. Blood 2003; 102: 2321-2333.
[29] Kang J, Rychahou PG, Ishola TA, Mourot JM, Evers BM, Chung DH. N-myc is a novel regulator of PI3K-mediated VEGF expression in neuroblastoma. Oncogene 2008; 27: 3999-4007.
[30] Garcia V, Garcia JM, Silva J, Peña C, Dominguez G, Lorenzo Y, Diaz R, Alonso I, Colas A, Hurtado A, Sanchez A, Bonilla F. Levels of VEGF-A mRNA in plasma from patients with colorectal carcinoma as possible surrogate marker of angiogenesis. J Cancer Res Clin Oncol 2008; 134: 1165-1171.
[31] Lu Y and Wahl LM. Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKKalpha/NF-kappaB pathway. J Leukoc Biol 2005; 78: 259-265.
[32] Gustin JA, Ozes ON, Akca H, Pincheira R, Mayo LD, Li Q, Guzman JR, Korgaonkar CK, Donner DB. Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. J Biol Chem 2004; 279: 1615-1620.