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Original Article

The expression status of ZIC2 as a prognostic marker for nasopharyngeal carcinoma

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Abstract: Zinc finger protein ZIC2 is a transcription factor encoded by the ZIC2 gene, which can interact with various DNAs and proteins. ZIC2 expression may promote cell proliferation and inhibit apoptosis. Recent studies have reported that ZIC2 acts as an oncogene in various cancers. The expression and distinct prognostic value of ZIC2 in NPC is not well established. The aim of this study was to investigate ZIC2 expression and its prognostic significance in nasopharyngeal carcinoma (NPC). The ZIC2 expressions at the mRNA levels in NPC tissues and normal tissues were investigated using Oncomine analysis. ZIC2 protein expression was analyzed in paraffin-embedded NPC tissues by immunohistochemistry. Statistical analyses were performed to evaluate the clinicopathological significance of ZIC2 expression. The result shows the expression of ZIC2 mRNA is significantly elevated in NPC tissue compared with normal nasopharynx tissues. In paraffin-embedded tissue samples, the immunoreactivity of ZIC2 was primarily seen in the nuclei and cytoplasms within tumor cells. High ZIC2 expression was obviously related to poor OS and DFS compared to low ZIC2 expression. In a multivariate analysis, the expression of ZIC2 was an independent prognostic factor for OS and DFS. ZIC2 is up-regulated in NPC and associated with histology and survival. ZIC2 may serve as a prognostic indicator for patients with NPC.

Keywords: NPC, ZIC2, overexpression, prognosis, nasopharyngeal carcinoma (NPC), Epstein-Barr virus

Introduction

Nasopharyngeal carcinoma (NPC), an Epstein-Barr virus-associated malignancy, arises from the nasopharynx and has an unbalanced distribution of incidence and morbidity in different regions of the world. It is well known that NPC is endemic in southern China, Hong Kong, North Africa [1], and Southeast Asia with an approximate incidence of 30 per 100,000, yet NPC is relatively rare among white populations [2-4]. What causes these variations in the incidence of NPC is unclear. Multiple factors, such as EBV infection, environmental factors, and genetic susceptibility are believed to account for NPC's geographical distribution [5]. Since EBV infection is ubiquitous in the B lymphocytes but rare in epithelial cells [6], it is generally accepted that other environmental and genetic factors are also important determinants for NPC risk.

Currently, radiotherapy and concurrent chemoradiotherapy (CCRT) are the preferred treatment strategies for NPC. Despite a great improvement in diagnosis and treatment, the 5-year overall survival for locally-advanced NPC has remained poor [7]. Therefore, a novel target is in urgent need for early diagnosis and an efficient therapeutic option for NPC. Zinc finger protein ZIC2 (ZIC2) is a transcription factor encoded by the ZIC2 gene, which contains C2H2 zinc fingers and can interact with various DNA and proteins [8].

ZIC2 expression can also promote cell proliferation and inhibit apoptosis during the development of pancreatic ductal adenocarcinoma [9]. Recent studies have reported that ZIC2 acts as an oncogene in various cancers, such as ovarian cancer [10], liver cancer [11], and pancreatic cancer [9]. However, the clinical significance of

Table 1. Correlation of ZIC2 expression with clinicopathological features

Characteristics	Total (n=108)	ZIC2 expression		*P* value
		Low (n=99)	High (n=9)	
Age (years)				0. 305
≤ 46	44 (40.7%)	42 (95.5%)	2 (4.5%)	
> 46	64 (59.3%)	57 (89.1%)	7 (10.9%)	
Gender				0.112
Male	78 (72.2%)	74 (94.9%)	4 (5.1%)	
Female	30 (27.8%)	25 (83.3%)	5 (16.7%)	
Histology				0.478
WHO I	0 (0%)	0 (0%)	0 (0%)	
WHO II	41 (38.0%)	39 (95.1%)	2 (6.9%)	
WHO III	67 (62.0%)	60 (89.6%)	7 (10.4%)	
Clinical stage (UICC)				0.683
I	3 (2.9%)	3 (100.0%)	0 (0.0%)	
II	23 (22.5%)	22 (95.7%)	1 (4.3%)	
III	47 (46.1%)	43 (91.5%)	4 (8.5%)	
IV	25 (24.5%)	21 (84.0%)	4 (16.0%)	
V	4 (3.9%)	4 (100.0%)	0 (0.0%)	
T classification				0.463
T1	11 (10.8%)	10 (90.9%)	1 (9.1%)	
T2	40 (39.2%)	36 (90.0%)	4 (10.0%)	
T3	31 (30.4%)	30 (96.8%)	1 (3.2%)	
T4	20 (19.6%)	17 (85.0%)	3 (15.0%)	
N classification				0.233
N0	29 (28.4%)	25 (86.2%)	4 (13.8%)	
N1	25 (24.5%)	25 (100.0%)	0 (0.0%)	
N2	38 (37.3%)	34 (89.5%)	4 (10.5%)	
N3	10 (9.8%)	9 (90.0%)	1 (10.0%)	
Carotid sheath involvement				0.617
No	15 (14.7%)	13 (86.7%)	2 (13.3%)	
Yes	87 (85.3%)	80 (92.0%)	7 (8.0%)	
Nasal cavity involvement				0.055
No	72 (70.6%)	63 (87.5%)	9 (12.5%)	
Yes	30 (29.4%)	30 (100.0%)	0 (0.0%)	
Maxillary sinus involvement				0.680
No	80 (78.4%)	72 (90.0%)	8 (10.0%)	
Yes	22 (21.6%)	21 (95.5%)	1 (4.5%)	
Neck lymph node level involvement				0.827
No	29 (28.4%)	25 (86.2%)	4 (13.8%)	
Level I-III	63 (61.8%)	59 (93.7%)	4 (6.3%)	
Level IV	10 (9.8%)	9 (90.0%)	1 (10.0%)	
Maximum neck lymph node diameter				0.488
<20 mm	52 (51.0%)	46 (88.5%)	6 (11.5%)	
≥20 mm	50 (49.0%)	47 (94.0%)	3 (6.0%)	

WHO, World Health Organization.

ZIC2 in NPC remains unclear. In the present study, we examined the ZIC2 expression in NPC tissue samples and revealed its significance in NPC prognosis and clinical pathology.

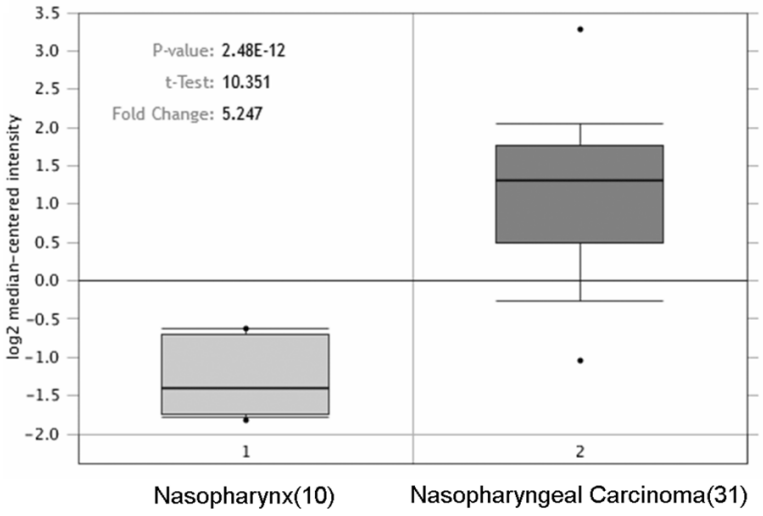


Figure 1. ZIC2 expression in NPC and normal nasopharynx tissues. The figure was derived from gene expression data in the Oncomine database comparing expression levels of ZIC2 in normal (left plot) and cancer tissue (right plot).

Material and methods

Oncomine analysis

Oncomine (www.oncomine.org) is an online database of cancer microarrays that aims at collecting, standardizing, and analyzing cancer transcriptome data to the biomedical research community and providing gene expression as well as clinical and pathological profiles for the major types of cancer contrasted with respective normal tissues [12]. It contains 65 gene expression datasets covering a wide variety of human malignant cancers [12]. In this study, we took advantage of this high-throughput database to mine ZIC2 expression in nasopharyngeal carcinoma tissues and normal tissues. The threshold value of this study was a 2.0 fold change, a cut-off *p*-value <0.05, and top 10% gene rank.

Patients and tissue specimens

All clinical samples used for the expression of ZIC2 studies by immunohistochemistry (IHC) assay were collected from the First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, between April 2010 and December 2017. The original site of NPC was involved in selecting retrievable biopsy samples. These cases included 78 (72.2%) men and 30 (27.8%) women, with a median age of 48 years (range, 16 to 80 years old). The median follow-up time was 46 months (range, 3.0 to 89 months). All the patients were followed from diagnosis until death, or relapse, or the last census date. The clinicopathologic characteristics for these patients including age, gender, histology, clinical stage T classification, N classification, carotid sheath involvement, nasal cavity involvement, maxillary sinus involvement, neck lymph node level involvement, and maximum neck lymph node diameter are summarized in **Table 1**. All the NPC patients were treated with standard radical radiotherapy, except 4 patients with distant metastasis at the time of diagnosis (with or without chemotherapy). The TNM stage was defined on the basis of the 8th AJCC (American Joint Committee on Cancer) Staging System. Based on the primary tumor sites and lymph nodes, there were 3, 23, 47, 25 and 4 patients belonging to the categories of stage I, II, III, IVa, and IVb respectively. For the use of these clinical materials on research purposes, prior consent was obtained from each of the patients, and the protocol of this study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University.

Immunohistochemistry (IHC) staining

Formalin-fixed and paraffin-embedded NPC samples were cut into sequential sections, each 4 μm thick. Then the slides were baked for 3 hours at 60°C. After being deparaffinized in xylenes and rehydrated through graded alcohol to distilled water, these slides were immersed into 3% hydrogen peroxide to block endogenous peroxidase activity at room temperature for 20 min, and then we boiled the slides in an ethylenediamine tetraacetic acid (EDTA) buffer (pH=8.0) for 3 min in a high-pressure cooker for antigen retrieval. After the retrieval solution returned to room temperature, the slides were incubated with diluted rabbit polyclonal anti-ZIC2 antibody (1:200 dilutions; Affinity, USA) in a moist chamber at 4°C overnight. Subsequently, after we washed the samples 3 times in ph-

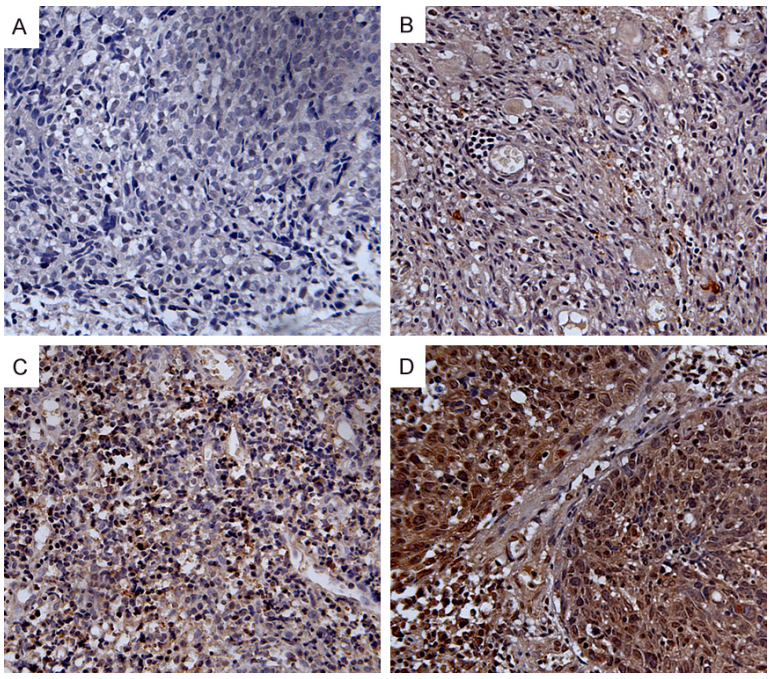


Figure 2. Expression analysis of ZIC2 protein as determined by immunohistochemistry (200×). ZIC2 expression was localized in the nuclei and cytoplasms of NPC cells. A. Negative staining of ZIC2 in NPC tissues. B. "+" (score 1-4, weakly positive) expression of ZIC2 in NPC tissues. C. "++" (score 5-8, positive) expression of ZIC2 in NPC tissues. D. "+++" (score 9-12, strongly positive) expression of ZIC2 in NPC tissues.

osphate buffered saline, we added Tween-20 (TBST), and then the slides were incubated with a secondary antibody at room temperature for 1 hour and then washed in PBST 3 times, and sequentially stained for 2 min in DAB (3,3-diaminobenzidine) at room temperature for protein detection. Finally, the slides were counterstained with Mayer's hematoxylin and dehydrated and mounted. A negative control was obtained by replacing the primary antibody with a normal rabbit IgG.

IHC evaluation

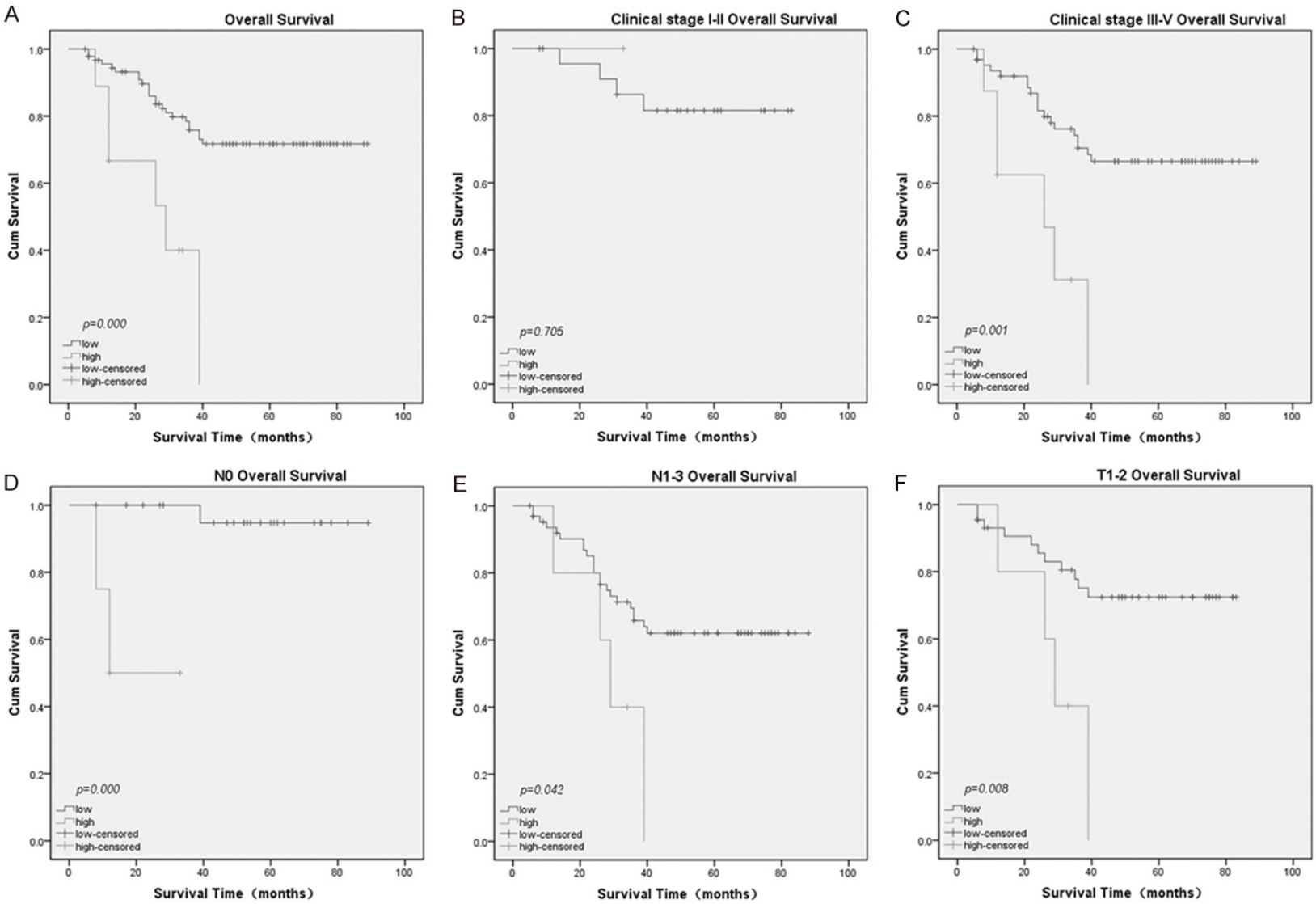
To ensure the accuracy and objectivity of the experiment, the evaluation of the IHC staining for each sample was conducted separately by three pathologists blinded to the clinicopathological data. The expression of ZIC2 was located in the nuclei and cytoplasms. Criteria were established such that tissue with BSG expression in more than 25% of tumor cells would be considered as BSG-positive. The immunostaining of EGFR and P53 expression was assessed according to the proportion of cells with a positive expression to the total number of cells. At least 10 typical fields in high magnification (40×40) from necrotic areas were randomly selected for counting. Results of the immunostaining were divided into four groups: (-) for negative staining, (+) for weak staining, (++) for moderate staining and (+++) for strong staining.

Evaluation of IHC

The tumor cell percentage was scored as follows: 0 for less than 10% positive cells; 1 for 10-25% positive cells; 2 for 26-50% positive cells; 3 for 51-75% positive cells; 4 for more than 75% positive cells. The staining intensity was categorized: 0 for no staining; 1 for weak staining; 2 for moderate staining and 3 for strong staining. The two individual parameters were multiplied to get a final immunoreactivity score (IRS) ranging from 0 to 12. An optimal cut-off value for the high and low expression of ZIC2 was decided on the basis of a measure of heterogeneity with a log-rank test statistical analysis with respect to the overall survival (OS). For ZIC2, the optimal cutoff value was determined: an IRS ≤ 4 was defined as low expression and an IRS > 4 was defined as high expression.

Statistical analysis

The statistical analysis was performed by the Statistical Program for Social Sciences (SPSS, Chicago, IL, USA; 19.0). The duration from the start of randomization to death caused by any event was defined as overall survival (OS). The duration from the start of randomization to tumor recurrence or relapse or death was defined as disease-free survival (DFS). The correlation between the ZIC2 expression and the clinicopathological characteristics of these NPC patients was assessed by a chi-square test. Survival curves for the ZIC2 high-expression and the ZIC2 low expression patients were plotted by the Kaplan-Meier analysis and a log-rank test. The univariate and multivariate survival analyses were performed using the Cox proportional hazard model to determine effect of the


A
Overall Survival
Cum Survival
Survival Time (months)
p=0.000
low
high
low-censored
high-censored
B
Clinical stage I-II Overall Survival
Cum Survival
Survival Time (months)
p=0.705
low
high
low-censored
high-censored
C
Clinical stage III-V Overall Survival
Cum Survival
Survival Time (months)
p=0.001
low
high
low-censored
high-censored
D
N0 Overall Survival
Cum Survival
Survival Time (months)
p=0.000
low
high
low-censored
high-censored
E
N1-3 Overall Survival
Cum Survival
Survival Time (months)
p=0.042
low
high
low-censored
high-censored
F
T1-2 Overall Survival
Cum Survival
Survival Time (months)
p=0.008
low
high
low-censored
high-censored

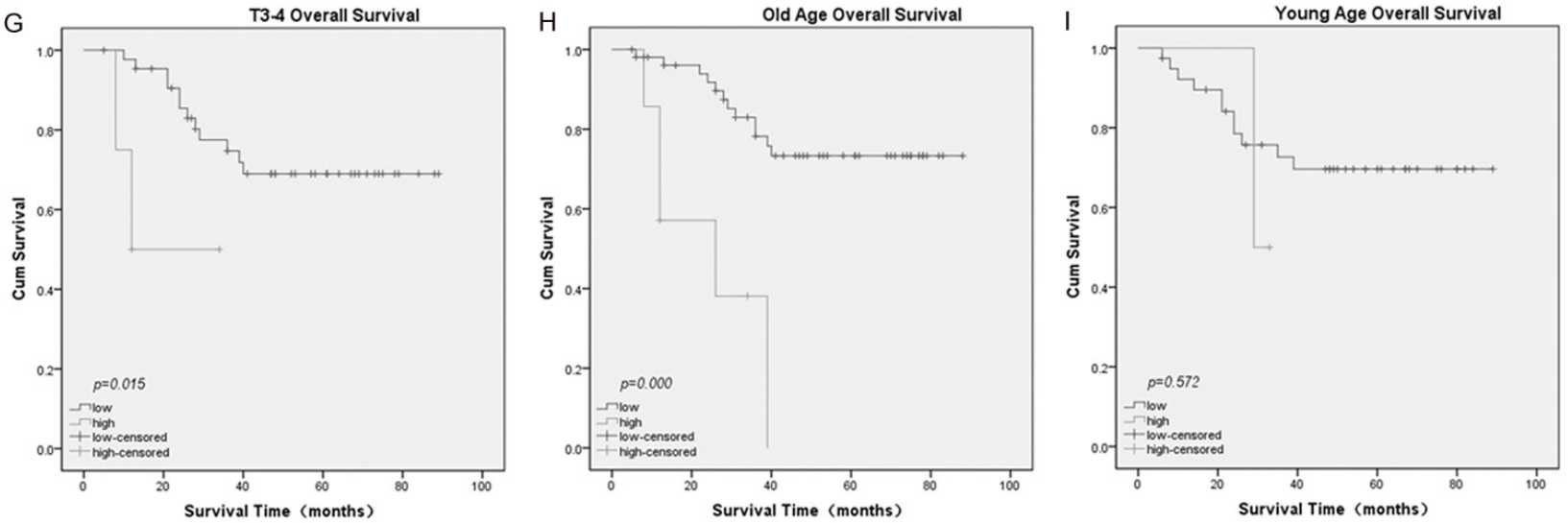


Figure 3. The prognostic value (OS) of ZIC2 expression analyzed using a Kaplan-Meier Plotter. A. OS rates for patients with high ZIC2 expression versus those with low ZIC2 expression levels. B. OS rate for patients with early clinical stage tumors (stage I/ II) with high ZIC2 expression versus those with low ZIC2 expression. C. OS rate for patients with late clinical stage tumors (stage III/ IV) with high ZIC2 expression versus those with low ZIC2 expression. D. OS rate for patients with N0 Stage tumors with high ZIC2 expression versus those patients with low ZIC2 expression. E. OS rate for patients with N1-3 Stage tumors with high ZIC2 expression versus those patients with low ZIC2 expression. F. OS rate for patients with T1-2 Stage tumors with high ZIC2 expression versus those patients with low ZIC2 expression. G. OS rate for patients with T3-4 Stage tumors with high ZIC2 expression versus those patients with low ZIC2 expression. H. OS rate for older patients with high ZIC2 expression versus those patients with low ZIC2 expression. I. OS rate for younger patients with high ZIC2 expression versus those patients with low ZIC2 expression.

particular prognostic factors on survival. All the *P*-values in the statistical analysis were two-tailed, and differences were considered significant if the *P*-value was <0.05.

Results

The expression level of ZIC2 was elevated in NPC patients

In the Oncomine database, the distribution of ZIC2 expression in NPC tissues and normal nasopharynx tissues were available from a microarray chips including 41 samples. We compared the expression levels of ZIC2 between NPC tissues and the normal nasopharynx tissue in the Oncomine database. The results showed that the expression of ZIC2 is significantly elevated in NPC tissue compared with normal nasopharynx tissues (**Figure 1**).

The expression patterns of ZIC2 in NPCs by IHC

To examine the expression pattern of ZIC2 in archived NPC tissues, we performed an IHC analysis with the specific antibody in 108 NPC tissues. Four representative images of immunostaining illustrated the ZIC2 expression pattern in the NPC tissues are shown in **Figure 2**. The Immunoreactivity of the ZIC2 expression was primarily seen in the nuclei and cytoplasms within the tumor cells. Among them, 29 (26.9%) cases showed weak intensity (**Figure 2A** and **2B**), 70 (64.8%) cases showed moderate staining (**Figure 2C**), and 9 (8.3%) cases showed strong intensity (**Figure 2D**).

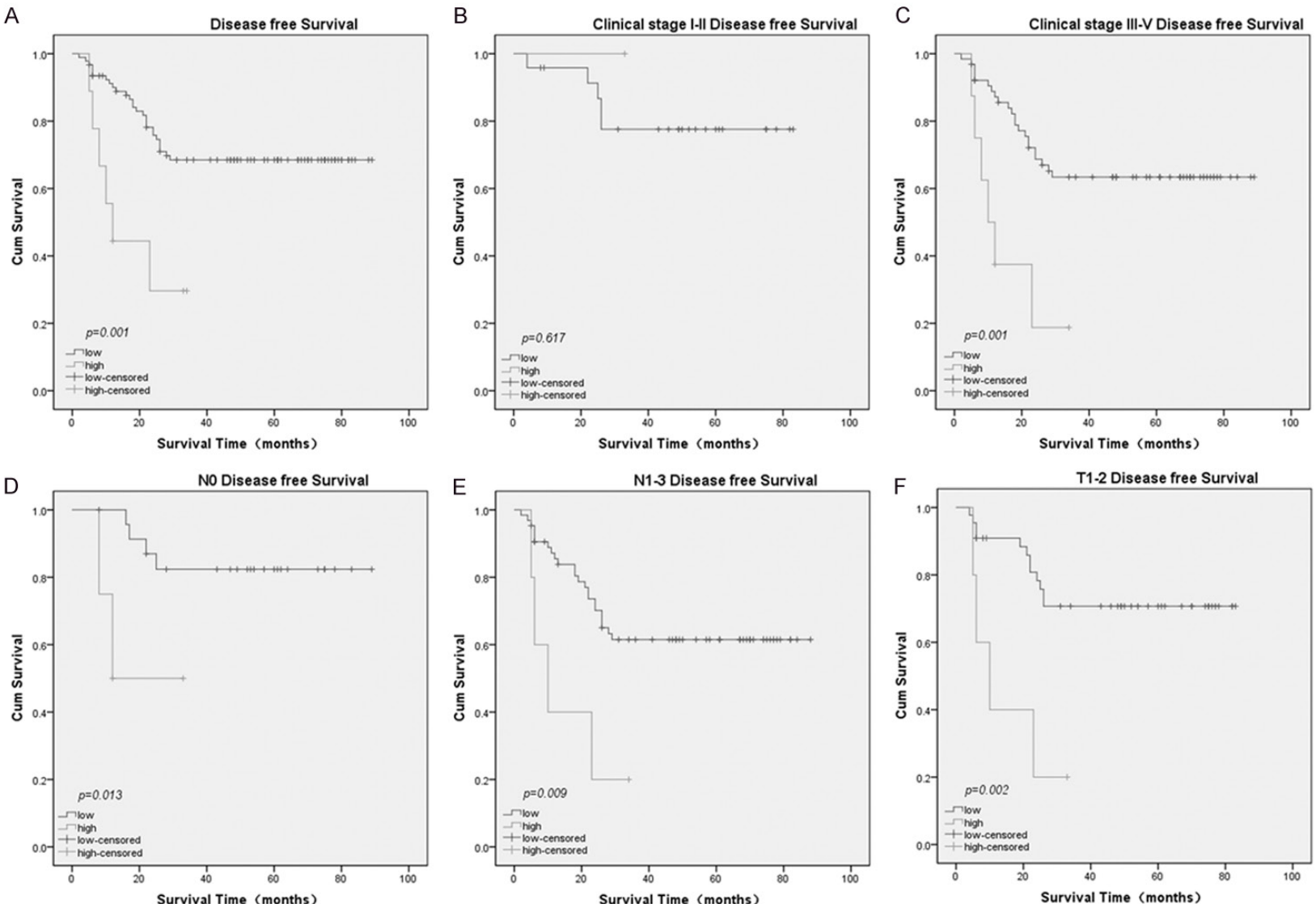
Furthermore, we investigated the relationship between ZIC2 expression and the clinicopathological parameters of NPC. Unfortunately, neither age, gender, clinical stage (UICC), Histology, T classification, N classification, carotid sheath involvement, nasal cavity involvement, maxillary sinus involvement, neck lymph node level involvement, or maximum neck lymph node diameter had any statistical relationship with the expression level of ZIC2 (**Table 1**).

The association between ZIC2 expression and patient prognosis

The prognostic value of ZIC2 was evaluated through estimation of OS and DFS using a Kaplan- Meier survival analysis (log-rank test). It is obviously shown that a high ZIC2 expression was significantly related to poor OS (P<0.001, **Figure 3A**) and DFS (P=0.001, **Figure 4A**) compared to low ZIC2 expression. In addition, a Univariate Cox proportional hazard regression analysis showed that clinical stage (UICC) (P<0.001), N classification (P=0.001), neck lymph node level involvement (P= 0.003), maximum neck lymph node diameter (P=0.020), and ZIC2 expression (P=0.001) were significantly associated with survival (**Table 2**). AS for DFS, clinical stage (8th AJCC) (P=0.026), N stage (P=0.010), neck lymph node level involvement (P=0.016), maximum neck lymph node diameter (P=0.045), and ZIC2 expression (P=0.003) were significantly associated with survival (**Table 3**).

After multivariate adjustment for the above significant clinicopathological features, the clinical stage (8^{th} AJCC) (HR: 0.020; 95% confidence interval: 0.003-0.134; P<0.001), maximum neck lymph node diameter (HR: 0.378; 95% confidence interval: 0.166-0.859; P=0.020). and ZIC2 expression (HR: 0.146; 95% confidence interval: 0.054-0.399; P<0.001) were independent prognostic factors for OS (**Table 2**), while only ZIC2 expression was an independent prognostic factor for DFS (HR: 0.187; 95% confidence interval: 0.070-0.498; P=0.001, **Table 3**).

Furthermore, we analyzed the prognostic value of ZIC2 in selective patient subgroups stratified by clinical stage (8^{th} AJCC), N stage, T stage, and age. The expression of ZIC2 was strongly associated with OS in patients of advanced stage tumor (**Figure 3C**, log-rank test, P=0.001), but not in patients of early stage tumor (**Figure 3B**, log-rank test, P=0.706). The expression of ZIC2 was also strongly associated with OS duration in the patients of both N0 tumors (**Figure 3D**, log-rank test, P<0.001) and N1-3 tumors (**Figure 3E**, log-rank test, P=0.042), and both T1-2 tumors (**Figure 3F**, log-rank test, P=0.008) and T3-4 tumors (**Figure 3G**, log-rank test, P=0.015). As for the age subgroup, the expression of ZIC2 was strongly associated with OS duration in older patients (**Figure 3H**, log-rank test, P<0.001), but not in younger patients (**Figure 3I**, log-rank test, P=0.572). When evaluated according to DFS, the expression of ZIC2 was strongly associated with DFS


A
Disease free Survival
Cum Survival
Survival Time (months)
p=0.001
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B
Clinical stage I-II Disease free Survival
Cum Survival
Survival Time (months)
p=0.617
low
high
low-censored
high-censored
C
Clinical stage III-V Disease free Survival
Cum Survival
Survival Time (months)
p=0.001
low
high
low-censored
high-censored
D
N0 Disease free Survival
Cum Survival
Survival Time (months)
p=0.013
low
high
low-censored
high-censored
E
N1-3 Disease free Survival
Cum Survival
Survival Time (months)
p=0.009
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high
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T1-2 Disease free Survival
Cum Survival
Survival Time (months)
p=0.002
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high-censored

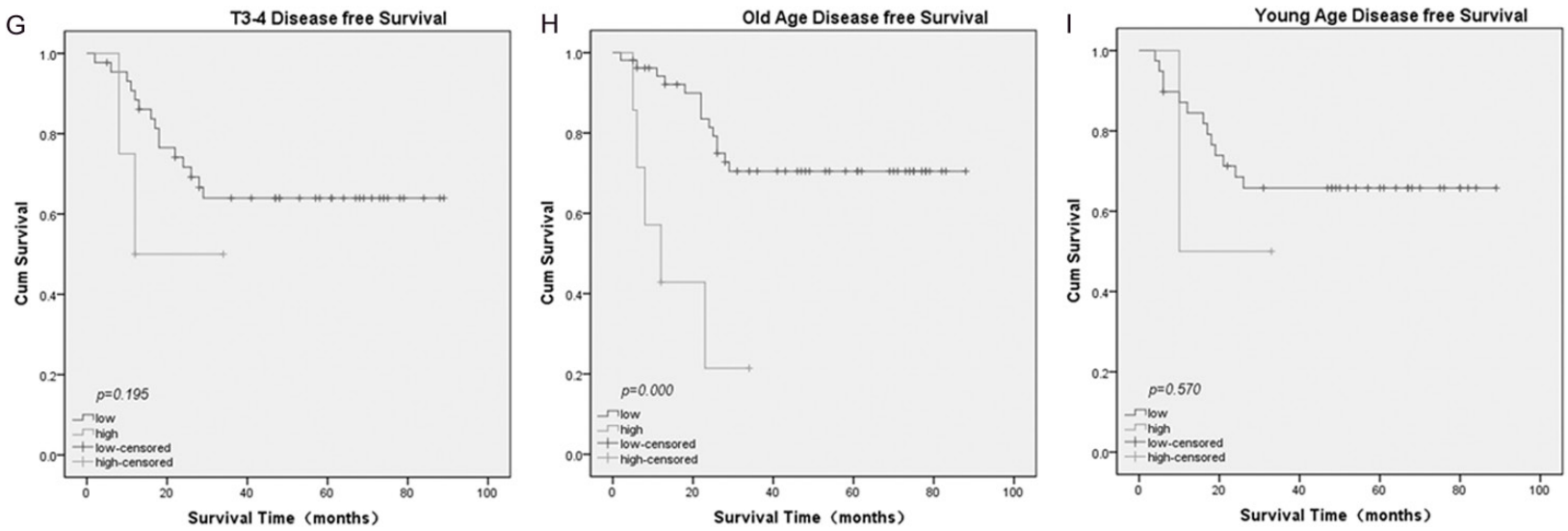

Figure 4. The prognostic value (DFS) of ZIC2 expression analyzed using a Kaplan-Meier plotter. A. DFS rates for patients with high ZIC2 expression versus those with low ZIC2 expression levels. B. DFS rates for patients with early clinical stage tumor (stage I/ II) with high ZIC2 expression versus those with low ZIC2 expression. C. DFS rate for patients with late clinical stage tumor (stage III/ IV) with high ZIC2 expression versus those with low ZIC2 expression. D. DFS rate for patients with N0 Stage tumor with high ZIC2 expression versus those patients with low ZIC2 expression. E. DFS rate for patients with N1-3 Stage tumor with high ZIC2 expression versus those patients with low ZIC2 expression. F. DFS rate for patients with T1-2 Stage tumor with high ZIC2 expression versus those patients with low ZIC2 expression. G. DFS rate for patients with T3-4 Stage tumor with high ZIC2 expression versus those patients with low ZIC2 expression. H. DFS rate for patients of old age with high ZIC2 expression versus those patients with low ZIC2 expression. I. DFS rate for patients of young age with high ZIC2 expression versus those patients with low ZIC2 expression.

Table 2. Cox-regression analysis of parameters associated with overall survival of all patients

Factor	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age				
≤ 46	Reference			
> 46	1.019 (0.487-2.134)	0.960	-	-
Gender				
Male	Reference			
Female	1.160 (0.495-2.718)	0.732	-	-
Histology				
WHO I				
WHO II	Reference		-	-
WHO III	1.456 (0.700-3.027)	0.315	-	-
Clinical stage (UICC)				
I	Reference	0.000	Reference	0.000
II	0.036 (0.006-0.221)	0.000	0.020 (0.003-0.134)	0.000
III	0.046 (0.009-0.239)	0.000	0.018 (0.003-0.111)	0.000
IV	0.147 (0.030-0.726)	0.019	0.073 (0.013-0.397)	0.002
T classification				
T1	Reference	0.263		
T2	1.103 (0.360-3.377)	0.863	-	-
T3	0.546 (0.215-1.385)	0.202	-	-
T4	0.438 (0.152-1.263)	0.126	-	-
N classification				
N0	Reference	0.001		
N1	0.081 (0.021-0.316)	0.000	-	-
N2	0.237 (0.083-0.682)	0.008	-	-
N3	0.257 (0.100-0.660)	0.005	-	-
Carotid sheath involvement				
No	Reference			
Yes	0.903 (0.314-2.594)	0.849	-	-
Nasal cavity involvement				
No	Reference			
Yes	0.740 (0.344-1.593)	0.441	-	-
Maxillary sinus involvement				
No	Reference			
Yes	0.754 (0.334-1.702)	0.496	-	-
Neck lymph node level involvement				
No	Reference	0.003		
Level I-III	0.211 (0.026-1.723)	0.147	-	-
Level IV-V	0.241 (0.094-0.617)	0.003	-	-
Maximum neck lymph node diameter				
<20 mm	Reference		Reference	
≥20 mm	0.394 (0.179-0.865)	0.020	0.378 (0.166-0.859)	0.020
ZIC2 expression				
Low	Reference		Reference	
High	0.219 (0.087-0.551)	0.001	0.146 (0.054-0.399)	0.000

duration in advanced-stage tumor patients (**Figure 4C**, log-rank test, P=0.001), but not in early-stage tumor patients (**Figure 4B**, log-rank test, P=0.617). The expression of ZIC2 was

Table 3. Cox-regression analysis of parameters associated with the disease free survival of all patients

Factor	Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Age				
≤ 46	Reference			
> 46	1.080 (0.542-2.155)	0.826	-	-
Gender				
Male	Reference			
Female	1.194 (0.539-2.648)	0.662	-	-
Histology				
WHO I				
WHO II	Reference		-	-
WHO III	1.362 (0.683-2.719)	0.380	-	-
Clinical stage (UICC)				
I	Reference	0.026		
II	1.038 (0.093-6.314)	0.931	-	-
III	6.490 (0.094-8.713)	0.929	-	-
IV	1.340 (0.334-1.979)	0.914	-	-
T classification				
T1	Reference	0.562		
T2	0.534 (0.185-1.539)	0.245	-	-
T3	0.574 (0.192-1.714)	0.320	-	-
T4	0.855 (0.279-2.613)	0.783	-	-
N classification				
N0	Reference	0.010		
N1	0.172 (0.057-0.514)	0.002	-	-
N2	0.290 (0.104-0.805)	0.017	-	-
N3	0.283 (0.110-0.724)	0.008	-	-
Carotid sheath involvement				
No	Reference			
Yes	0.780 (0.274-2.219)	0.642	-	-
Nasal cavity involvement				
No	Reference			
Yes	0.594 (0.295-1.195)	0.144	-	-
Maxillary sinus involvement				
No	Reference			
Yes	0.739 (0.343-1.591)	0.439	-	-
Neck lymph node level involvement				
No	Reference	0.016		
Level I-III	0.172 (0.057-0.514)	0.002	-	-
Maximum neck lymph node diameter				
<20 mm	Reference			
≥20 mm	0.483 (0.237-0.982)	0.045	-	-
ZIC2 expression				
Low	Reference		Reference	
High	0.251 (0.102-0.615)	0.003	0.187 (0.070-0.498)	0.001

also strongly associated with DFS duration in the patients of both N0 tumors (**Figure 4D**, log-rank test, P=0.013) and N1-3 tumors (**Figure 4E**, log-rank test, P=0.009). As for the T Stage subgroup, the expression of ZIC2 was strongly associated with DFS duration in patients with T1-2 tumors (**Figure 4F**, log-rank test, P=0.002), but not in patients with T3-4 tumors (**Figure 4G**, log-rank test, P=0.195). In older patients, the expression of ZIC2 was strongly associated with DFS duration (**Figure 4H**, log-rank test, P<0.001), but not in younger patients (**Figure 4I**, log-rank test, P=0.570).

Discussion

The human ZIC (zinc finger of the cerebellum) proteins-ZIC1, ZIC2, ZIC3, ZIC4, and ZIC5, which share five highly-conserved Cys2His2 zinc-finger motifs, function in biological processes [13] as transcription factors (TFs) which contribute to the pathogenesis of multiple human cancers [14]. As zinc finger transcription factors, these proteins can bind with the GC-rich sequence in target genes [15]. ZIC proteins are critical to the developing vertebrate embryo and to human cancers [16]. The Cys2His2 zinc-finger motifs interact with the Gli family proteins via homologous structures in both an antagonistic and synergistic manner, which are quite essential for the development of human nervous system [17]. These five proteins, which are homologues of the Drosophila odd-paired gene (OPA), are structurally similar to each other, which imply that the ZIC proteins share some, but not all, functions [8]. ZIC genes play important roles in a wide array of developmental systems, such as the central nervous system, muscle and skeletal development [18]. In recent years, growing evidence has indicated that ZICs may play different roles in the pathological events of various diseases, including cancer.

ZIC1 has been found to inhibit the growth and development of various carcinomas, including GC [19], colorectal cancer [20], medulloblastoma [21], thyroid cancer, mesenchymal neoplasms [22], breast cancer [23] and endometrial cancer [24], and has also become a putative indicator of good prognosis [20, 25-28]. Genome-wide analysis of CpG island methylation in pTa-bladder cancer suggested that ZIC4 [29] was correlated with poor progression [29, 30]. Pavlova et al. [31] also identified that methylation of ZIC4 might participate in the development of breast cancer. However, ZIC2, ZIC3, and ZIC5 are overexpressed in cancer cells and may function as oncogenes by promoting the proliferation and inhibiting the apoptosis of tumor cells [32-34]. But the clinicopathologic and prognostic significance of the ZIC proteins still requires further illumination.

ZIC2 is only found in the testis and brain in normal tissues, but it is widely expressed in tumors. It has C2H2 zinc fingers and can interact with various DNAs and proteins [8]. ZIC2 expression could also promote cell proliferation and inhibit apoptosis during the development of pancreatic ductal adenocarcinoma [9] Recent studies have reported that ZIC2 acts as an oncogene in various cancers, such as ovarian and cervical cancer [10, 35], liver cancer [11], pancreatic cancer [9], and small cell lung carcinoma [32]. Marchini et al. have reported that the overexpression of ZIC2 increased the growth rate and foci formation of ovarian cancer and stimulated anchorage- independent colony formation [10]. Chan and colleagues have revealed that in cervical cancer, ZIC2 could promote cell growth by enhancing Hedgehog signaling [35]. It can also up-regulate the expression of OCT4 by recruiting the nuclear remodeling factor complex (NURF) to the OCT4 promoter in liver cancer cells [11]. ZIC2 can enhance cellular proliferation and reduce apoptosis in pancreatic cancer via activating the transcription of fibroblast growth factor receptor 3 (FGFR3) and Annexin A8 (ANXA8) [9]. ZIC2 expression seems to increase stepwise during tumor progression and targeting ZIC2 should be a promising strategy for the clinical management of multiple cancers. However, the roles of ZIC2 in the development of cancer have not yet been fully elucidated, and the clinical significance of ZIC2 in NPC remains unclear. A comparison of ZIC2 expression and the clinical significance in NPC is required.

In this study, we compared the expression level of ZIC2 between NPC and normal nasopharynx tissues in the Oncomine database and found that the expression of ZIC2 is significantly elevated in NPC tissue compared with normal nasopharynx tissues. So, we consider that ZIC2 is an important molecular marker of NPC that can help provide precise diagnoses. ZIC2 overexpression in NPC may reflect the aberrant

regulation of transcription factors (TFs). However, to understand the precise signaling pathways of ZIC2 in NPC requires further studies.

Reports have proved the prognostic value of ZIC2 in human cancers. Marchini et al. have reported that the upregulation of ZIC2 in stage I ovarian cancer was related to a poorer outcome [10]. Chan et al. also demonstrated its over-expression in cervical cancer was associated with a worse overall survival of patients [35]. Bidus et al. have illustrated that ZIC2 overexpression was found in endometrial cancer patients with metastasis in lymph nodes [36]. However, the prognostic implication of ZIC2 in NPC has not been investigated. In our study, a high ZIC2 expression was significantly related to poor OS and DFS, compared to low ZIC2 expression group. In addition, a Cox regression revealed that clinical stage (UICC), maximum neck lymph node diameter and ZIC2 expression were independent prognostic factors for OS, while only ZIC2 expression was an independent prognostic factor for DFS. This finding indicates the possibility of using ZIC2 as a predictor for prognosis and survival. A sub-group analysis revealed that ZIC2 overexpression patients with an obviously poor OS among patients whose tumors demonstrated the features of late stage disease, and old age. When evaluated according to DFS, the expression of ZIC2 was strongly associated with DFS duration in late stage tumor patients, T1-2 tumors, and older patients.

In conclusion, to our knowledge, this is the first report addressing ZIC2 expression and its clinicopathological and prognostic significance in NPC. Our findings suggest that ZIC2 is up-regulated in NPC and closely correlated with histology, and a Cox regression analysis revealed that ZIC2 might be an independent molecular biomarker for the prediction of NPC prognosis and survival. ZIC2 may serve as a prognostic indicator for patients with NPC.

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Disclosure of conflict of interest

None.

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