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Original Article

Increased expression of IRS-1 is associated with lymph node metastasis in nasopharyngeal carcinoma

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Abstract: Nasopharyngeal carcinoma (NPC) is a head and neck malignant tumor rare throughout most of the world but common in Southeast Asia, especially in Southern China, which is with characteristics of early cervical lymph node metastasis and high incidence rate of distant metastasis. Insulin receptor substrate 1 (IRS-1) is a signaling adapter protein that is encoded by the IRS-1 gene in humans, plays an important role in the development, progression, invasion and metastasis of tumors. The aim of the present study was to investigate the association between the expression of IRS-1 protein and clinicopathological characteristics in NPC by immunohistochemistry. The results showed that the expression level of IRS-1 was significant higher in NPC than that in the control nasopharyngeal epithelia (P = 0.042). The positive percentage of IRS-1 expression in NPC with lymph node metastasis was also significantly higher than those without lymph node metastasis (P = 0.008). Positive expression of IRS-1 was proved to be the independent predicted factor for lymph node metastasis of NPC (P = 0.025) regardless of age, gender, histological type and clinical stages by multivariate logistic regression analysis. In addition, results showed higher sensitivity and agreement rate of IRS-1 for predicting lymph node metastasis of NPC patients. Taken together, high expression of IRS-1 might be closely correlated with lymph node metastasis in NPC and positive expression of IRS-1 could be used as an independent biomarker for predicting lymph node metastasis of NPC.

Keywords: Insulin receptor substrate (IRS), insulin receptor substrate 1 (IRS-1), nasopharyngeal carcinoma (NPC), metastasis, biomarker

Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck malignant tumor rare throughout most of the world but common in Southeast Asia, especially in Southern China, which is derived from nasopharyngeal epithelium with characteristics of early cervical lymph node metastasis and high incidence rate of distant metastasis. Epstein-Barr virus (EBV) infection, chemical carcinogens, genetic variation or spontaneous mutation are closely associated with its occurrence and development [1, 2]. Radiation therapy is the major therapeutic modality used to treat NPC, and most NPC patients can be cured if the disease is diagnosed and treated at an early stage, whereas, in advanced stages, local recurrence and metastasis are still the leading cause of mortality [3]. Therefore, further elucidation of the molecular mechanism and novel targets underlying NPC is vital for the development of new effective therapeutic agents.

The insulin receptor substrate (IRS) proteins are cytoplasmic adaptor proteins that function as essential signaling intermediates downstream of activated cell surface receptors, many of which have been implicated in cancer [4]. The IRS proteins play a central role in mediating the signals from the IR/IGF-1R that control tumor cell metabolism [5]. Insulin receptor substrate 1 (IRS-1) is one member of the insulin receptor substrate family, which is associated with tumor initiation and progression. Overexpression of IRS-1 promotes cells growth, inhibits basal

autophagy, reduces oxidative stress-induced autophagy, and diminishes oxidative stress-mediated autophagy-dependent cell death [6]. Interestingly, recent evidence shows that IRS-1 exhibits increased expression in hepatocellular, pancreatic, prostatic, breast, ovarian and colorectal cancers [7-13].

However, so far whether the alteration of the expression of IRS-1 is associated with development and progression or clinicopathological/prognostical implication for NPC has not been reported. In the present study we investigated the expression of IRS-1 protein and clinicopathological correlations in NPC by Immunohistochemistry (IHC). We found that positive percentage of IRS-1 expression in NPC was significantly higher than that in the non-cancerous nasopharyngeal control tissue. Also, higher expression of IRS-1 was associated with the lymph node metastasis in NPC. Therefore, our results indicated that IRS-1 might play an important role in promoting the metastasis of NPC. Moreover, multivariate analysis confirmed that increased expression of IRS-1 protein might be an independent predictive factor for lymph node metastasis in NPC.

Materials and methods

Tissue samples and clinical data

Two hundred and forty-five (245) paraffin-embedded NPC cases from the primary NPC patient with their age from 16 to 78 year (median, 45.56 years), also 30 cases of non-cancerous nasopharyngeal epithelial control specimen from independent patients with chronic inflammation of nasopharyngeal mucosa, were obtained from The Second Xiangya Hospital of Central South University (Changsha, China). No patient had previously been treated with chemotherapy and radiotherapy at the time of original biopsy. Complete clinical record and follow-up data were available for all patients. Also, written informed consent was obtained from all patients. This study protocol, specimen usage, and data retrieval were approved by the Institutional Human Experiment and Ethics Committee of the Second Xiangya Hospital of Central South University (approval number S-02-2000). All specimens had been confirmed pathological diagnosis according to the 2005 WHO histological classification of NPC. The patients were staged according to the UICC/AJCC 1997 staging system of NPC.

The histological patterns and clinical stages of NPC were classified as follows: 222 cases of undifferentiated non-keratinizing carcinomas and 23 cases of differentiated non-keratinizing carcinomas; 8 cases of clinical stage I, 81 cases of stage II, 108 cases of stage III, and 48 cases of stage IV. Among these patients included in the research, 177 patients were positive for cervical lymph node metastasis and 68 patients were negative. Among 245 patients, 16 patients were treated by chemotherapy alone, 91 patients by radiotherapy alone, and 138 patients by combined radiotherapy and chemotherapy. Complete clinical record and follow-up data of all patients were available. Overall survival time was calculated from the data of diagnosis to the date of death or the data last known alive. A total of 154 patients (65.1%) were alive with a mean follow-up period of 56.8 months (5-120 months).

IHC and scores

The IHC staining for IRS-1 protein in NPC sections was carried out using ready-to use Envision TM+ Dual Link System-HRP methods (Dako; Carpintrria, CA). The staining condition for antibody was adjusted according to our laboratory experience. Briefly, each NPC section was deparaffinized and rehydrated, and high-temperature antigen retrieval was achieved by heating the samples in 0.01 M citrate buffer in a domestic microwave oven at full power (750 Watts) for 30 minutes, then the samples were immersed into methanol containing 0.3% H_2O_2 to inactivate endogenous peroxidase at room temperature (RT) for 30 minutes. To eliminate nonspecific staining, the slides were incubated with appropriate preimmune serum for 30 minutes at RT. After incubation with a 1: 100 dilution of primary antibody to IRS-1 protein (Rabbit Monoclonal IgG, Catalog#: 1692-1, Clone ID: EP263Y, Epitomics, Inc.) at 4°C overnight, slides washed with physiological phosphate buffered saline (PBS) three times for five minutes each and second antibody conjugated with a labeled polymer-HRP was added according to the manufacturer's instructions and incubated at 37°C for 30 minutes. Color reaction was developed by using 3-Amino-9-Ethylcarbazole (AEC) chromogen solution. All slides were counterstained with hematoxylin. Positive control slides were included in every experiment in addition to the internal positive control.

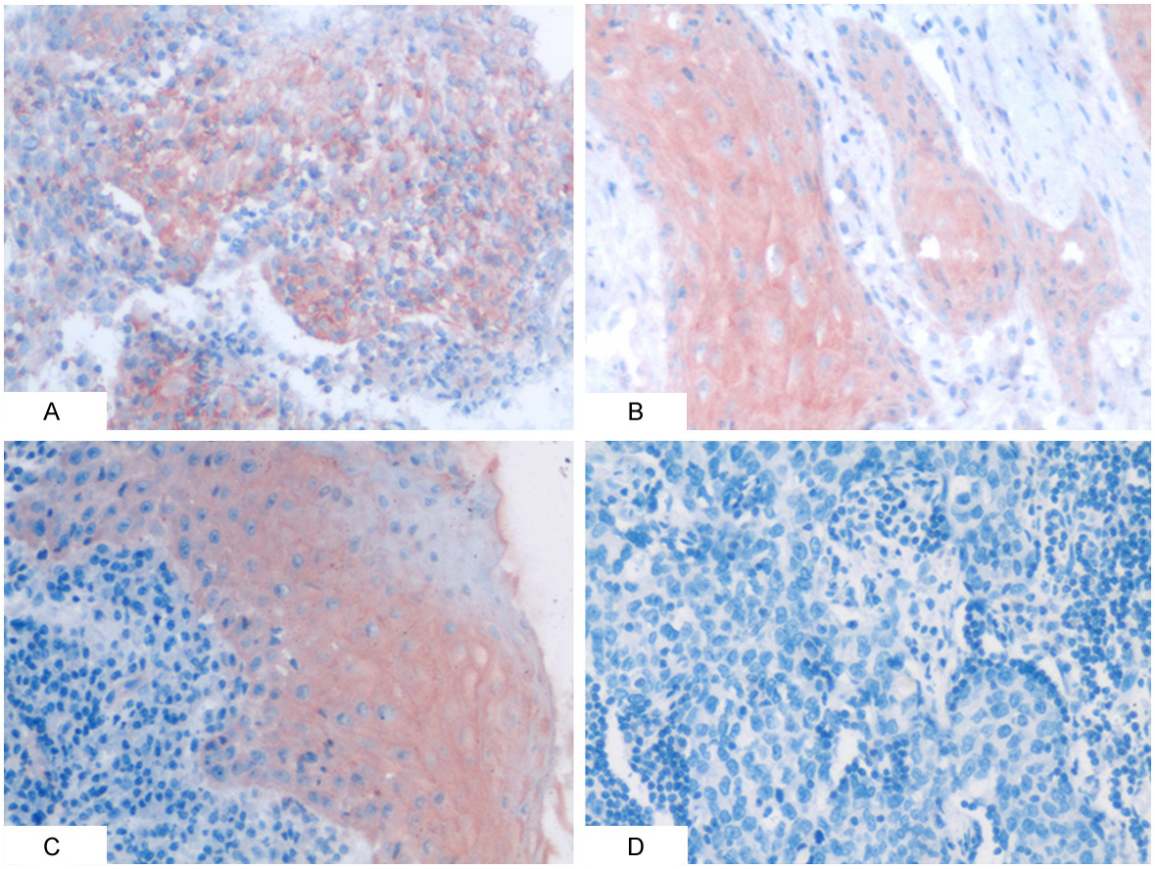

Figure 1. Expression of IRS-1 in NPC and non-cancerous nasopharyngeal control tissue was detected by IHC. The expression of IRS-1 was detected by IHC using specific antibody as described in the section of materials and methods. Strong positive staining of IRS-1 was identified on the membrane and cytoplasm of NPC cells (A and B, 20×, IHC, AEC staining), also (A) showed positive staining IRS-1 on the membrane and cytoplasm in a small subset of lymphocytes infiltrating NPC tissues. Membrane and cytoplasm positive staining of IRS-1 was showed in the normal nasopharyngeal epithelial cells (C, 20×, IHC, AEC staining). Negative staining of IRS-1 in the NPC (D, 20×, IHC, AEC staining).

The specificity of the antibody was determined with matched IgG isotype antibody as a negative control.

Immunohistochemical staining was evaluated independently by QW and XL who were blinded to the clinicopathological data, at 200× magnification light microscopy. Positive expression of IRS-1 protein was identified on the membrane and cytoplasm of NPC and control nasopharyngeal epithelial cells. A semi-quantitative evaluation of IRS-1 was performed using a method described in the literature [14] as follows: the percentage of positive cells was divided into five grades (percentage scores): ≤ 10% (0), 11-25% (1), 26-50% (2), 51-75% (3), and > 75% (4). The intensity of staining was divided into four grades (intensity scores): no staining (0), light brown (1), brown (2), and dark brown (3). Staining positivity was determined by the formula: overall scores = percentage score × intensity score. The total score ranged from 0 to 12, with negative staining (0-1) and positive expression (2-12). Agreement between the two evaluators was 98%, and all scoring discrepancies were resolved through discussion between the two evaluators.

Statistical analyses

All statistical analyses were performed using SPSS 19.0. The chi-square test was used to analyze the relationship between the expression of IRS-1 protein and clinicopathological characteristics of NPC. Kaplan-Meier analysis was performed for overall survival curves and statistical significance was assessed using the log-rank test. Overall survival was defined as the time from the treatment initiation (diagnosis) to the date of death. To identify whether

Table 1. Analysis of the association between expression of IRS-1 protein and clinicopathological features of NPC (n = 245)

Clinicopathological features (n)	IRS-1 expression		*P*-value
	Positive (%)	Negative (%)	
Age (years)			
≤ 40 (n = 160)	111 (69.4)	49 (30.6)	
> 40 (n = 85)	57 (67.1)	28 (32.9)	0.710
Gender			
Female (n = 57)	34 (59.6)	23 (44.4)	
Male (n = 188)	134 (71.3)	54 (28.7)	0.098
Clinical stages			
Stage I-II (n = 89)	61 (68.5)	28 (31.5)	
Stage III-IV (n = 156)	107 (68.6)	49 (31.4)	0.993
Lymph node status			
LNM (n = 177)	130 (73.4)	47 (26.6)	
No LNM (n = 68)	38 (55.9)	30 (44.1)	0.008*
Histological type			
DNKC (n = 222)	150 (74.9)	18 (25.1)	
UNKC (n = 23)	18 (80.0)	5 (20.0)	0.293
Survival status			
Alive (n = 154)	101 (65.6)	53 (34.4)	
Death (n = 91)	67 (73.6)	24 (26.4)	0.190

*Chi-square test, statistically significant difference ($P < 0.05$). DNKC, Differentiated non-keratinized carcinoma; UNKC, Undifferentiated non-keratinized carcinoma; LNM, lymph node metastasis.

expression of IRS-1 protein is an independent factor for lymph node metastasis of NPC, the multivariate logistic regression analysis was performed. The sensitivity, specificity, positive predictive value, negative predictive value and agreement rate of IRS-1 staining were hired to evaluate the validation of IRS-1 in predicting lymph node metastasis of NPC patients. All *P* values were based on the two-sided statistical analysis and *P*-value less than 0.05 was considered to be statistically significant.

Results

Association between expression of IRS-1 protein and clinicopathological features of NPC

We examined the positive expression and cellular location of IRS-1 in NPC and non-cancerous nasopharyngeal control tissues by IHC. Positive expression of IRS-1 protein was identified on the membrane and cytoplasm of NPC cells (**Figure 1A**, **1B**) and control nasopharyngeal epithelial cells (**Figure 1C**), also **Figure 1A** showed positive staining IRS-1 on the membrane and cytoplasm in a small subset of lymphocytes infiltrating NPC tissues.

The positive percentage of IRS-1 expression in the NPC and control nasopharyngeal epithelia were 68.6% (168/245) and 50.0% (15/30), respectively. There was significantly higher expression of IRS-1 protein in NPC compared to the non-cancerous nasopharyngeal control tissues ($P = 0.042$). We also analyzed the associations between the expression of IRS-1 protein and clinicopathological features of NPC including patients' age, gender, clinical stages, histological types, lymph node metastasis, and survival status by univariate Chi-Square Test. Date shown in **Table 1** indicated that NPC with lymph node metastasis presented significantly higher positive expression of IRS-1 ($P = 0.008$) than those without lymph node metastasis. However, no correlation was found between the expression of IRS-1 protein and age, gender, clinical stages, histological Types and survival status of NPC patients ($P > 0.05$, respectively).

Impact of expression of IRS-1 protein on the survival of NPC patients

To further evaluate the impact of expression of IRS-1 on the survival of NPC, we employed the Kaplan-Meier analysis to plot the survival curve of all 245 NPC patients, and statistical significance was assessed using the log-rank test.

Figure 2 illustrated the Kaplan-Meier survival plots for NPC patients with different expression of IRS-1 protein (**Figure 2A**). Univariate survival (long-rank test) analysis showed that there was no significant difference between the overall survival rate of NPC patients and expression of IRS-1 protein ($P > 0.05$). We also plotted the survival curve for NPC patients with the conventional prognostic parameters, including clinical stages, lymph nodal status and different therapy strategies. As shown in **Figure 2B-D**, the NPC patients with advanced stage NPC (stage III and IV), lymph node metastasis and radiotherapy/chemotherapy alone had lower overall survival than that patients with early stage NPC

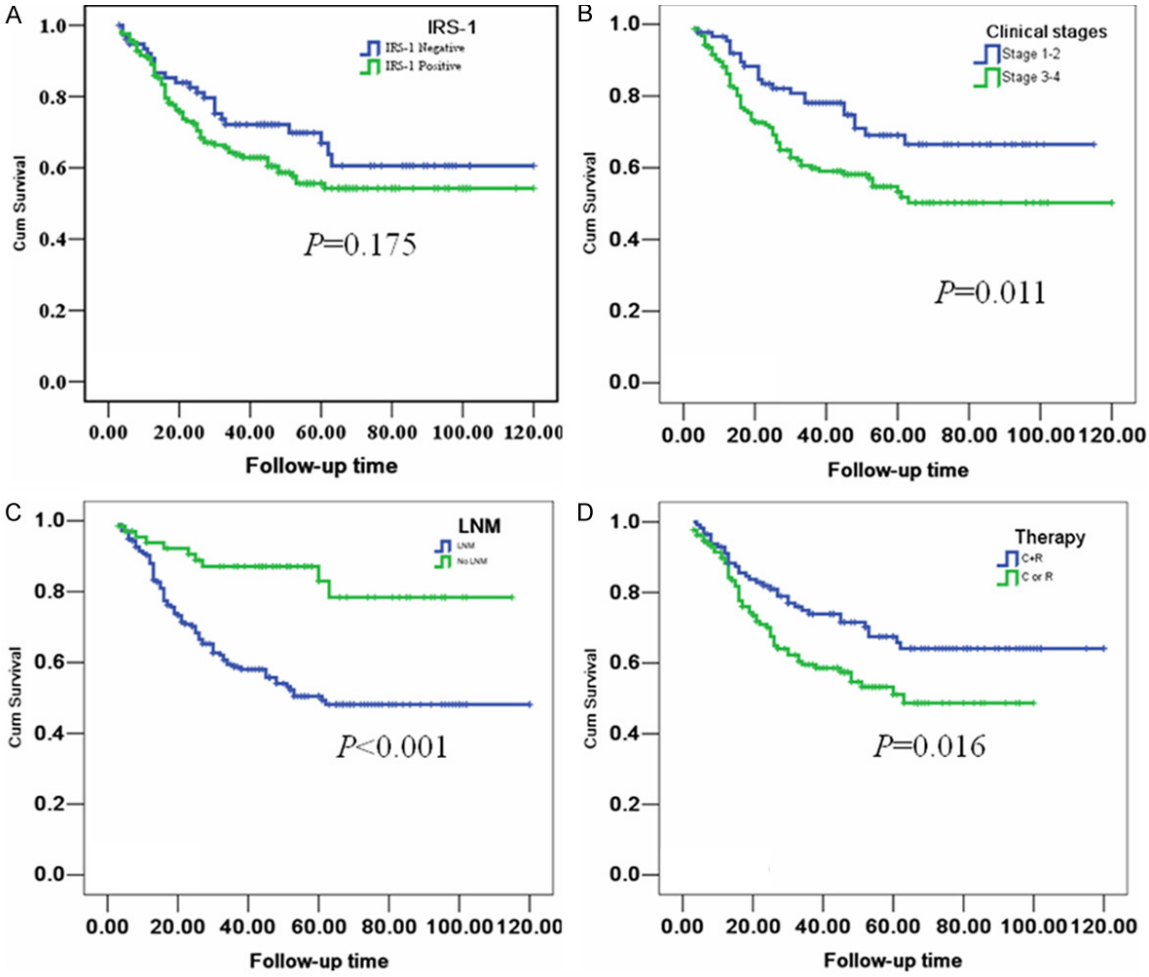

Figure 2. Kaplan-Meier overall survival curves of NPC patients with expression of IRS-1 and different clinicopathological characteristics. Kaplan-Meier analysis to plot the survival curve of 245 cases of NPC patients with expression of IRS-1 protein and clinicopathological characteristics and statistical significance were assessed using the log-rank test. A: Positive expression of IRS-1 had no significantly correlation with overall survival rates of NPC patients ($P > 0.05$, two sided). B: NPC patients with clinical stage III and IV were significantly related to poor prognosis compared to those patients with clinical stage I and II ($P = 0.011$, two sided). C: NPC patients with lymph node metastasis (LNM) were significantly related to poor prognosis compared to those patients without lymph node metastasis ($P < 0.001$, two sided). D: NPC patients with combination chemotherapy and radiotherapy were significantly related to good prognosis compared to patients with chemotherapy alone or radiotherapy alone ($P = 0.016$, two sided).

(stage I and II), without lymph node metastasis and with combination of radiation and chemotherapy ($P < 0.05$, **Figure 2B**, $P < 0.01$, **Figure 2C**, and $P < 0.05$, **Figure 2D**, respectively).

Assessment of IRS-1 protein as an independent factor predicting lymph node metastasis in NPC

Multivariate logistic regression analysis was carried out to further evaluate whether expression of IRS-1 was the independent predicted biomarker for the lymph node metastasis of NPC. As mentioned in the **Table 2**, the positive expression of IRS-1, clinical stages, and survival status were independent predicted parameters for lymph node metastasis in NPC, and positive expression of IRS-1 showed the significantly higher risk assessment of exp (β) = 2.049 (**Table 2**) ($P = 0.014$), which was compared with clinical stage and survival status of NPC. These results of multivariate analysis proved that high expression of IRS-1 was an independent factor for lymph node metastasis of NPC regardless of patient's clinical stages, age, gender and therapeutic regimens.

Validation of IRS-1 in predicting lymph node metastasis of patients with NPC

In order to evaluate the validation of IRS-1 in predicting lymph node metastasis of NPC

Table 2. Multivariate logistic regression analysis of lymph node metastasis factors in NPC patients (n = 245)

Variables	B	S.E.	Wald	Sig.	Exp (B)	95.0% CI for Exp (B)	
						Lower	Upper
Clinical stages	.713	.317	5.058	.025*	2.041	1.096	3.801
Histological patterns	-1.270	.498	6.516	.011*	.281	.106	.745
Survival status	-1.493	.389	14.746	.000*	.225	.105	.482
Age (years)	.292	.329	.788	.375	1.340	.702	2.555
Gender	-.396	.353	1.262	.261	.673	.337	1.343
IRS-1	.804	.326	6.063	.014*	2.234	1.178	4.234

95% CI, 95% confidence interval. *$P < 0.05$.

Table 3. Summary of statistical analysis of IRS-1 protein expression for the detection of NPC patients with lymph node metastasis

Sensitivity (n [%])	130/177 (73.4)
Specificity (n [%])	30/68 (44.1)
PPV (n [%])	130/168 (77.4)
NPV (n [%])	30/77 (39.0)
Agreement rate (n [%])	160/245 (65.3)
*P**	0.008

*Calculated using χ^2 test. PPV, positive predictive value; NPV, negative presictive value.

patients, the sensitivity (= true positive/[true positive + false negative]), specificity (= true negative/[false positive + true negative]), positive predictive value (= true positive/[true positive + false positive]), and negative predictive value (= true negative/[true negative + false negative]), agreement rate (= [true positive + true negative]/[true positive + false positive + true negative + false negative]) were hired to analyze the expression of IRS-1 protein in NPC. The results in **Table 3** showed that positive expression of IRS-1 protein was one predictive factor for lymph node metastasis of NPC with good sensitivity (73.4%). The positive predictive value and negative predictive value of IRS-1 for lymph node metastasis of NPC was 77.4% and 39.0%, respectively. The agreement rate of IRS-1 for the assessment of lymph node metastasis of NPC was more than 65.3%. Furthermore, there was statistically significant difference between the positive expression of IRS-1 in the NPC and status of lymph node metastasis ($P = 0.008$).

Discussion

IRS proteins are positioned to play a pivotal role in regulating the response of tumor cells to many different microenvironmental stimuli and regulating cancer cell survival, proliferation, and motility [15]. IRS-1, the first and most important (IRS) family member, is an adaptor protein that integrates multiple transmebrane signals from growth factors and hormones, to regulate cell growth, survival, differentiation and metabolism [16, 17]. IRS-1 is highly expressed in many cancers with constitutive stabilization of beta-catenin, such as colorectal carcinomas and ovarian endometrioid adenocarcinomas. Also, it has been proved that there is a higher expression of IRS-1 in metastatic rectal cancer tissues than in non-metastatic ones. There are growing body of data implicating IRS-1 as a critical signaling component in cancer development and progression and IRS-1 might be a novel target gene for tumor treatment [18, 19]. Overexpression of IRS-1 in human hepatocellular carcinoma cells prevents transforming growth factor β1-induced apoptosis [17]. High expression of IRS-1 is involved in breast ductal carcinoma in situ (DCIS), while IRS-2 is presented low expression in DCIS and increased its expression with an enhanced invasive properties of cancer cells, also, evidences are accumulated that IRS-1 and IRS-2 act as different roles in the development and progression of tumor, that is IRS-1 and IRS-2 can promote proliferation and metastasis, respectively, in breast cancer [15, 21]. Suppression of IRS-1 promotes mammary tumor metastasis, in which IRS-1 works as a monitor keeping on the movements of tumor cells [15]. However, another study shows that progression of breast cancer is accompanied by a reduction of IGF-IR/IRS-1 expression and that IGF-IR/IRS-1 expression inversely correlates with high proliferation rate in dedifferentiated breast cancers [21]. In addition, research discovers that IRS-1 may suppress tumor cells growing with the fact of loss expression of IRS-1 in early non-small cell lung cancer [22]. The ability of migration of

prostate cancer cells with positive expression of IRS-1 has decreased. Also, the IRS-1/IRS-2 ratio is lower in the prostate cancer and atypical hyperplasia than in the control normal prostate [23]. Our previous study showed that down-regulation of IRS-1 gene expression had a positive correlation with lymph node metastasis of squamous cell carcinoma of the head and neck in our previous research [24]. In the present study, there was significantly higher expression of IRS-1 protein in NPC compared to the non-cancerous nasopharyngeal control tissues. Also, NPC with lymph node metastasis presented significantly higher positive expression of IRS-1 than those without lymph node metastasis. All the studies indicate that IRS-1 protein plays an important role in the development and progression of tumor, while it displays different function in different tumors. The possible mechanism may be that IRS-1 participates in multiple signaling pathways, especially PI3K and Ras/ERK pathways, to regulate cell division, death, metabolism, growth, differentiation and variation. In addition, IRS-1 is involved in the interaction between EGFR and IGF-IR pathways via the regulation of epidermal growth factor (EGF) [17].

Tumor invasion and metastasis occurs by way of a complex series of sequential steps in which malignant cells first invade and occupy adjacent tissues (primary invasion) and penetrate into body cavities, the lymphatics and/or blood circulatory systems. As an important factor of poor prognosis, lymph node metastasis denotes a striking significance for NPC patients to get timely and effective diagnosis and treatment. In this study, our results indicated that expression of IRS-1 protein in NPC was significantly positive correlation with lymph node metastasis. We also found that high expression of IRS-1 was independent impact factor of lymph node metastasis after a multivariate logistic regression analysis was carried out. These results suggest that high expression of IRS-1 maybe play a critical role in promoting metastasis of NPC.

The sensitivity evaluates the ability of IRS-1 protein in diagnosis correctly with the lymph node metastasis of NPC, and the specificity evaluates its ability about judgment in NPC patients without lymph node metastasis. The agreement rate indicates the authenticity of IRS-1 protein for lymph node metastasis of NPC, the more the numerical is, the greater the authenticity is. The positive predictive value indicates the possibility of diagnosis of NPC with lymph node metastasis when the expression of IRS-1 protein is positive. The negative predictive value indicates the possibility of diagnosis of NPC without lymph node metastasis when the expression of IRS-1 protein is negative. In this study, our results showed higher sensitivity and agreement rate of IRS-1 for predicting lymph node metastasis of NPC patients. These results indicated that IRS-1 might act as a novel valuable biomarker for predicting lymph node metastasis in patients with NPC.

In summary, we first reported that high expression of IRS-1 was closely associated with lymph node metastasis in NPC and positive expression of IRS-1 could be used as an independent biomarker for predicting lymph node metastasis of NPC.

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Disclosure of conflict of interest

None.

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