

Original Article

Serum *IRS-1* acts as a novel biomarker for diagnosis in patients with nasopharyngeal carcinoma

Xiuxia Sun¹, Yongbao Chen², Jifang Tan³, Xuanchang Qi¹

¹Department of Otorhinolaryngology, Affiliated Hospital of Weifang Medical College, Weifang, Shandong, China; ²Department of Otorhinolaryngology, Yishui Central Hospital of Linyi, Shandong, China; ³Department of Otorhinolaryngology, Air force Jinan to Recruit Students in Flight Training, Jinan, Shandong, China

Received October 12, 2015; Accepted November 25, 2015; Epub July 1, 2018; Published July 15, 2018

Abstract: Background: Nasopharyngeal carcinoma (NPC) is a major head and neck cancer with high occurrence in Southeast Asia and southern China. Insulin receptor substrate 1 (*IRS-1*) plays an important role in the development, progression, invasion and metastasis of tumors. The purpose of this study was to evaluate whether *IRS-1* could be used as biomarkers for the diagnosis of NPC through measuring their expression and assess their relationship with clinical pathological factors. Methods: Quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) and Western blot were used to analyze the expression of *IRS-1* in 133 NPC patients and 104 healthy controls. The relationship between *IRS-1* expression and clinicopathological characteristics in NPC was estimated through chi-square test. We calculated diagnostic values of serum *IRS-1* expression by receiver operating characteristic (ROC) curve. Results: This study reports that *IRS-1* protein was weakly expressed in NPC specimens, but highly in healthy controls. Serum *IRS-1* were up-regulation in NPC patients compared with healthy controls. Their up-regulation was significantly correlated with lymph node status ($P=0.029$). Furthermore, the value of the area under the receiver-operating characteristic curve (AUC-ROC) was 0.907. The optimal cutoff value was 2.255, providing a sensitivity of 88.0% and a specificity of 77.9% in differentiating NPC patients from healthy controls. Conclusion: Our data indicates that serum *IRS-1* might increase the sensitivity and accuracy in diagnosis of NPC, and may be a potential target for diagnosis and gene therapy.

Keywords: Insulin receptor substrate-1, diagnosis, nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is characterized by peculiar epidemiologic and clinicopathologic features, affecting primarily middle-aged individuals [1]. NPC is a leading lethal malignancy that is most prevalent in Southeast Asia, especially in the Cantonese region of southern China [2, 3]. Despite growing incidence and awareness in the relevant populations, NPC is still characterized by diagnosed at a late stage, leading to a high mortality rate, usually through nasopharyngoscopy, a procedure that is subjective, skill-dependent, and expensive to maintain. The standard treatment for NPC is radiochemotherapy and 5-year survival rates have increased to approximately 60 to 70 % with the improvements in radiotherapy and chemotherapy regimens [4]. However, locoregional recurrence and distant metastasis following radio-

therapy still have deleterious effects on the survival rate of patients with NPC [5]. The clinical symptoms of NPC are usually nonspecific, but its location is somewhat special and examination of nasopharynx area requires expertise, which render early detection of NPC very difficult. To date, it remains a challenge to find efficient biomarkers for early detection/diagnosis and prognosis of this type of malignant disease.

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. Until now, four IRS proteins (*IRS-1* to *IRS-4*) have been identified [6]. 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 [7]. Recently, it is reported that *IRS-1* exhibits increased expression in variety of tumors, including hepatocellular, pancreatic, prostatic, breast, ovarian and colorectal cancers [8-14].

However, so far whether the alteration of the expression of *IRS-1* is associated with development and progression or clinicopathological/diagnostic implication for NPC has not been reported. In the present study we performed the *IRS-1* expression profiling in NPC serum compared with healthy controls using qRT-PCR and constructed the receiver operating characteristic (ROC) curves in order to identify whether specific *IRS-1* could discriminate between NPC patients and healthy controls.

Methods and materials

Patients and blood collection

All blood samples were collected from patients at Affiliated Hospital of Weifang Medical College (Fuzhou, China) between 2014 and 2015. A total of 133 patients with diagnosed NPC were recruited. Age, gender, diagnosis, clinical stage (stage I-stage IV), histology and lymph node status of these patients at diagnosis were recorded. The patients received a uniform protocol of image-guided intensity modulated radiotherapy (IMRT). A total of 104 healthy individuals served as controls. These were healthy volunteers without cancerous disease.

This study was approved by the institutional ethical review boards of Affiliated Hospital of Weifang Medical College, and written informed consent was obtained from all patients. All blood specimens were processed within 6 hr after blood withdrawal. Briefly, whole blood was drawn into EDTA-containing tubes and separated into plasma by centrifugation at 1,500 g for 10 min.

RNA preparation, reverse transcription, and quantitative real-time PCR

Total RNAs were extracted from blood samples using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Extracted RNA samples were reverse transcription to

cDNA as soon as possible, using an All-in-One First-Strand cDNA Synthesis Kit (Genecopoeia). Amplification of the appropriate product was confirmed by melting curve analysis following amplification. The *IRS-1* expression profile was quantified by TaqMan miRNA assays (Applied Biosystems), according to manufacturer's instructions. Relative expression of *IRS-1* was calculated using the comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control to normalize the data.

Protein extraction and Western blot analysis

For protein extraction, miRVana PARIS kit (Ambion) was used according to the manufacturer's instructions. Protein concentration was determined by the Bradford method using Coomassie brilliant blue (Biofer, Italy). Samples were separated on 12.5% SDS-PAGE gels and electrotransferred to PVDF membranes (Millipore). Membranes were blocked in 5% non-fat milk and incubated with a rabbit anti-*IRS-1* antibody (1:200, Boster, China) overnight at 4°C. The membranes were then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000, Boster, China) for 1 h at RT. Proteins of interest were detected and visualized by autoradiography after various exposure times. The *GAPDH* was used as internal control.

Statistical analysis

Graphical plotting was done using Origin Pro 9.0 software and statistical evaluations were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). All the above tests were unpaired and two-tailed. Receiver operating characteristic (ROC) curves were constructed, and the area under the ROC curve (AUC) was reported to assess the sensitivity and specificity. Chi-squared test was used to assess the correlation between the expression level of serum *IRS-1* and clinical pathological factors of NPC patients. *P* value <0.05 was considered to be statistically significant.

Results

Serum expression protein and mRNA level of IRS-1 in NPC and healthy controls

QRT-PCR and western blot analysis was utilized to investigate the *IRS-1* mRNA and protein

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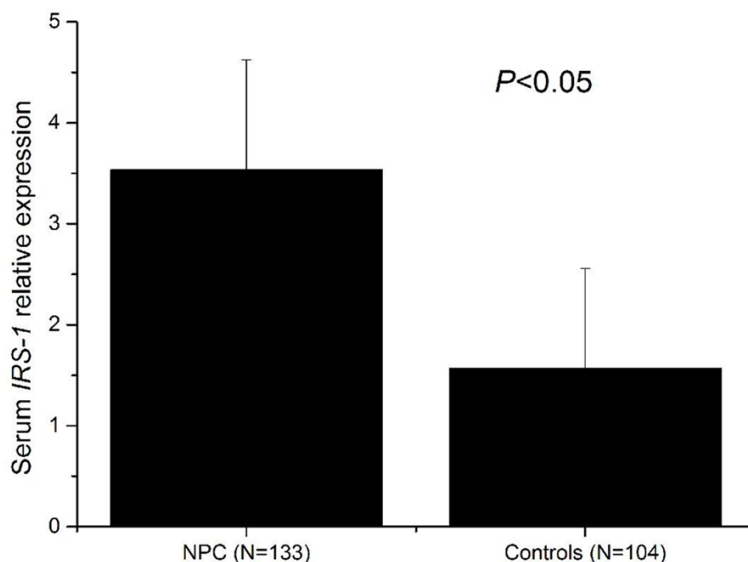


Figure 1. Serum *IRS-1* levels are up-regulated in NPC patients. qRT-PCR assay for the relative serum *IRS-1* expression to *GAPDH*, in NPC patients (n=133) and healthy volunteers (n=104).

Table 1. Differential protein expression of *IRS-1* between 133 NPC and 104 healthy controls

Group	No. of cases	<i>IRS-1</i> expression		χ^2	P values
		Negative	Positive		
NPC	133	44	89	47.000	0.000
Healthy controls	104	81	23		

expression in 133 cases of NPC serum and 104 healthy volunteer's blood. Using RNA isolated from serum, we performed RT-qPCR to detect the expression levels of *IRS-1* mRNA. Using *GAPDH* as normalization control, serum *IRS-1* expression was significantly higher in NPC compared with healthy controls ($P<0.05$, **Figure 1**).

Based on the above study, we further detected the expression of *IRS-1* at protein level by Western blot assays in NPC and healthy controls. Results revealed that 89 cases (66.9%) exhibited the positive *IRS-1* protein expression among all the 133 cases of NPC serum, whereas only 23 cases (22.1%) displayed the positive *IRS-1* protein expression in healthy volunteers blood (**Table 1**). Compared with healthy controls, there was significantly increase in *IRS-1* protein level in NPC ($P<0.05$), in line with the result of qRT-PCR. These results indicated that *IRS-1* might act as oncogene in NPC.

Relationship between *IRS-1* mRNA level and clinicopathological characteristics of NPC

Our investigation revealed that *IRS-1* protein expression was increased in NPC, which indicated that *IRS-1* might be carcinogenesis. Therefore, we further investigated the association of *IRS-1* expression with the clinicopathological characteristics of the patients to explore the potential role of *IRS-1* in NPC progression. The statistical analysis results, shown in **Table 2**, indicated that increased *IRS-1* protein expression was associated with lymph node status ($P=0.029$). However, there were no relationships with other features, including age, gender, clinical stages and pathology (all $P>0.05$).

Diagnostic value of *IRS-1* for NPC

The ROC curve was plotted to identify a cut-off value that could distinguish NPC from healthy controls. Analysis revealed that the area under the ROC curves (AUC) for serum *IRS-1* was 0.907 (95% confidence interval 0.870-0.944). At an optimal cut-off value of 2.255, the sensitivity and specificity was 88.0% and 77.9%, respectively (**Figure 2**).

Discussion

NPC is relatively rare on a global scale, but it is endemic in a few well-defined populations [15]. It occurs commonly in men, and in the productive age between 35-50 years. There are several host factors, including tobacco smoking, consumption of salt-preserved fish, and history of chronic respiratory tract diseases. In addition, Epstein-Barr virus (EBV) infection is another well-established risk factor for NPC [3, 16]. The majority of NPC patients have a variety of EBV antigens, and anti-EBV antibody serological testing has become an important tool for NPC diagnosis [17, 18]. Traditional assays of anti-EBV antibodies have been very useful in

Table 2. IRS-1 mRNA expression and clinicopathological features in NPC patients

Features	No. of cases (n=133)	IRS-1 expression		P values
		Low (n=54)	High (n=79)	
Age (years)				
<45	101	42	59	0.682
≥45	32	12	20	
Gender				
Male	94	41	53	0.272
Female	39	13	26	
Clinical stages				
I-II	46	21	25	0.388
III-IV	87	33	54	
Pathology				
Differentiated	102	45	57	0.134
Undifferentiated	31	9	22	
Lymph node status				
LNM	96	45	51	0.029
No LNM	37	9	26	

LNM: lymph node metastasis.

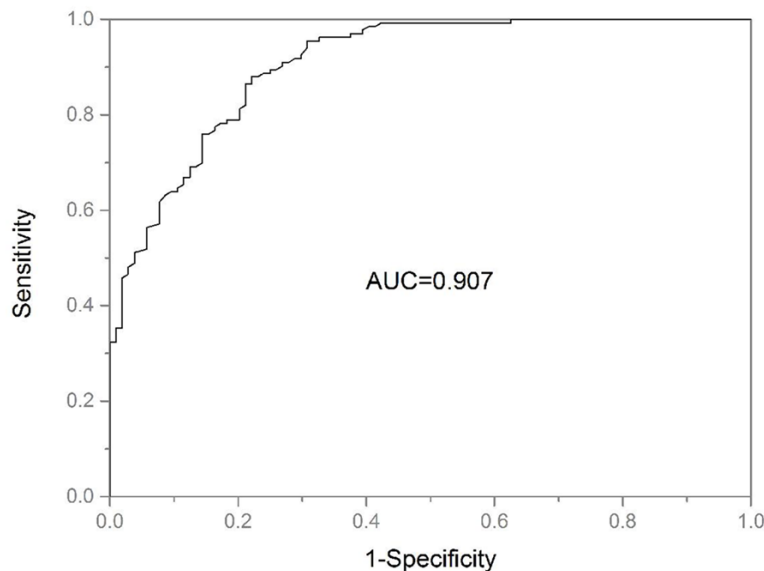


Figure 2. ROC analysis for evaluation of the accuracy of serum IRS-1 to discriminate patients with NPC from healthy controls. ROC: receiver operating characteristic, AUC: area under the curve.

clinical diagnosis of NPC [19]. However, EBV could proliferate in the lymphocytes and make the cells transform, and could long term subculture. The virus-infected cells have the genome of EBV and can produce various EBV-related antigens, such as EBV nuclear antigen (EBNA), early antigen (EA), membrane antigen (MA), viral capsid antigen (VCA), and lymphocyte

membrane antigen (LYDMA). To date, the high false positive rate of the screening biomarker and low sensitivity of the diagnosis biomarker make accurate early diagnosis of NPC difficult [20]. EBV DNA is considered to be a state-of-the-art quantitative blood biomarker in current NPC research. However, it has been reported that EBV DNA load is independent of serological parameters and does not reflect the number of intact tumor cells [21]. Therefore, investigation of the pathogenesis and identification of molecular markers of NPC may facilitate early diagnosis, prediction, and development of effective therapeutic strategies for NPC patients.

IRS proteins are positioned to play a pivotal role in regulating the response of tumor cells to many different micro-environmental stimuli and regulating cancer cell survival, proliferation, and motility [22]. IRS-1, the first and most important (IRS) family member, is highly expressed in many cancers. For example, Surmacz *et al.* reported that IRS-1 overexpression has been associated with tumor development, hormone independence and antioestrogen resistance in breast cancer [23]. Rocha *et al.* found a lack of correlation between IRS-1 expression and mitotic activity in cancer cells assessed by evaluation of the S-phase

fraction, but they suggested that higher IRS-1 levels enhance cancer growth and make earlier relapse possible [11]. IRS-1 is an estrogen-regulated gene frequently expressed in ER positive breast cancer cells [24] where it has been involved in anchorage-independent growth, cell survival [23, 25], and estrogen independent growth [26].

In the current study we compared the profile of *IRS-1* in serum from 133 NPC and 104 healthy controls and demonstrated that serum expression of *IRS-1* strongly differentiated the breast cancer patients from healthy controls. A highly significant increase was found in serum *IRS-1* of NPC compared with that of healthy individuals. Moreover, receiver operating curve analysis indicated that the AUC of *IRS-1* was 0.907 and the sensitivity and specificity at optimal cutoff being 88.0% and 77.9%, indicating that it might be potential biomarkers in the diagnosis of NPC Luo et al. revealed that the expression level of *IRS-1* was significant higher in NPC than that in the control nasopharyngeal epithelia [27]. Our results were consistent with previous studies.

We also analyzed the serum expression levels of *IRS-1* in relation to the different clinical pathologic characteristics in NPC patients. The result showed that serum *IRS-1* expression was relationship with lymph node status. However, there were no significant relationships between serum *IRS-1* expression and other features, including age, gender, clinical stages and pathology.

In the present study, the results showed the expression levels of *IRS-1* in NPC patients were higher than that of *IRS-1* in healthy controls. And the ROC results displayed significant diagnostic accuracy of *IRS-1*. The findings indicated serum *IRS-1* appeared to be potentially useful biomarkers for NPC detection. Further study with larger sample involving in validation and optimizing improvement should be conducted to confirm our results.

Disclosure of conflict of interest

None.

Address correspondence to: Xuanchang Qi, Department of Otorhinolaryngology, Affiliated Hospital of Weifang Medical College, Weifang 261000, Shandong, China. E-mail: xuchang14@126.com

References

[1] Li ZQ, Xia YF, Liu Q, Yi W, Liu XF, Han F, Luo W and Lu TX. Radiotherapy-related typing in 842 patients in canton with nasopharyngeal carcinoma. *Int J Radiat Oncol Biol Phys* 2006; 66: 1011-1016.

- [2] Wei WI and Sham JS. Nasopharyngeal carcinoma. *Lancet* 2005; 365: 2041-2054.
- [3] Chang ET and Adami HO. The enigmatic epidemiology of nasopharyngeal carcinoma. *Cancer Epidemiol Biomarkers Prev* 2006; 15: 1765-1777.
- [4] Zhang L, Zhao C, Ghimire B, Hong MH, Liu Q, Zhang Y, Guo Y, Huang YJ and Guan ZZ. The role of concurrent chemoradiotherapy in the treatment of locoregionally advanced nasopharyngeal carcinoma among endemic population: a meta-analysis of the phase III randomized trials. *BMC Cancer* 2010; 10: 558.
- [5] Lee AW, Poon YF, Foo W, Law SC, Cheung FK, Chan DK, Tung SY, Thaw M and Ho JH. Retrospective analysis of 5037 patients with nasopharyngeal carcinoma treated during 1976-1985: overall survival and patterns of failure. *Int J Radiat Oncol Biol Phys* 1992; 23: 261-270.
- [6] Lee YH and White MF. Insulin receptor substrate proteins and diabetes. *Arch Pharm Res* 2004; 27: 361-370.
- [7] Chan SH, Kikkawa U, Matsuzaki H, Chen JH and Chang WC. Insulin receptor substrate-1 prevents autophagy-dependent cell death caused by oxidative stress in mouse NIH/3T3 cells. *J Biomed Sci* 2012; 19: 64.
- [8] Bergmann U, Funatomi H, Kornmann M, Beger HG and Korc M. Increased expression of insulin receptor substrate-1 in human pancreatic cancer. *Biochem Biophys Res Commun* 1996; 220: 886-890.
- [9] Kornmann M, Maruyama H, Bergmann U, Tangvoranuntakul P, Beger HG, White MF and Korc M. Enhanced expression of the insulin receptor substrate-2 docking protein in human pancreatic cancer. *Cancer Res* 1998; 58: 4250-4254.
- [10] Hellawell GO, Turner GD, Davies DR, Poulson R, Brewster SF and Macaulay VM. Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease. *Cancer Res* 2002; 62: 2942-2950.
- [11] Rocha RL, Hilsenbeck SG, Jackson JG, VanDenBerg CL, Weng C, Lee AV and Yee D. Insulin-like growth factor binding protein-3 and insulin receptor substrate-1 in breast cancer: correlation with clinical parameters and disease-free survival. *Clin Cancer Res* 1997; 3: 103-109.
- [12] Koda M, Sulkowska M, Kanczuga-Koda L and Sulkowski S. Expression of insulin receptor substrate 1 in primary breast cancer and lymph node metastases. *J Clin Pathol* 2005; 58: 645-649.
- [13] Ravikumar S, Perez-Liz G, Del Vale L, Soprano DR and Soprano KJ. Insulin receptor sub-

IRS-1, a diagnostic biomarker of NPC

- strate-1 is an important mediator of ovarian cancer cell growth suppression by all-trans retinoic acid. *Cancer Res* 2007; 67: 9266-9275.
- [14] Esposito DL, Aru F, Lattanzio R, Morgano A, Abbondanza M, Malekzadeh R, Bishehsari F, Valanzano R, Russo A, Piantelli M, Moschetta A, Lotti LV and Mariani-Costantini R. The insulin receptor substrate 1 (IRS1) in intestinal epithelial differentiation and in colorectal cancer. *PLoS One* 2012; 7: e36190.
- [15] Parkin DM, Bray F, Ferlay J and Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; 55: 74-108.
- [16] O'Neil JD, Owen TJ, Wood VH, Date KL, Valentine R, Chukwuma MB, Arrand JR, Dawson CW and Young LS. Epstein-Barr virus-encoded EBNA1 modulates the AP-1 transcription factor pathway in nasopharyngeal carcinoma cells and enhances angiogenesis in vitro. *J Gen Virol* 2008; 89: 2833-2842.
- [17] Ai P, Wang T, Zhang H, Wang Y, Song C, Zhang L, Li Z and Hu H. Determination of antibodies directed at EBV proteins expressed in both latent and lytic cycles in nasopharyngeal carcinoma. *Oral Oncol* 2013; 49: 326-331.
- [18] Ng MH, Chan KH, Ng SP and Zong YS. Epstein-Barr virus serology in early detection and screening of nasopharyngeal carcinoma. *Ai Zheng* 2006; 25: 250-256.
- [19] Chang KP, Hsu CL, Chang YL, Tsang NM, Chen CK, Lee TJ, Tsao KC, Huang CG, Chang YS, Yu JS and Hao SP. Complementary serum test of antibodies to Epstein-Barr virus nuclear antigen-1 and early antigen: a possible alternative for primary screening of nasopharyngeal carcinoma. *Oral Oncol* 2008; 44: 784-792.
- [20] Tsang RK, Vlantis AC, Ho RW, Tam JS, To KF and van Hasselt CA. Sensitivity and specificity of Epstein-Barr virus IGA titer in the diagnosis of nasopharyngeal carcinoma: a three-year institutional review. *Head Neck* 2004; 26: 598-602.
- [21] Lin SY, Tsang NM, Kao SC, Hsieh YL, Chen YP, Tsai CS, Kuo TT, Hao SP, Chen IH and Hong JH. Presence of Epstein-Barr virus latent membrane protein 1 gene in the nasopharyngeal swabs from patients with nasopharyngeal carcinoma. *Head Neck* 2001; 23: 194-200.
- [22] Porter HA, Perry A, Kingsley C, Tran NL and Keegan AD. IRS1 is highly expressed in localized breast tumors and regulates the sensitivity of breast cancer cells to chemotherapy, while IRS2 is highly expressed in invasive breast tumors. *Cancer Lett* 2013; 338: 239-248.
- [23] Surmacz E. Function of the IGF-I receptor in breast cancer. *J Mammary Gland Biol Neoplasia* 2000; 5: 95-105.
- [24] Jackson JG, White MF and Yee D. Insulin receptor substrate-1 is the predominant signaling molecule activated by insulin-like growth factor-I, insulin, and interleukin-4 in estrogen receptor-positive human breast cancer cells. *J Biol Chem* 1998; 273: 9994-10003.
- [25] Nolan MK, Jankowska L, Prisco M, Xu S, Guvakova MA and Surmacz E. Differential roles of IRS-1 and SHC signaling pathways in breast cancer cells. *Int J Cancer* 1997; 72: 828-834.
- [26] Surmacz E and Burgaud JL. Overexpression of insulin receptor substrate 1 (IRS-1) in the human breast cancer cell line MCF-7 induces loss of estrogen requirements for growth and transformation. *Clin Cancer Res* 1995; 1: 1429-1436.
- [27] Luo J, Wen Q, Li J, Xu L, Chu S, Wang W, Shi L, Xie G, Huang D and Fan S. Increased expression of IRS-1 is associated with lymph node metastasis in nasopharyngeal carcinoma. *Int J Clin Exp Pathol* 2014; 7: 6117-6124.