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Original Article

Relationship between dysregulation of STAT4 and clinicopathological significance in nasopharyngeal carcinoma

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Abstract: Background: Aberrant expression of STAT4 has been validated in different cancers. However, its role in nasopharyngeal carcinoma (NPC) remains unidentified. The purpose of the current study was to investigate the clinical significance of STAT4 and its potential role in the carcinogenesis and deterioration of NPC. Materials and Methods: Expression of STAT4, as well as EGFR and P53 in 393 NPC patients and 54 benign nasopharyngeal diseases was detected by immunohistochemistry. Meanwhile, the correlation of STAT4 expression with a variety of clinicopathological features was analyzed. Results: The expression rate of STAT4 was significantly higher in NPC tissues (78.6%) than non-cancerous nasopharyngeal tissues (27.8%, $Z = -7.836$, $P < 0.001$). The area under curve (AUC) of STAT4 was 0.712 (95% CI: 0.611-0.813, $P < 0.001$) to diagnose squamous cell carcinoma of NPC. Expression of STAT4 was positively correlated to tumor infiltration ($r = 0.392$, $P < 0.001$), lymphatic metastasis ($r = 0.289$, $P = 0.002$), TNM clinical stage ($r = 0.345$, $P < 0.001$), EGFR ($r = 0.267$, $P = 0.001$) and P53 ($r = 0.378$, $P < 0.001$). Conclusion: STAT4 can be regarded as a potential diagnostic biomarker for NPC. It might be involved in the carcinogenesis and deterioration of NPC.

Keywords: STAT4, NPC, IHC, tumor infiltration, metastasis, clinical TNM stage

Introduction

Nasopharyngeal carcinoma (NPC), occurring predominantly in the Eustachian tube and fossa of Rosenmüller [1], is a highly metastatic cancer that arises from the squamous epithelial cells which cover the surface of nasopharynx [2]. It differs significantly from other head and neck malignancies in its epidemiology, etiology, pathology, clinical manifestation and treatment. NPC is endemic in Southeast of Asia, Southern China, North Africa, and Alaska [3]. Take China for instance, the annual incidence of NPC is about 80 cases per 100,000 [4]. The etiology of NPC involves miscellaneous factors, such as ethnics, genetic susceptibility, exposure to chemical carcinogens, Epstein-Barr virus (EBV) infection [5, 6]. According to the World Health Organization (WHO) classification, the pathological type of NPC can be divided into type I keratinizing squamous cell carcinoma (well differentiated), type II non-keratinizing squamous cell carcinoma (moderately differentiated), and type III undifferentiated carcinoma (poorly differentiated) [7]. In clinic, NPC is diagnosed and staged via biopsy together with computed tomography (CT) and positron emission tomography (PET) [2]. According to the National Comprehensive Cancer Network (NCCN) guidelines, the standard treatments for NPC are radiotherapy or radiochemotherapy rather than surgical resection [8, 9]. While NPC tends to be much more sensitive to radiation compared with other cancers, the curative effects depend mostly on the tumor stage, however, most NPC patients are in the advanced stage when diagnosed as the primary lesion lies in the silent painless area [10]. The 5-year survival rate for stage I and II NPC patients ranges from 72 to 90%. However, the 5-year survival rate for stages III and IV NPC patients drops to around 55% and 30%, respectively, due to a relatively higher potential of locoregional recurrence or metastasis [2, 11]. Therefore, it arouses our interest to

Table 1. Relationship between STAT4 expression and clinicopathological parameters

Parameters	Total (n)	Expression of STAT4 n (%)		Z	*P*
		Negative	Positive		
Tissue					
Normal tissue	54	39 (72.2%)	15 (27.8%)	-7.836	< 0.001
NPC	393	84 (21.4%)	309 (78.6%)		
Histological type					
Adenocarcinoma	4	0	4 (100%)	-1.047	0.295
Squamous carcinoma	389	84 (21.6%)	305 (78.4%)		
Squamous carcinoma					
Differentiated	250	62 (24.8%)	188 (75.2%)	-2.058	0.040
Undifferentiated	139	22 (15.8%)	117 (84.2%)		
Gender					
Female	121	27 (22.3%)	94 (77.7%)	-0.303	0.762
Male	272	57 (21.0%)	215 (79.0%)		
Age					
≤ 40	89	14 (15.7%)	75 (84.3%)	-1.475	0.140
> 40	304	70 (23.0%)	234 (77.0%)		
Tumor infiltration					
T1	30	14 (46.7%)	16 (53.3%)	H = 17.682[a]	0.001
T2	32	10 (31.2%)	22 (68.8%)		
T3	27	2 (7.4%)	25 (92.6%)		
T4	22	1 (4.5%)	21 (95.5%)		
Tumor infiltration					
T1-T2	62	24 (38.7%)	38 (61.3%)	-3.956	< 0.001
T3-T4	49	3 (6.1%)	46 (93.9%)		
Lymphatic metastasis					
N0	6	2 (33.3%)	4 (66.7%)	H = 11.371[a]	0.010
N1	22	11 (50%)	11 (50%)		
N2	62	12 (19.4%)	50 (80.6%)		
N3	21	2 (9.5%)	19 (90.5%)		
Lymphatic metastasis					
N0-N1	28	13 (46.4%)	15 (53.6%)	-3.138	0.002
N2-N3	83	14 (16.9%)	69 (83.1%)		
TNM clinical stage					
I	6	2 (33.3%)	4 (66.7%)	H = 14.608[a]	0.002
II	22	11 (50.0%)	11 (50.0%)		
III	48	12 (25.0%)	36 (75.0%)		
IV	35	2 (5.7%)	33 (94.3%)		
TNM clinical stage					
I/II	28	13 (46.4%)	15(53.6%)	-3.138	0.002
III/IV	83	14 (16.9%)	69 (83.1%)		
EGFR					
-	14	5(35.7%)	9 (64.3%)	H = 10.376[a]	0.016
+	40	9 (22.5%)	31 (77.5%)		
++	19	9 (47.4%)	10 (52.6%)		
+++	38	4 (10.5%)	34 (89.5%)		
P53					
-	12	3 (25.0%)	9 (75.0%)	H = 11.003[a]	0.012
+	36	14 (38.9%)	22 (61.1%)		
++	34	9 (26.5%)	25 (73.5%)		
+++	29	1 (3.4%)	28 (96.6%)		

[a]Kruskal-Wallis test was performed to analyze the difference. Mann-Whitney U test was used in the rest of statistics.

find novel robust diagnostic biomarkers to diagnose NPC in an early stage.

Signal transducers and activators of transcription (STATs) have influence on interacting tumor cells with their immune microenvironment by suppressing or inducing the specific cytokines and growth factors [12]. Dysregulation of STAT4 has been implicated to play a role in the pathogenesis for a variety of cancers, including cutaneous T-cell lymphoma (CTCL) [13], colorectal cancer [14] and hepatocellular carcinoma [15]. However, up till now, no published study has focused on the relationship of STAT4 and NPC. Hence, we primarily concentrate on studying the association between the expression of STAT4 and other clinicopathological parameters in NPC patients in our current study.

Material and methods

Patients and tissue samples

A total number of 447 patients (142 females and 305 males, aged from 18 to 85 with a mean age of 59.6) were recruited in the current study. These patients were admitted to the First Affiliated Hospital of Guangxi Medical University (Nanning Guangxi, China) between January, 2011 and December, 2013. A total of 447 tissues were collected from each patient. Among them, 393 cases

were NPC tissues and were identified as retrievable biopsy tissues, either from metastatic nodes or from primary sites. Fifty-four were non-cancerous nasopharyngeal tissues, collected from those who were diagnosed with rhinopolyp or chronic nasopharyngitis. Moreover, among the 393 NPC patients, those 111 individuals without distant metastasis, their primary tumor tissues along with the regional lymph nodes were further staged by the seventh edition of UICC Staging System for NPC. As a result, 6 cases (5.4%) were classified as stage I, 22 cases (19.8%) as stage II, 48 cases (43.2%) as stage III and 35 cases (31.5%) as stage IV. All of the 447 samples were preserved in paraffin blocks and were assembled for tissue microarrays (TMAs). The clinicopathological parameters of these NPC patients were summarized in **Table 1**. The current study was approved by The Ethical Committee of First Affiliated Hospital of Guangxi Medical University, China. Written informed consents had been obtained from all participating patients.

Immunohistochemical staining and scores

The immunohistochemical staining (IHC) method was performed on the formalin-fixed, paraffin embedded (FFPE) 4-μm-thick tissue samples to detect the expression of STAT4. Deparaffinization of all tissues was performed with a series of xylene baths. Rehydration was conducted, with a range of alcohol solutions of graded concentrations. As for immune-staining, we used the following primary antibodies: mouse monoclonal antihuman STAT4 antibody (Santa Cruz Biotech Company, PL-68, CA, USA, 1:300 dilution); mouse monoclonal anti-EGFR (Beijing Jinqiao Biological Co. LTD.) and mouse monoclonal anti-P53 (Beijing Jinqiao Biological Co. LTD.). A standard detection by means of avidin-biotin immunoperoxidase complexes was performed as previously described [16, 17]. After the immunodetection, all the tissues were reviewed by two independent pathologists without knowing any clinical information of patients. Percentage of positive cells was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), 3 (strong). The final score of each sample was calculated by the multiplication of the positive cell score and the intensity score. It would be considered as STAT4 positive if the final score was more than 2. Expression of EGFR and P53 was calculated with the formula which was number of positive cells/total number of the cells × 100%. We counted at least 10 random representative fields at high magnification (40 × 40) and the results were recorded as- (negative staining), + (weak staining), ++ (moderate staining) and +++ (strong staining) [18].

Statistical analysis

Statistical analysis was performed with SPSS 20.0. In the study, Kruskal-Wallis test (two-independent-samples test) and Mann-Whitney U test (test for several independent samples) were used to analyze the difference of STAT4 expression with clinicopathological parameters of NPC. Effectiveness of STAT4, EGFR and p53 to diagnose NPC was generated by receiver operating characteristic (ROC) curves. For all the statistic results, *P*-value less than 0.05 (two-tailed test) was significant.

Results

Expression of STAT4 in NPC samples

Among the 393 NPC tissue samples, 309 samples (78.6%) showed positive expression of STAT4 while only 15 cases (27.8%) in the 54 normal tissues were STAT4 positive. By statistical analysis, there was a significant difference between normal tissues and NPC tissues in the expression of STAT4 (Z = -7.836, P < 0.001, **Figure 1**). No relationship was found between histological type and STAT4 expression (Z = -1.047, P = 0.295). However, it showed significant difference between the differentiated and undifferentiated squamous cell carcinoma (Z = -2.058, P = 0.040) as 188 (75.2%) of the 250 differentiated squamous cell carcinoma showed positive STAT4 expression, meanwhile, 117 (84.2%) of the 139 undifferentiated squamous cell carcinoma showed positive STAT4 expression. In addition, ROC curve was performed, and diagnostic value of STAT4 was found in squamous cell carcinoma of NPC with the area under the curve (AUC) of 0.712 (95% CI: 0.611-0.813, P < 0.001), in NPC infiltration (T3-4) with the AUC of 0.663 (95% CI: 0.563-0.763, P = 0.003), in lymphatic metastasis with the AUC of 0.648 (95% CI: 0.522-0.773, P = 0.020), and in T3-T4 TNM stage with the AUC of STAT4 was 0.648 (95% CI: 0.522-0.733, P = 0.020).

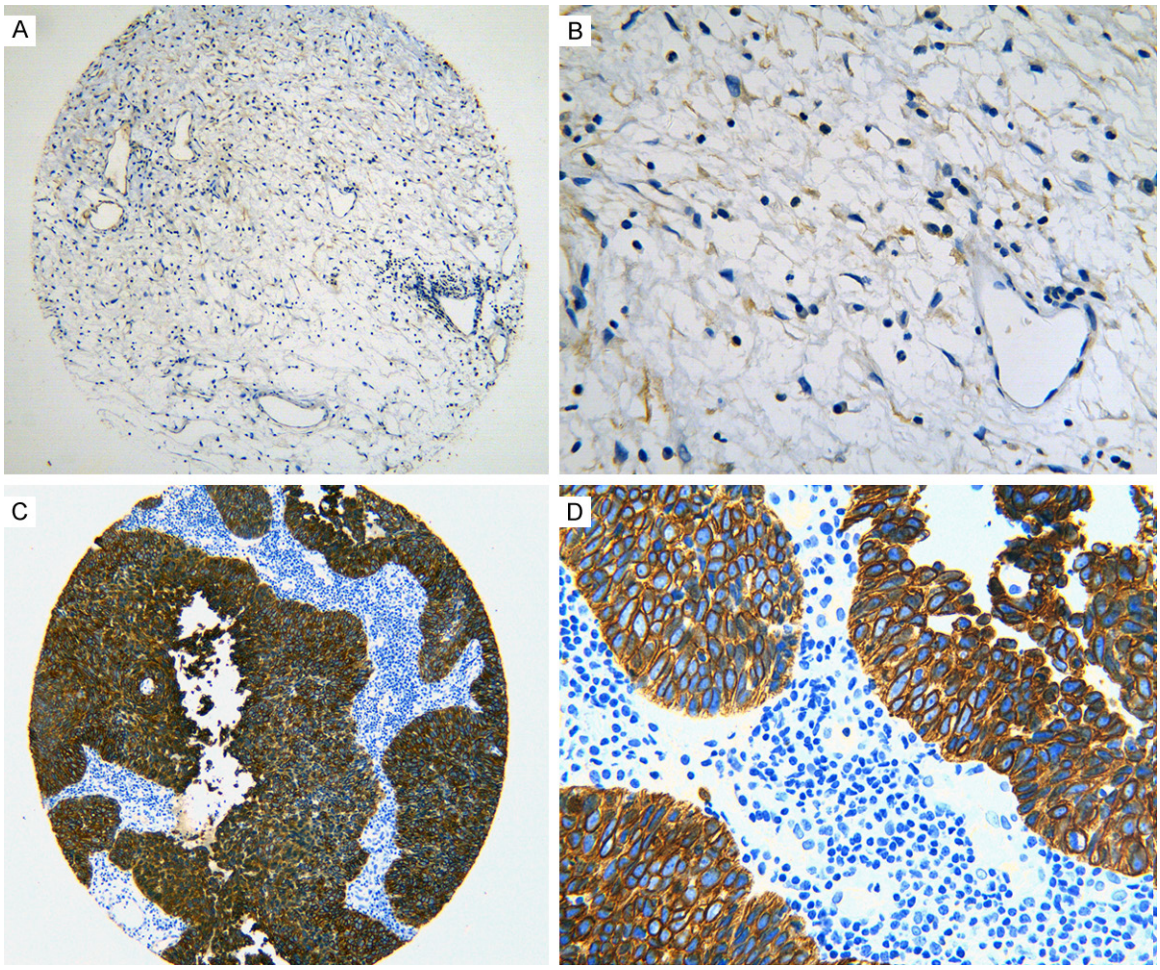

Figure 1. Expression of STAT4 in normal nasopharyngeal tissues and NPC tissues. IHC was performed to detect the expression of STAT4. There were sporadically positive cells in part of the interstitial fibrous tissues while the expression of STAT4 was negative in epithelium of non-cancerous nasopharyngeal tissues (A × 100, B × 400). The expression of STAT4 was strongly positive in NPC tissues. Positive signal can be found in cytoplasm and cytomembrane (C × 100, D × 400).

Table 2. Spearman correlation of Stat4 and clinicopathological parameters in NPC

	r	*P*
Gender	-0.027	0.573
Age	0.051	0.278
Tumor infiltration (T)	0.392	< 0.001
Lymphatic metastasis (N)	0.289	0.002
TNM clinical stage	0.345	< 0.001
Histological type	-0.053	0.296
EGFR	0.267	0.001
P53	0.378	< 0.001

Relationship between STAT4 and other clinicopathological features

Varies of potential parameters in clinic were selected to investigate the relationship between the aberrant expression of STAT4 and clinical NPC outcomes. As shown in **Table 1**, STAT4 expression did not vary significantly with gender (Z = -0.303, P = 0.762) or age (Z = -1.475, P = 0.140). Positive rate of STAT4 was perceptibly increased as the tumor infiltration degree aggravated (H = 17.682, P = 0.001). NPC patients in T3-T4 infiltration stage (93.9%, 46/49) showed a remarkably higher level of STAT4 than those in T1-T2 (61.3%, 38/62, P < 0.001). Lymphatic metastasis stage was correlated with the expression of STAT4 (H = 11.371, P = 0.010). Moreover, according to N category, a notably higher level of STAT4 emerged among patients in N2 and N3 (83.1%, 69/83) than that in N0 and N1 (53.6%, 15/28, P = 0.002). As for NPC patients in their advanced clinical stage, STAT4 demonstrated a stronger expression (H = 14.608, P = 0.002). Compared to those in early

stage (stage I and II, 53.6%, 15/28), STAT4 of those in advanced stage (stage III and IV, 83.1%, 69/83, $P = 0.002$) showed a considerably higher expression level.

Relationships between STAT4 and other biochemical indicators in NPC

The ratio of positive STAT4 expression was 77.3% (75/97) in the positive group of EGFR, significantly higher than the negative group of EGFR (64.3%, 9/14, $P = 0.016$). Comparing the four sequential group, patients with higher EGFR expression exhibited remarkably stronger positive rate of STAT4 ($H = 10.376^a$, $P = 0.016$). It was similar with P53 that STAT4 was significantly higher in the P53- positive group (75.8%, 75/99) than in the P53-negative group (75%, 9/12, $P = 0.012$). As P53 expression level increased, the positive rate of STAT4 went higher as well ($H = 11.003$, $P = 0.012$). In addition, spearman analysis indicated that EGFR was positively correlated with P53 ($r = 0.780$, $P < 0.001$) **Table 2**.

Spearman correlation of STAT4 and other clinicopathological features

STAT4 expression was associated with the following clinical features: tumor infiltration ($r = 0.392$, $P < 0.001$), lymphatic metastasis ($r = 0.289$, $P = 0.002$), TNM clinical stage ($r = 0.345$, $P < 0.001$), EGFR expression ($r = 0.267$, $P = 0.001$) and P53 expression ($r = 0.378$, $P < 0.001$). No correlation was found between STAT4 and gender, age and histological type. The above results were listed in **Table 2**.

Discussion

As a member of STAT family, STAT4 is involved in transmitting signals and plays a role in regulating various biological responses, including cell development, differentiation, proliferation and survival [19, 20]. Previous studies have casted light on the relationship between STAT4 and some diseases. Dobrian et al validated that the deletion of STAT4 reduced inflammation in peri-vascular and visceral adipose tissue [21]. Netchiporouk et al revealed in their study that constitutive activation of STAT (including STAT3, STAT4 and STAT5) was observed in cutaneous T-cell lymphoma (CTCL) [13]. Litvinov et al concluded that loss of STAT4 expression was associated with the acquisition of Th2 cell phenotype and could be a vital prognostic marker for CTCL progression [22]. Aavikko et al suggested in their study that STAT4 was a potential classic Kaposi sarcoma (cKS) predisposition gene by whole-genome sequencing [23]. Cheng et al indicated that STAT4 might be a potential target for the treatment of colorectal cancer (CRC) as it was positively related with the depth of CRC invasion [14]. Wang et al proposed that decreased STAT4 enhanced cell proliferation and indicated poor prognosis in hepatocellular carcinoma [24]. Thus, we hypothesized that STAT4 might play a role in carcinogenesis in various malignancies. Chen et al detected activated nuclear STAT4 in the NPC cell line HK666 and EBV positive cell line Rael [25]. However, no other publication was available with respect to the relationship between STAT4 and NPC. Therefore, we conducted the current research to scrutinize the expression of STAT4 in NPC patients and validate the correlation between STAT4 and NPC progression.

According to our results, an increased expression level of STAT4 was observed in NPC tissues compared to the non-cancerous tissues taken from benign nasopharyngeal disease patients. Moreover, non-keratinizing undifferentiated carcinoma represented the major pathological type of NPC in southern China [26]. Samples in our current study was also taken from patients in southern China, and statistical analysis showed an overexpression of STAT4 in undifferentiated group compared with differentiated group, which suggested that STAT4 might be a risk factor for Chinese ethnics.

The entire study cohort included both adenocarcinoma and squamous cell carcinoma of NPC, but no significant difference was found of the STAT4 expression between different histological types. This may due to the limited sample scale (only 4 cases of adenocarcinoma), a further study of larger samples and subgroup analysis for adenocarcinoma was needed. Significant variance of STAT4 was found with regard to tumor infiltration, lymphatic metastasis and TNM clinical stage yet. Additionally, ROC curve was performed to validate the diagnostic role of STAT4 in histological type of NPC, NPC infiltration, lymphatic metastasis, as well as TNM stage. These results suggested that STAT4 played a vital role in the progression of NPC and the STAT4 expression level of nasopharyn-

geal tissue could become a diagnostic biomarker in NPC. Netkiporouk et al found that STAT4 signaling was critical for T helper 1 (Th1) phenotype differentiation, and the loss of STAT4 resulted in a switch from Th1 to Th2 in malignant T cells [13]. As being reported by Möhnle et al, T cell functions must be tightly controlled in order to keep the balance between proinflammatory activity and detrimental overactivation [27]. Thus, we held the belief that detecting the expression level of STAT4 can assist to diagnose NPC and its progression as we found statistical significance in the ROC curve.

The epidermal growth factor receptor (EGFR), encoded by c-erbB-1 proto- oncogene, is one of the ErbB protein tyrosine kinase receptor family member. EGFR is involved in several carcinomas for its role in the regulation of cellular growth, metabolism, differentiation and migration [28]. Zhang et al found the expression rate of EGFR in NPC was 89.5%, and its expression indicated poor prognosis in NPC patients [28]. Ma et al concluded that EGFR was involved in the development of NPC and played a role as a prognostic biomarker for NPC in his meta-analysis [29]. In our cohort, the expression of STAT4 showed a consistent association with the accumulation of EGFR, which suggested that STAT4 was closely related to EGFR and may somehow be connected to the prognosis of NPC patients. Previous studies also revealed that P53, a tumor suppressive gene, could activate the promoter of EGFR [30, 31], and P53 was closely related to the carcinogenesis of NPC [32]. In our current study, positive P53 expression was correlated to the positive expression of STAT4, which also indicated that STAT4 played a role in NPC development. Above all, there might be a signaling pathway which link EGFR, P53 and STAT4 together and their expression may affect each other. As a result, deregulation of STAT4 may have influence on the carcinogenesis of NPC. However, further researches are needed to confirm the hypothesis.

Based on the conclusion drew by other researchers and the results of our own study, we concluded that STAT4 might be a potential biomarker to diagnose NPC, and its overexpression could indicate deterioration of NPC and may lead to dismal outcomes for NPC patients. Further studies with larger scale of patients and experiments *in vitro* and *in vivo* are needed to investigate the potential mechanisms of STAT4.

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Disclosure of conflict of interest

None.

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