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ORIGINAL ARTICLE

The Infiltration of ICOS$^+$ Cells in Nasopharyngeal Carcinoma is Beneficial for Improved Prognosis

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Abstract

Nasopharyngeal carcinoma (NPC) is a highly malignant tumor, associated with poor patient prognoses, and high rates of morbidity and mortality. Currently, immune checkpoint therapy has brought new treatment strategy for NPC. The inducible T cell co-stimulator (ICOS) belongs to the B7-CD28 immunoglobulin superfamily, which is currently the subject of intense study due to great successes gained in treatment of different malignancies by disrupting their family members. However, the role of ICOS played in NPC remains poorly understood. Immunohistochemistry (IHC) was stained with the ICOS specific antibody and ICOS expression is decreased in patients with either lymphatic or distant metastasis and inversely associated with TNM stage of NPC patients. Importantly, high ICOS expression is significantly correlated with overall survival (OS) of NPC patients ($N = 185$, $p < 0.001$), and ICOS expression is also proved to be an independent prognostic factor by multivariate analysis. Surgical excised fresh NPC specimens ($N = 185$) were homogenized to analyze the specific cytokine expression by ELISA assay. ICOS expression level is associated with increased cytotoxic T lymphocyte number and high interferon IFNγ expression, the characteristics of Th1 cells. In addition, the correlation between the percentage of ICOS$^+$ T cells in tumor tissue and survival was detected. Conclusively, expression of ICOS is associated with improved survival in NPC and percentage of ICOS$^+$ cells acting as Th1 cells in primary tumor tissue may be a clinical biomarker for good prognosis of NPC patients.

Keywords Nasopharyngeal carcinoma · Survival · Inducible T cell co-stimulator · IFNγ · Prognosis

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic malignancy in southern China, with a peak annual incidence approaching 30 per 100,000 persons [1]. More than 70% of patients with newly diagnosed NPC are classified as having locoregionally advanced disease [2]. With the advent of concurrent chemo-radiotherapy, intensity-modulated radiotherapy (IMRT), and imaging techniques, locoregional control has substantially improved and distant metastasis is now the main source of treatment failure for NPC [3]. Although several biomarkers were identified for evaluating the prognosis of recurrent NPC, the overall survival (OS) rate of patients is still not improved with 5-year OS rate being only 30% [4, 5], making the management of recurrent NPC being a big challenge in clinic [6, 7]. Therefore, seeking reliable prognostic markers as well as effective treatments are urgently required.

B7-CD28 family mediates the critically bidirectional signals on regulation of T cell function: positive second signals that promote and sustain T cell responses and negative second signals that regulate T cell tolerance [8, 9]. The significance of this family was highlighted by the FDA approval of ipilimumab, an antibody blocking CTLA-4, for cancer in 2011, which was reinforced by successes achieved by targeting CD28 family member PD-1 in different malignancies [10].

The CD28 family member ICOS, which interacts with a ligand ICOSL, was first reported on activated human T cells [11]. This pathway can promote T cell production of several cytokines including IL-10, IL-4, IL-5, IFNγ and IL-17, depending on which cell type the effect dominates [9]. A recent study shows that the ICOS mediates interaction between tumor-infiltrating CD4$^+$ T cells and plasmacytoid dendritic cells (pDCs), which leads to the amplification of regulatory T cells (Tregs) and interleukin-10 secretion, then Tregs and pDCs that infiltrate primary breast tumors impair patient

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survival [12, 13]. However, what is the role of ICOS signal in NPC has not been determined.

In this study, we utilized hundreds of NPC tumor sections to investigate the expression of ICOS in NPC and its clinical significance. The expression pattern and function of ICOS signal were further examined in surgical excised tumor tissue of NPC patients. We demonstrated that ICOS mainly expresses on Th1 cells and $ICOS^+$ cell correlates with beneficial prognosis of NPC patients.

Materials and Methods

Patients and Samples

This study was approved by the independent ethics committee, Tianjin Medical University General Hospital. Consent was waived and patient records were deidentified and anonymized prior to analysis.

For this study, 270 NPC patients were consecutively sampled by our hospital from March 2012 to May 2015. Patients were selected based on the following criteria: 1) histologically proven locoregionally advanced NPC with available biopsy specimens; 2) Karnofsky score ≥ 70; 3) receiving radical IMRT and concurrent cisplatin-based chemotherapy at initial diagnosis; 4) no previous malignancy or other concomitant malignant disease; and 5) ICOS staining was detectable in the tumor tissues. Therefore, there were 185 patients who qualified for this study. All patients underwent disease staging using the American Joint Committee on Cancer (AJCC) 2010 staging system. The clinical characteristics are listed in Table 1. All the enrolled patients received similar treatment strategies, which were IMRT combined with cisplatin-based chemotherapy. The biopsy specimens were obtained by nasal endoscopy for pathological analysis.

Table 1 Patient characteristics and significance of ICOS expression in clinical parameters

Characteristic	Cytomembrane high group ($n = 81$)	Cytomembrane low group ($n = 104$)	*P* value
Sex			0.638
Male	56	68	
Female	25	36	
Age			0.275
≧50	49	72	
<50	32	32	
BMI (kg/m^2)			0.881
≧23	33	41	
<23	48	63	
WHO histologic type			0.624
Differentiated	39	51	
Undifferentiated	42	53	
EBV infection			0.423
Positive	59	69	
Negative	22	35	
T classification			0.001
T1+ T2	53	42	
T3+ T4	28	62	
Lymph node metastasis			P < 0.001
Absent	56	37	
Present	25	67	
Distant metastasis			0.001
Absent	73	73	
Present	8	31	
Overall stage			P < 0.001
I + II	57	43	
III + IV	24	61	

Abbreviation: *BMI* body mass index, *EBV* Epstein-Barr virus

Immunohistochemistry (IHC) Staining

Immunohistochemical staining of 5 μm sections from formalin-fixed paraffin-embedded nasopharyngeal biopsies specimens were performed in the Department of Pathology of our hospital with the antibody 1:200 anti-ICOS (abcam, Danvers, MA, USA) using the standard protocol for routine diagnostic specimens. Hematoxylin and eosin sections were also reviewed for the presence of tumors. ICOS expression was evaluated according to the extent and intensity of staining: the percentage of tumor cells with cytomembranic positivity (0, ≤5%; 1, 6 to ≤15%; 2, 16 to ≤30%; 3, 31 to ≤50%; 4, >50%) was added with the intensity of staining (0, negative; 1, weak; 2, moderate; 3, strong), and a final score was created to determine the cut-off value for low and high expression group by using grades of the extent × grades of intensity staining. Then, the protein expression was sorted into four categories: "-" for a score of 0–3, "+" for a score of 4–6, "++" for a score of 7–9 and "+++" for a score of >9; low expression was defined as a final score < 6 and high expression with a final score ≥ 6.

ELISA to Analyze the Cytokines in the Tumor Tissues

Previous studies proved that ICOS signals exerts its effects mainly through regulating cytokine production by activated and effect T cells [14, 15], and it can induce T cells to produce cytokines, such as IFNγ (Th1 marker), IL4 (Th2 marker), IL17 (Th17 marker) and IL10 (Treg marker). Aim to clarify the expression pattern of these cytokines in the NPC tumors, ELISA assay has been used to test their concentration. Briefly, these four cytokine ELISA kits were purchased from Abcam Inc. (California, America), and fresh tumor tissues was

homogenized by PBS with 1:20 (weight: volume), the PBS homogenized solution was added into 96-well plate, and commercial menu was followed to get the cytokine concentration with the help of standard curve. All the cytokine concentration was calculated with per 100 μg total protein.

Statistical Analysis

The Statistical Package for Social Sciences, version 17.0 (SPSS, Chicago, IL, USA), software was used for statistical analysis. The disease-free survival (DFS) and overall survival (OS) were estimated by use of the Kaplan–Meier method. DFS and OS were measured from Day 1 of treatment to the date of the event. Log-rank test was used in univariate analysis. $\chi 2$ and Fisher's exact tests were used to compare the differences between the ICOS high group and the ICOS low group. Multivariate analysis was performed using the Cox proportional hazards model. All statistical tests were two sided, and $P < 0.05$ was considered to be statistically significant.

Results

General Information

Among the 185 patients enrolled, including 124 males and 61 females, the median age was 50 years (range from 20 to 73 years). The median body mass index (BMI) was 22.5 kg/m2 (range, 16.1–32.2 kg/m2). Total 128 patients had Epstein-Barr virus (EBV) infection, and the infection ratio was 69.2%. All tumors were classified as having nonkeratinizing phenotype. After a median follow-up duration of 70 months, 18 (9.7%) patients died and 13 (7.0%), 9 (4.9%), and 12 (6.5%) patients suffered from local failure, regional failure, and distant metastasis, respectively. The 5-year DFS, and OS rates were 54.0%, and 53.2%, respectively. The detailed patient characteristics was shown in Table 1.

Clinicopathologic Correlations with ICOS

ICOS staining was detectable in 129 patients (69.7%, 129 of 185 patients) and was mainly located at the membrane region in the tumor tissues, most ICOS+ cells are located into the nuclei-condensed region. ICOS staining was classified as high expression in 81 patients. Representative staining of ICOS in NPC is shown in Fig. 1. In this study, high expression of ICOS staining was not significantly correlated with the clinicopathological parameters of age, sex, BMI and EBV infection, but significantly related with clinical stage at diagnosis, and lymphocyte metastasis and distant metastasis. Detailed data are summarized in Table 1.

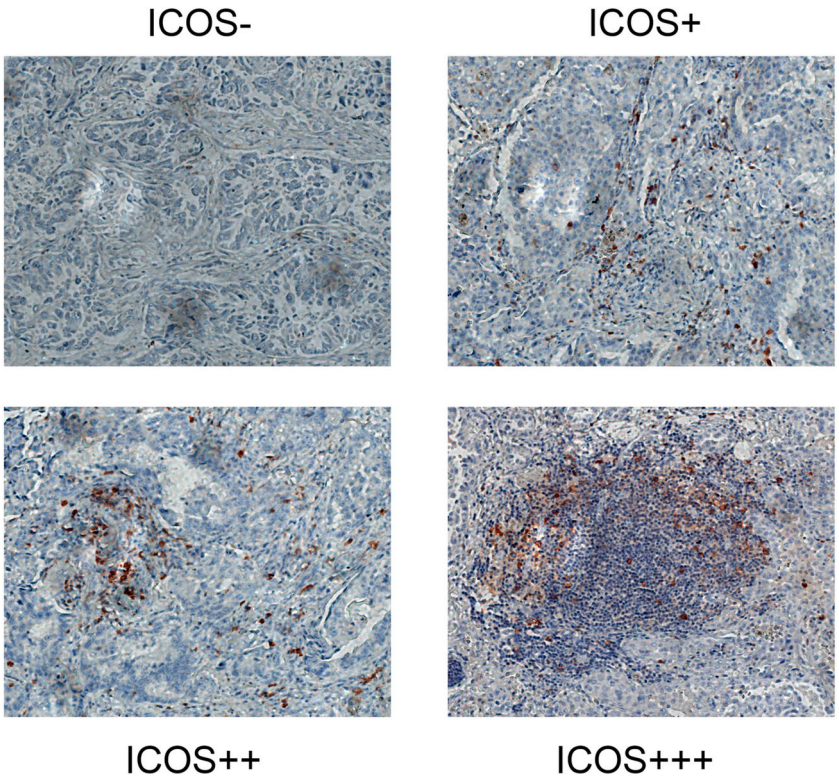

Fig. 1 Immunohistochemical staining for ICOS in patients with NPC, and classification of different intensity of ICOS staining

Table 2 Univariate and multivariate analyses of prognostic parameters for survival in 185 NPC patients

	Univariate analysis			Multivariate analysis		
Prognostic parameter	HR	95% CI	P value	HR	95% CI	P value
Expression of ICOS (low vs. high)	0.365	0.284–0.621	0.001	0.475	0.321–0.701	0.001
Age (y)	1.487	0.841–1.654	0.079	–	–	–
Sex (male vs female)	1.194	0.821–1.422	0.102	–	–	–
Tumor differentiation	1.245	0.813–1.534	0.156	1.457	0.756–1.875	0.147
T classification	2.221	1.574–2.897	0.014	1.875	0.941–2.356	0.034
Lymphatic metastasis (absent vs. present)	3.256	1.987–4.214	0.003	2.985	1.269–3.877	0.001
Distant metastasis (absent vs. present)	4.521	2.338–6.145	0.001	4.125	2.476–5.031	0.001

Abbreviations: *BMI* body mass index, *HR* Hazard ratio, *CI* Confidence interval

Prognostic Values Related with ICOS

The values of various potential prognostic factors including age, sex, BMI, overall stage, and ICOS on predicting OS and DFS were evaluated. The outcomes of univariate and multivariate analysis are shown in Table 2. Our results revealed that a high expression of ICOS was correlated with improved OS (5y-OS: 52.4% vs 75.6%, $P = 0.0003$, Fig. 2). In the univariate and multivariate analysis, ICOS was suggested to be an independent prognostic factor for OS (hazard ratio: 0.365 for univariate analysis, and 0.475 for multivariate analysis, both $P < 0.01$), detailed data was shown in Table 2.

ELISA Assay Demonstrated that ICOS Expression is Positive Correlated with IFNγ Level in NPC Tumors

As shown in Fig. 3, in the ICOS expression group, the IFNγ and IL17 concentration from tumor tissues were significantly higher than those from ICOS lower expression group. On the contrary, the IL4 and IL10 is remarkably lower in ICOS high expression group compared with lower ICOS expression group. By the regression analysis, the IFNγ was positively correlated with $ICOS^+$ cell infiltration.

Discussion

In our study, higher cytomembranic ICOS was mainly expressed in low stage NPC patients, about 70.4% among patients in stage 1 and stage 2; while only 29.6% of patients from advanced tumors has high expression of cytomembranic ICOS. Based on my knowledge, it is first time that our results revealed NPC patients with high cytomembranic ICOS expression had a significantly increased survival outcome, which provides a beneficial biomarker to predict the prognosis of NPC.

Our results demonstrated that the expression rate of ICOS in NPC tumor tissues significantly correlated with an improved prognosis of overall survival and disease-free survival. Based on known immunology studies, most $ICOS^+$ cells are tumor-infiltrating immune cells, including T effector cells and Treg cells [16, 17]. ICOS strong immune staining positive correlation with improved survival also suggest that these $ICOS^+$ cells are also possible CD^{8+} cells, the beneficial mechanism of ICOS higher expression is due to improved immunity which cytotoxic lymphocytes number increased.

Several previous studies have suggested that $ICOS^+$ cell, acting as Th1 cells, is associated with improved survival of colorectal cancer patients [8]. In contrast to other co-stimulatory receptors of CD28 in this family, the effects of ICOS on activation of naive T cells and T cell proliferation are modest [8, 18, 19] . ICOS signal seem to play an important role on modulating cytokines production by activated and effector T cells, particularly Th1 and Th2 effector responses [15]. Since Th1 and Th2 cells play almost opposite roles in the immune system, disruption of ICOS may induce contrasting results depending on which effector function ICOS dominates. Our data showed that $ICOS^+$ higher tumor tissues expressed higher level of INFγ and IL17, and less IL10 and

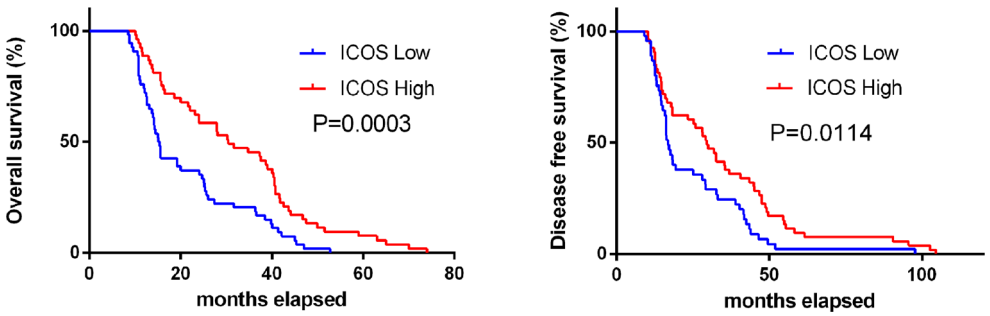

Fig. 2 Kaplan–Meier curves stratified by the patterns of Immunohistochemical staining for cytomembranic ICOS (low vs high)

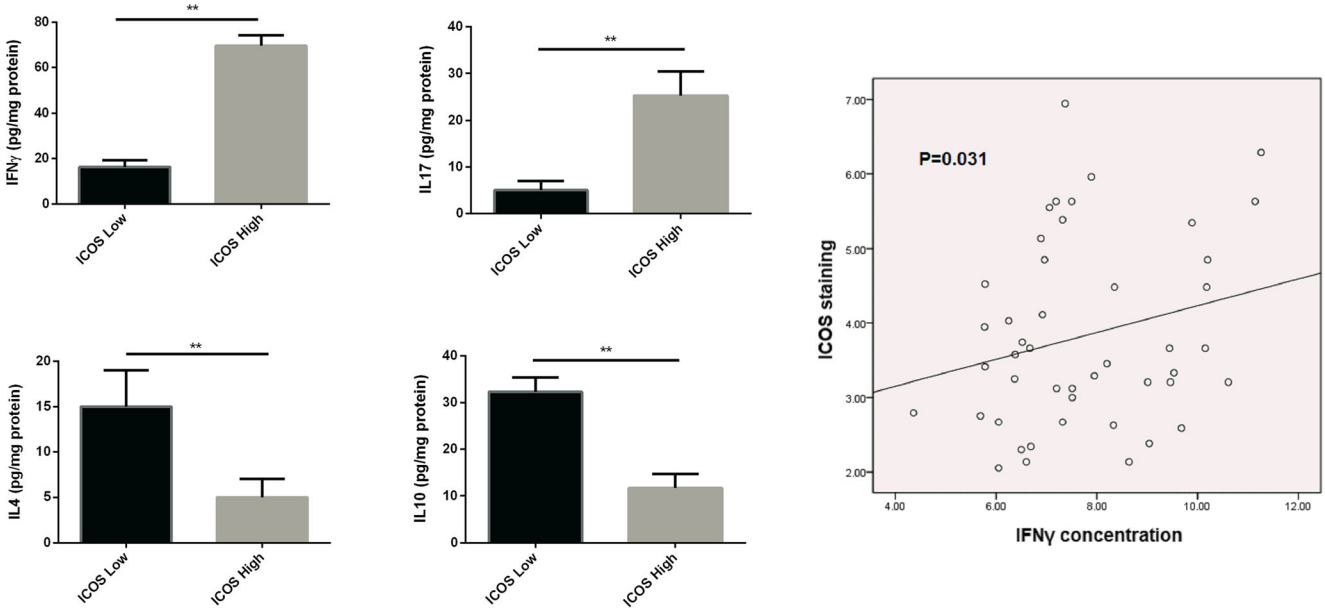

Fig. 3 Four kinds of cytokines concentration from tumor tissues in ICOS higher or lower groups by ELISA analysis; and ICOS expression was positively correlated with IFNγ concentration in tumor tissues

IL4, compared to ICOS+ lower tumor tissues, suggesting that ICOS promotes Th1 effector response in NPC patients. Previous study reported that Th1 cells inhibit tumor cell invasion and metastasis by communicating with tumor associated myeloid cells, including TAMs and MDSCs [20], which may contribute to the improved survival of NPC patients with high ICOS expression. The mechanism on how ICOS signal regulates Th1 effector response need to be clarified in future work.

There are several limitations in the current study, including the inclusion of patients with cytomembranic ICOS staining who completed treatment only, the retrospective nature of the study design, and the limited number of patients with Stage T4 or N3 disease, and these could affect the outcomes. Nevertheless, our report is noteworthy because this is the first study to evaluate cytomembranic ICOS expression in NPC.

So far, ICOS has already been a drug target for cancer immunotherapy. Several agonist antibodies have already moved into phase 1 or 2 clinical trial, such as JTX-2011 (Jounce Therapeutics) [21] and GSK3359609 (GlaxoSmithKline) [22]. Upon administration, anti-ICOS agonist antibody will target and bind to ICOS expressed on tumor infiltrating CD4+ T cells. This stimulates ICOS+ T cell proliferation, enhances cytotoxic T lymphocyte (CTL) survival and increases CTL-mediated immune responses against tumor cells.

Conclusion

ICOS expression levels correlated with OS in NPC, and this might be due to enhanced TH1 cytotoxicity effect by its co-stimulatory effect. ICOS might be a potential therapeutic target for NPC in the future.

Compliance with Ethical Standards

Conflict of Interest None.

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