

## Original Article

# Prognostic value of TROP2 in human nasopharyngeal carcinoma

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**Abstract:** Background: There is increasing evidence demonstrating the role of human trophoblast cell surface antigen 2 (TROP2) in cancer development and progression. However, their prognostic value in Epstein-Barr virus (EBV) associated nasopharyngeal carcinoma (NPC) remains to be elucidated. Method: The prognostic significances of TROP2 and Ki-67 were determined by immunohistochemistry in 58 NPC samples. TROP2 mRNA expression level and biological functions were evaluated. The presence of EBV was assessed using in situ hybridization. Analyses were conducted on the association between each of these variables as well as clinical outcome. Results: TROP2 was exhibited over expression in 64% of NPC samples and significantly associated with highly proliferative tumor cells ( $P = 0.05$ ) and lymph node metastases ( $P=0.03$ ). Overexpression of TROP2 significantly correlated with worse overall survival ( $P = 0.026$ ) and poor disease-free survival ( $P = 0.021$ ). By univariate analysis, high expression of TROP2 significantly correlated with patients with distant metastases, Ki-67 and EBV infection. Multivariate analysis further revealed that TROP2 along with Ki-67 and distant metastasis are independent prognostic predictors for NPC patients. Conclusion: Our findings have demonstrated that overexpression of TROP2 appears to be an independent predictor for poor clinical outcome in NPC. The strong correlation of overexpression of TROP2 with Ki-67 and distant metastases indicates a potentially therapeutic strategies targeting TROP2 for NPC patients.

**Keywords:** Nasopharyngeal carcinoma, TROP2, EBV, prognostic predictor, clinical outcome

## Introduction

Head and neck squamous cell carcinoma is the sixth most common malignant tumor worldwide [1], among of which, Nasopharyngeal carcinoma (NPC) represents a unique epithelial malignancy, with distinct biological and epidemiologic features from other cancers of the head and neck region. More than 80% of nasopharyngeal carcinomas harbor Epstein-Barr virus (EBV) and majority of these patients distribute in Southeast Asia regions [2]. Ionizing radiation therapy is the major curative treatment modality for early disease. Despite progress in combination with chemotherapy for more advanced stages, 30-40% of patients will still develop distant metastases within 4 years [2], underlining the importance to better understand the molecular basis of tumor characteristics and to search for new tumor mediators that contribute to disease progression and developing novel therapeutic strategies.

There is increasing evidence supporting the role for TROP2 (human trophoblast cell surface antigen 2) in tumor development and progression. TROP2 is a cell surface transmembrane glycoprotein receptor, which is also known as Tumor-Associated Calcium Signal Transducer 2 (TACSTD2). It belongs to a family with at least two closely related genes (TACSTD1-EPCAM, and TACSTD2-TROP2) [3]. Both of them share sequence homology with both thyroglobulin type I and interleukin-2 receptors [4]. TROP2 was first discovered in human trophoblast cells, and subsequently it has been found significantly different expression between cancer cells and normal epithelial cells. The higher expression of TROP2 was found in the majority of human epithelial carcinomas, including gastric [5], colorectal [6], cervical [7], oral cavity [8] and laryngeal carcinomas [9]. In recent years, overexpression of TROP2 has been recognized to closely associate with cancer progression

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**Table 1.** Characteristics of Patients

Characteristic	N = 58	(%)
Sex		
Male	42	(72.4)
Female	16	(27.6)
Age (years)		
Range	24-7	
Median	45	
Tumor stage		
T1-2	19	(32.8)
T3-4	39	(67.2)
N0	13	(22.4)
N1-3	45	(77.6)
M1	12	(20.7)
Lymph node metastasis		
No	21	(36.2)
Yes	37	(63.8)
Distant metastasis		
No	46	(79.3)
Yes	12	(20.7)
TROP2 membrane expression		
Low (0-1.5)	21	(36.2)
High (2-3)	37	(63.8)
Ki-67		
≤ 10%	23	(39.7)
> 10%	35	(60.3)
EBER		
0-1	17	(29.3)
2-3	41	(70.7)

and poor prognosis [7, 8, 10] and it has emerged as an attractive cancer therapy target.

Although the function of TROP2 is not well understood, early studies have shown that TROP2 not only acts as a calcium signal transducer but also plays a role in regulating the growth of carcinoma cells [11]. To date, the role of TROP2 in NPC remains unclear, we therefore investigated TROP2 expression and its correlation with clinicopathologic features in nasopharyngeal carcinoma samples.

## Material and methods

### Patient characteristics

This study was conducted according to the regulations of the local Medical Ethics Committee. A total of 58 nasopharyngeal carcinoma

punch or cone formalin-fixed and paraffin-embedded biopsy samples were retrieved during the period of 2001-2011. The clinical, demographic and tumor-related characteristics of the patients have been described in **Table 1**. The majority of patients were male (42 or 72.4%), median age was 45 years. 77.6% patients had lymph node metastasis at the diagnosis and 67% patients had locally advanced disease with stage III and IV. All 58 patients shared an average follow-up time of 96 months. During the follow-up period, 26 patients were dead (44.9%) and all of the patients were not treated to either radiotherapy or chemotherapy prior to the biopsy. All studied tumour specimens were assessed on hematoxylin-and-eosin-stained slides using standard diagnostic criteria. This study was approved from the Second Hospital of Jilin University and all patients have consented for the study.

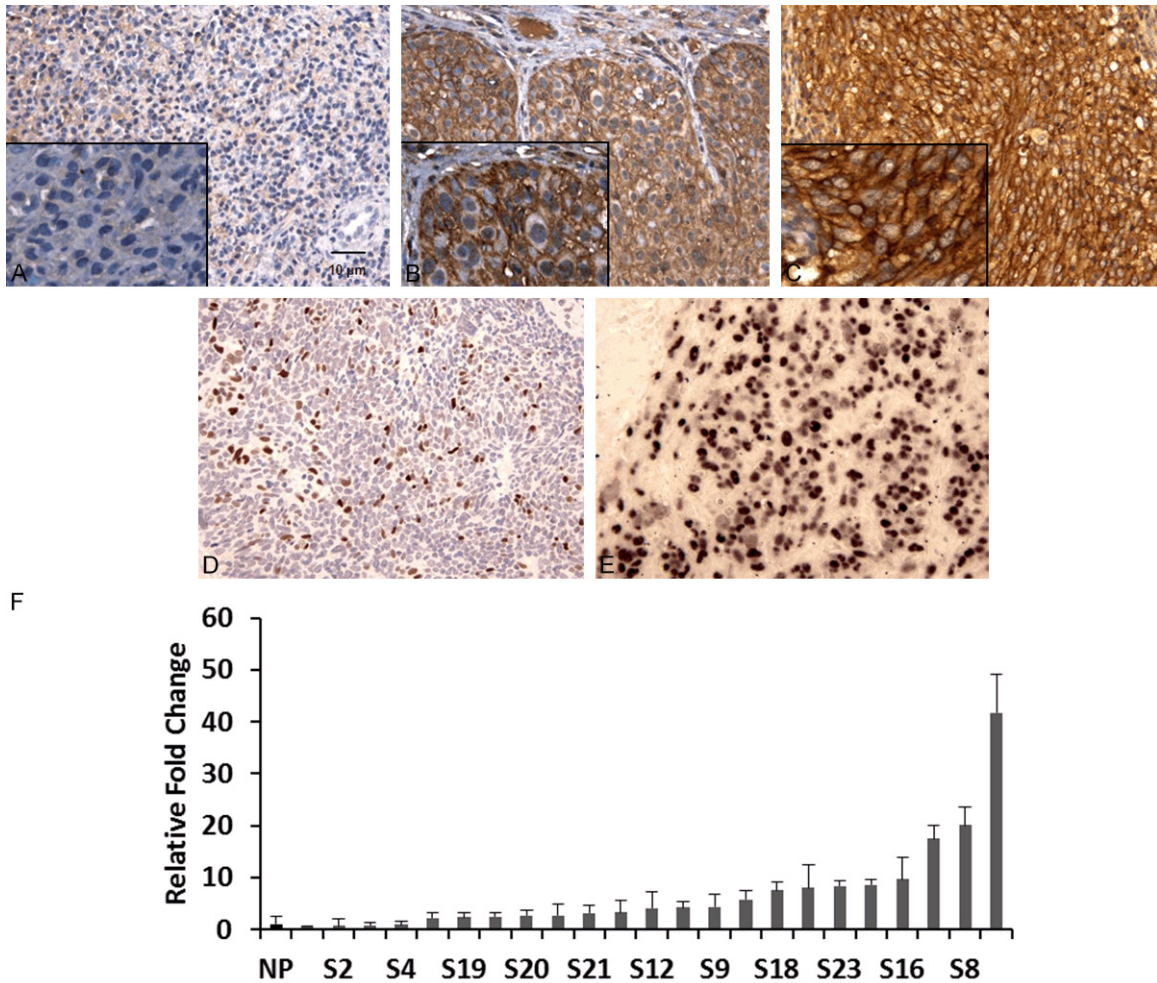
### Immunohistochemistry

The expression of TROP2 was determined by immunohistochemistry using a purified goat polyclonal antibody (AF650, R&D Systems, Inc., Minneapolis, MN, USA), which identifies TROP2 extracellular domain as described previously [12]. Ki-67 expression was evaluated by using the monoclonal antibody (Dako Cytomation). Briefly, 4- $\mu$ m sections were deparaffinised and rehydrated, antigens were retrieved by using citrate acid buffer in a microwave oven. Endogenous peroxidase was treated with 3% hydrogen peroxide and non-specific binding was blocked with normal goat serum. A 1:50 dilution of goat anti-TROP2 or an anti-Ki-67 (1:100) antibody was then applied on the sections overnight at 4°C. In combination with the Universal LSAB™2 detective system (Dako Cytomation), the positive signal was detected by 3,3'-diaminobenzidine in chromogenic solution (DAB) with haematoxylin as a counterstain. Sections without primary antibodies were used as negative controls.

### Immuno-activity evaluation

TROP2 expression was evaluated by two individuals using light microscopy with a double-blind method. Positive TROP2 expression was assessed by calculating the tumor cell membrane staining proportion and intensity, as described previously [5, 10]. The proportion of positive cells was scored as: 0 = none; 1 < 10%;

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**Figure 1.** Immunohistochemical staining of TROP2, Ki-67 and in situ hybridization detection of EBV in nasopharyngeal carcinoma. A. None or weak membrane expression of TROP2. B, C. Moderate and strong TROP2 expression in tumor cells (magnification 200× or 400×). D. Ki-67 expression in tumor cells (magnification 200×). E. Purple-blue signal showed EBV infection in NPC tumor cells (magnifications 200×). F. TROP2 over-expressed in 19 out of 23 NPCs by QRT-PCR.  $P < 0.0001$ .

2 = 10-50%; 3  $\geq$  50%. Staining intensity was defined as: 0 = no staining; 1 = weak; 2 = moderate; 3 = strong. The overall score was a combination of staining proportion and intensity. Low = score 0-1.5; high = score 2-3. Immunostaining of Ki-67 was evaluated by scanning the entire section at low power ( $\times 100$ ) and then counting at least 500 tumor cells in the five most densely staining fields at high power ( $\times 400$ ). The percentage of positively staining tumor nuclei was calculated. Ki-67 positive was considered when  $> 10\%$  of tumor cells with nuclear staining.

### Cells lines and transfection

CNE-1, nasopharyngeal carcinoma cells were purchased from Shanghai Aiyuan Biotechnology

Co., Ltd. and maintained at 37°C, 5% CO<sub>2</sub> in RPMI 1640 complete medium (GIBCO, USA) with 10% fetal bovine serum (FBS, GIBCO, USA). The normal human fibroblast cells (GM05757) were purchased from Coriell Institute (USA) and cultured in the Eagle's Minimum Essential Medium. To deplete endogenous TROP2 expression, as described previously [7], a pair of SIRNA sequences targeting TROP2 were designed and synthesized by Genepharma Co., Ltd (Shanghai, China). These sequences were: Forward: 5'-GCACGCUCAUCUAUUACCUTT-3', Reward: 5'-AGGUAAUAGAUGAGCGUGCTT-3'; Negative scramble control sequences were: 5'-UUCUCCGAACGUGUCACGUTT-3', 5'-ACGUG-ACACGUUCGGAGAATT-3'. Lipofectamine 2000 (Invitrogen) was used to transfect the tumor cells with either scramble (SC) negative control

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**Table 2.** TROP2 expression with clinicopathological parameters

Parameter	TROP2 expression		P value
	Low (%)	High (%)	
Sex (n = 58)			
Male	14 (33.3)	28 (66.7)	0.86
Female	5 (31.3)	11 (68.7)	
Age (median)			
< 45	10 (34.5)	19 (60.9)	0.97
≥ 45	9 (31.0)	20 (69.0)	
Tumor stage			
T1-2	7 (36.8)	12 (63.2)	0.87
T3-4	12 (30.8)	27 (69.2)	
Lymph node status			
No	11 (52.4)	10 (47.6)	0.035
Yes	8 (21.6)	29 (78.4)	
Distant metastasis			
No	14 (30.4)	32 (69.6)	0.69
Yes	5 (41.7)	7 (58.3)	
Ki-67			
≤ 10	12 (52.2)	11 (47.8)	0.023
> 10	7 (20.0)	28 (80.0)	
EBER			
0-1	5 (29.4)	12 (70.6)	0.91
2-3	14 (34.1)	27 (65.9)	

or SIRNA (life technologies) by using the reverse transfection protocol, according to the manufacturer's instructions.

### Quantification of TROP2 expression

In order to evaluate TROP2 expression in mRNA level, Quantitative real-time PCR (QRT-PCR) was utilized. The primers were designed with Primer 4.0 and the forward primer was 3'-gagattccccgaagttctc-5' and the reverse primer was 3'-aactccccagttccttgat-5'. Total RNA was extracted from 23 available primary tumor samples or from cell lines using Recover All Total Nucleic Acid Isolation kit (Ambion). The reverse transcription was performed using Super Script III Reverse Transcriptase (Invitrogen Corp.) according to the manufacturer's recommendations. QRT-PCR analyses were performed using SYBR Green Master Mix (Applied Biosystems) and the ABI PRISM 7900 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA). The relative fold change in mRNA expression was calculated using the 2- $\Delta\Delta$ Ct method, where the average of  $\Delta$ Ct val-

ues for the amplicon of interest was normalized to that of an endogenous gene (GAPDH), compared with control specimens. Each experiment was performed triplicate.

Western blot was used to detect TROP2 expression in cell lines. The cells were transfected with either SC or SIRNA. The cells were collected and lysed at 48 hours after. As described previously [13]. Blots were incubated with anti-TROP2 antibody (1:1000) for overnight followed by incubation with the second antibodies. Signals were visualized using the ECL Western blotting substrate kit (Pierce, USA).

### Cell proliferation assay

The cell proliferation and cytotoxicity of introducing anti-TROP2 SIRNA were assessed by using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) staining kit (Sigma, USA). Cells were reverse transfected with either SC control, anti-TROP2 SIRNA or Lipofectamine 2000 and seeded onto 96-well plates ( $5 \times 10^3$  cells/per well). Cell viability was measured at 24, 48 and 72 hours post-transfection.

### Epstein-Barr virus (EBER) detection

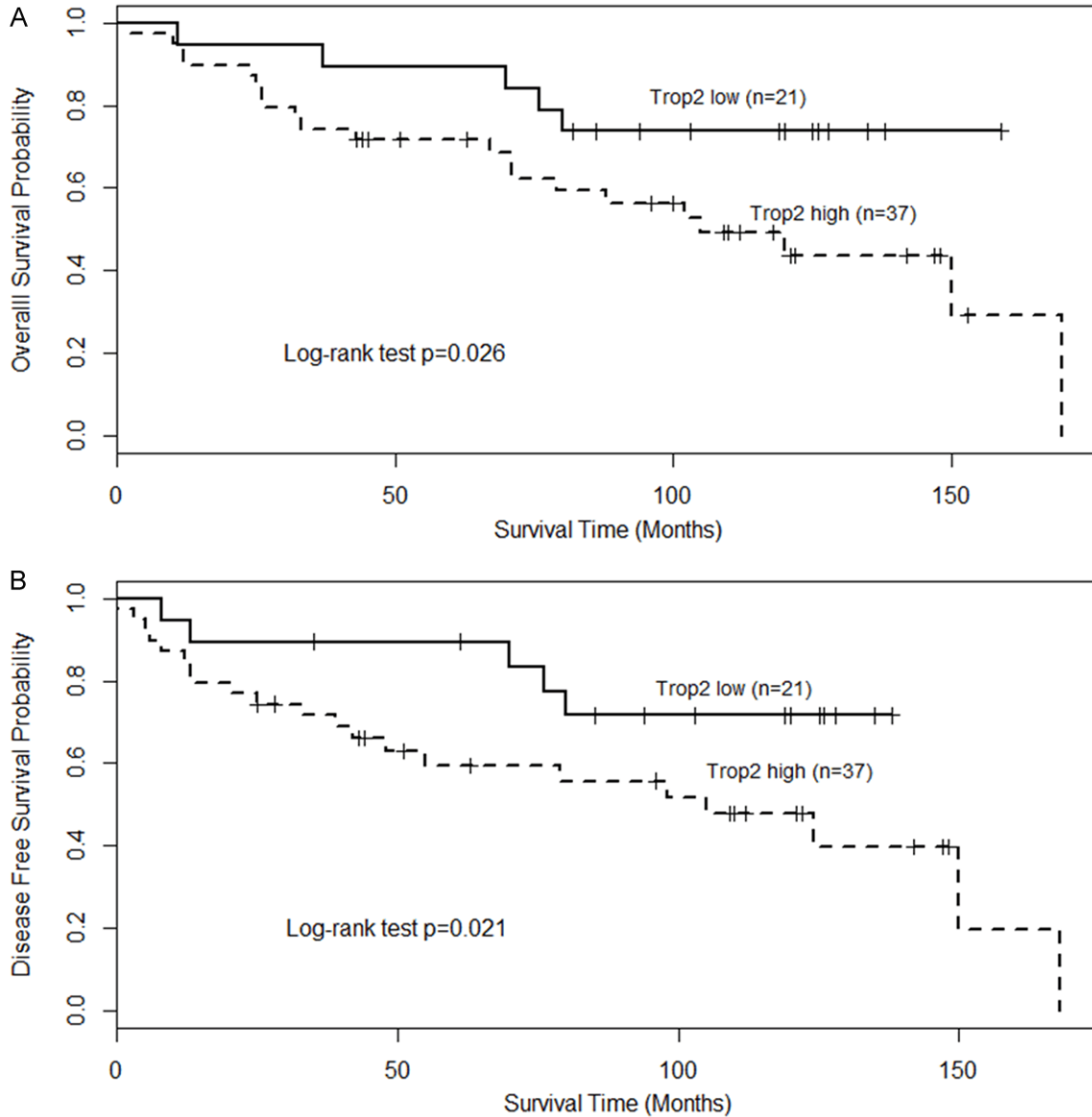
By using a commercial available probe, the presence of latent EBV infection (EBER) in NPC was detected by in situ hybridization on formalin-fixed, paraffin-embedded tissue sections in accordance with the manufacturer's instruction. Briefly, 4- $\mu$ m sections were deparaffinised and rehydrated, after predigested with proteinase K, a fluorescein-conjugated EBV (EBER) peptide nucleic acid probe (DAKO-Agilent technologies, China) was applied on the section for 2 hours at 55°C. Hybridization signals were detected by alkaline phosphatase-conjugated antiluorescein, then by 5-bromo-4-chloro-3-indolylphosphate nitroblue tetrazolium as a chromogen. The sample was defined as positive if dark brown staining signal was observed in the tumor nuclei. Based on the staining proportion and intensity, a grade was assigned as 0 (negative); 1+ (< 10% of positive staining); 2+ (10-50% positivity) and 3+ (> 50% positivity).

### Statistical analysis

Overall survival (OS) was defined from the time of diagnosis to date of death. Disease-free survival (DFS) was defined from the time of diagno-



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**Figure 2.** Prognostic significance of TROP2 in patients with nasopharyngeal carcinoma. Kaplan-Meier analysis was performed for overall survival and disease free survival. TROP2 low: score as 0-1; TROP2 high (over-expression): score as 2-3. Patients with high expression of TROP2 exhibited significantly worse overall survival (A) and shortened disease-free survival (B) than that with low expression of TROP2 as defined by log-rank test.

sis to the date of first failure. Survival curves were generated using the Kaplan-Meier method and compared by means of the log-rank test. Associations between TROP2 expression and clinicopathological variables were assessed with the Chi-test. In the univariate model, if factors with prognostic significance, they were then further analysed in a multivariate Cox's proportional hazards regression model. A P-value of less than 0.05 was considered to be statistically significant.

### Results

#### *Evaluation of TROP2 expression in NPC patients*

TROP2 immuno-activity was determined in 58 archival formalin-fixed and paraffin-embedded NPC samples using immunohistochemistry. TROP2 expression was assessed based on the combination of positive tumor cell staining proportion and intensity. Our observations were

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**Table 3.** Univariate analysis of prognostic parameters

Parameter	OS	DFS
	P value	P value
Sex		
(Male/female)	0.561	0.503
Age		
(≥ 45 vs. < 45)	0.109	0.167
T stage		
(pI-II vs. pIII-IV)	0.221	0.084
lymph node status		
(Y/N)	0.740	0.602
Distant metastasis		
(Y/N)	0.029	0.009
TROP2 overexpression		
(Y/N)	0.026	0.021
Ki-67 overexpression		
(Y/N)	0.019	0.012
EBER expression		
(Y/N)	0.025	0.057

DFS-disease-free survival, OS-overall survival.

consistent with previously reported, when comparing with adjacent normal epithelial cells (showed no or weak staining), the majority of cancer cells showed a homogeneous moderate to strong membranous expression of TROP2. Moderate or strong immuno-activity was observed in 37 of the 58 (63.8%) specimens and none or weak expression was in 21 (36%) specimens. Overall, TROP2 was exhibited overexpression in 64% of NPC samples (**Table 1; Figure 1A-C**).

Among these 58 samples, TROP2 transcript mRNA expression was further evaluated by QRT-PCR in 23 available samples (with sufficient RNA). By comparing with 6 normal tissues pooled RNA (squamous mucosa with chronic inflammation and lymphoid hyperplasia), 19 out of 23 cases had 2.3- to 41.8- fold higher expression levels of TROP2 in NPC samples (**Figure 1F**), demonstrating that TROP2 was indeed significantly overexpressed in NPC at both protein and mRNA levels.

### Expression of Ki-67 and EBV in NPC

Tumor nuclei immuostaining of Ki-67 was observed in 35 of 58 (60.3%) of NPC specimens (**Table 1; Figure 1D**). Overexpression of Ki-67 was correlated significantly with mem-

brane accumulation of TROP2 protein in NPC tumor cells (**Table 2; P = 0.02**), 28 of 39 (71.7%) of the highly TROP2 expressed samples have increased expression of Ki-67, indicating TROP2 more likely expressed in the highly proliferative tumor cells.

NPC has a strong correlation with EBV infection [14]. Our in situ hybridization (ISH) data demonstrated that the majority of tumors were EBER positive (41 of 58; 70.7%) (**Table 1; Figure 1E**) (defined as ≥ 10% positively signal in tumor nuclei). However, there was not a statistical association between overexpression of TROP2 and positive EBV status in this cohort (**Table 2; P = 0.91**).

### Correlation of TROP2 expression with clinicopathological features

Using univariate analysis, TROP2 status was observed to be correlated significant for overall survival (OS) and disease-free survival (DFS). As observed in **Figure 2**, Kaplan-Meier survival curves showed that patients with high TROP2 expression had worse clinical outcome than those with low TROP2 expression by log-rank test ( $P = 0.026$  and  $P = 0.021$ , respectively). When compared TROP2 expression to gender, age, tumor stage, lymph node metastases, distant metastases, Ki-67 and EBV expression, overexpression of TROP2 significantly correlated with patients with distant metastases, proliferation indicator and EBV infection, but not with sex, age and lymph node status (**Table 3**). By using Cox proportional hazard regression model, multivariate analysis was performed. TROP2 overexpression together with Ki-67 and distant metastases were independent prognostic factors for poor disease-free survival (**Table 4; TROP2; P = 0.031; Hazard ratio: 4.81; 95% CI between 2.097 to 21.07**). Likewise, TROP2 was also significantly correlated with overall survival when controlled for the clinical prognostic factors (**Table 4; P = 0.04**). The hazard ratio for the effect of TROP2 on survival when controlled for clinical factors was 4.56.

### TROP2 promotes cell proliferation in NPC cells

Given the evidence that TROP2 overexpressed in NPC samples and was associated with poor clinical outcomes, we further investigated the biological significance of depletion of TROP2. CNE-1 cells were transfected with scramble

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**Table 4.** Multiivariate analyses of prognostic parameters

Parameter	DFS		OS	
	P value	HR (95% CI)	P value	HR (95% CI)
T stage				
(pI-II vs. pIII-IV)	0.096	2.59 (0.843-7.98)	0.233	2.02 (0.643-6.257)
lymph node status				
(Y/N)	0.604	1.35 (0.441-4.163)	0.741	1.21 (0.391-3.764)
Distant metastasis				
(Y/N)	0.016	3.78 (1.278-11.18)	0.040	2.87 (1.048-7.885)
TROP2 overexpression				
(Y/N)	0.031	4.81 (1.097-21.07)	0.037	4.56 (1.043-19.92)
Ki-67 overexpression				
(Y/N)	0.020	3.79 (1.242-11.62)	0.033	3.45 (1.26-10.54)
EBER expression				
(Y/N)	0.065	0.42 (0.163-1.057)	0.030	0.36 (0.143-0.923)

DFS-disease-free survival, OS-overall survival, HR-Hazard ratio, CI-confidence interval.

controls (SC) or anti-TROP2 SIRNA. First, we demonstrated that untreated CNE-1 cells exhibited elevated TROP2 expression compared with a normal cell control (**Figure 3A**), 3.8-folds increasing of TROP2 expression were observed, consistent with a previously study [7]. Furthermore, introduction of anti-TROP2 SIRNA caused significant reduction of TROP2 expression in both mRNA and protein levels (**Figure 3A, 3B**).

The SIRNA transfected cells showed significantly decreased cell viability compared to controls starting from 24 hours post-transfection, continuously declined at 48 (55%) hours and 72 (62%) hours post-transfection (**Figure 3C**).

### Discussion

In the present study, we observe for the first time that a correlation between TROP2 expression with patient clinical outcome in nasopharyngeal carcinoma. We provide evidence that TROP2 was overexpressed in 64% of NPC patients, with none or low expression in normal, suggesting that TROP2 plays an important role in this aggressive malignancy and associates with the development of NPC. Moreover, overexpression of TROP2 significantly correlates with poor overall survival and disease-free survival. Particularly, patients with higher TROP2 expression exhibit a closely relationship with higher proliferation index (Ki-67) and promote tumor cell proliferation *in vitro*. Furthermore, overexpression of TROP2 along with Ki-67 and distant metastasis have been con-

sidered as independent prognostic predictors for NPC patients. Our data further confirm the observations in many other studies in various cancer phenotypes.

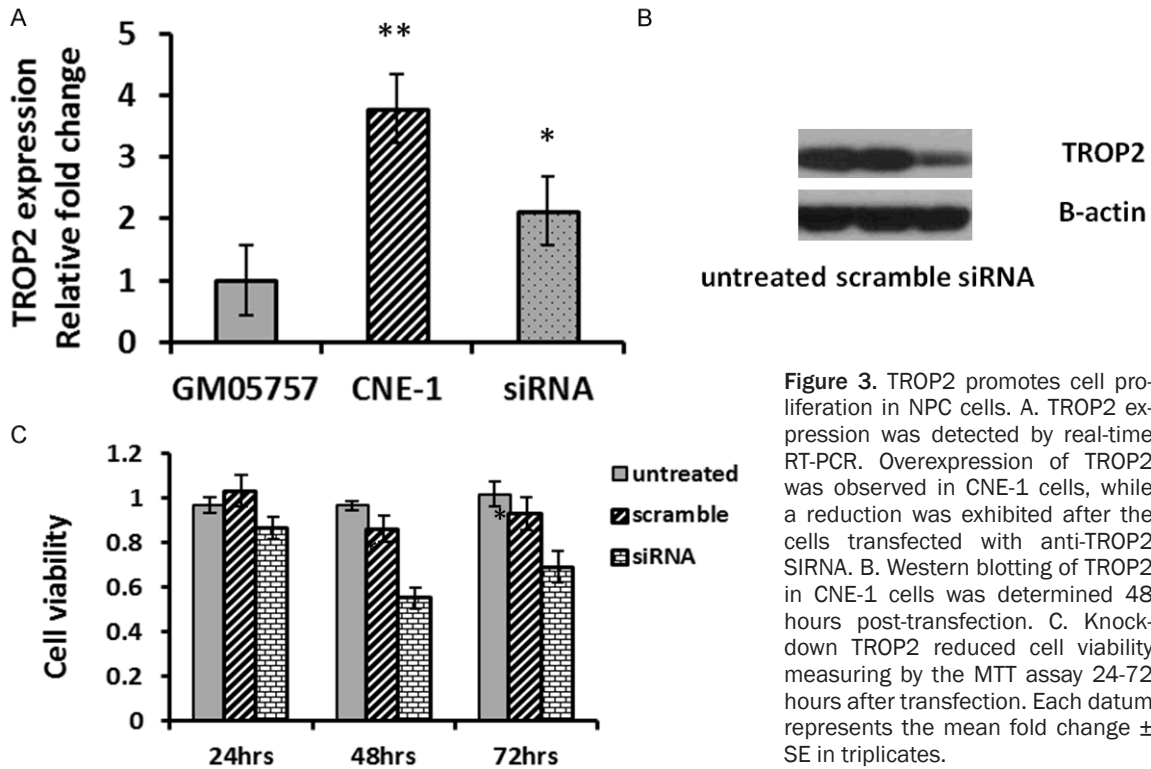
Ki-67 has been reported extensively in numerous of cancers as a tumor proliferation index. Liu T at al. [7] showed that TROP2 was overexpressed in cervical cancer and the overexpression was closely related to the high

expression of Ki-67 and poor prognosis. Our data in line with their observations, suggesting that TROP2 expression could correlate with the cancer aggressive behaviour. However, the precise mechanism of TROP2 in the development and progression of NPC remains unclear.

TROP2 plays a critical role for tumor growth. It can be phosphorylated by protein kinase C (PKC) [15], resulting in transient increase in intracellular calcium levels [16] and leading to cell proliferation. Another mechanism is that TROP2 may control cleavage of membrane-bound proteins [17]. Upon cleavage, the intracellular domain can enter the nucleus and regulate target genes (e.g. c-myc) to promote cell proliferation [18]. Extensive studies have shown that TROP2 regulates proliferation not only by effecting ERK pathway, but also mediating their downstream transcription cell cycle genes [19], such as cyclin D1, cyclin E, cyclin-dependent kinase (CDK) 4, CDK2, and up-regulation of NF-KB [20]. This regulating capacity relies on the phosphorylation on an S303 site of protein kinase C in the cytoplasmic region of TROP2 [20], which leads to the activation of MAPK/ERK pathway, resulting in tumor invasion and metastasis [21].

Over expression of TROP2 has been correlated to increased metastasis in many cancers, such as colorectal cancer [6], pancreatic cancer [10], lung cancer [19], laryngeal squamous cell carcinoma [9], and prostate cancer [22]. In prostate cancer, overexpression of TROP2 in

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**Figure 3.** TROP2 promotes cell proliferation in NPC cells. A. TROP2 expression was detected by real-time RT-PCR. Overexpression of TROP2 was observed in CNE-1 cells, while a reduction was exhibited after the cells transfected with anti-TROP2 siRNA. B. Western blotting of TROP2 in CNE-1 cells was determined 48 hours post-transfection. C. Knock-down TROP2 reduced cell viability measuring by the MTT assay 24-72 hours after transfection. Each datum represents the mean fold change  $\pm$  SE in triplicates.

cancer cell lines relocates RACK1 and interacts with integrin  $\beta$ 1, resulting in decreased cell adhesion to fibronectin [22]. Our results are in line with their observation, in that knock down TROP2 cause significant reduction of cell viability (Figure 3C). TROP2 has been proposed to be a potential therapeutic target either alone or in combination with other agents [23]. Anti-TROP2 antibody (hRS7) has been used in pre-clinical trials [24].

TROP2 signaling is a rapidly expanding area. There is likely a biologic basis for Epstein-Barr virus (EBV) infection with TROP2 overexpression. EBV status has closely association with non-keratinizing nasopharyngeal carcinomas [25]. Genetic alterations in host cells [26], oncogenic properties of EBER1, multiple EBV latent genes infections [27] and inflammatory stroma stimulated by TGF- $\beta$ 1 [28] or other genes have been reported to be crucial events in NPC. In the current cohort of 58 patients, EBV status has statistically significant for improved overall survival compared to EBV negative patients ( $P = 0.025$ ), but no significant for disease free survival ( $P = 0.06$ ). However, there is bore no relationship to TROP2 over expression ( $P = 0.91$ ). One explanation may be

related to the small sample size or the criteria used to define over-expression. Nonetheless, further studies in a large cohort of samples may validate the exploratory findings.

In conclusion, our findings have demonstrated that overexpression of TROP2 appears to be an independent predictor for poor clinical outcome in NPC. The strong correlation of overexpression of TROP2 with Ki-67 and distant metastases indicates a potentially therapeutic strategies targeting TROP2 for NPC patients.

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### Disclosure of conflict of interest

None.

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