RESEARCH ARTICLE

High Cytoplasmic Expression of the Orphan Nuclear Receptor NR4A2 Predicts Poor Survival in Nasopharyngeal Carcinoma

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Abstract

Objective: This study aimed at investigating whether the orphan nuclear receptor NR4A2 is significantly associated with clinicopathologic features and overall survival of patients with nasopharyngeal carcinoma (NPC). Methods: Immunohistochemistry was performed to determine NR4A2 protein expression in 84 NPC tissues and 20 non-cancerous nasopharyngeal (NP) tissues. The prognostic significance of NR4A2 protein expression was evaluated using Cox proportional hazards regression models and Kaplan-Meier survival analysis. Results: We did not find a significant association between total NR4A2 expression and clinicopathological variables in 84 patients with NPC. However, we observed that high cytoplasmic expression of NR4A2 was significantly associated with tumor size (T classification) ($P = 0.006$), lymph node metastasis (N classification) ($P = 0.002$) and clinical stage ($P = 0.017$). Patients with higher cytoplasmic NR4A2 expression had a significantly lower survival rate than those with lower cytoplasmic NR4A2 expression ($P = 0.004$). Multivariate Cox regression analysis analysis suggested that the level of cytoplasmic NR4A2 expression was an independent prognostic indicator for overall survival of patients with NPC ($P = 0.033$). Conclusions: High cytoplasmic expression of NR4A2 is a potential unfavorable prognostic factor for patients with NPC.

Keywords: NR4A2 - nasopharyngeal carcinoma - prognosis - cytoplasmic overexpression

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Introduction

Nasopharyngeal carcinoma (NPC) is a tumor arising from the epithelial cells that cover the surface and line the nasopharynx in epidemic areas of Southern China and Southeast Asia (Brennan, 2006; Razak et al., 2010). The multifactorial pathogenesis identified for NPC relies on germ line genetic susceptibility, acquired cellular genetic and epigenetic alterations, including the influence of diet, carcinogens and Epstein-Barr virus (EBV) infection (Chan, 2010). When the disease is diagnosed and treated at an early stage, most NPC patients can be cured. However, approximately 70% of patients with the initial diagnosed NPC present with locally advanced-stage due to its deep location of tumor growth and vague symptoms at early stages (Cao et al., 2012). Despite recent advances in radiation techniques and chemotherapy, local failure and distant metastasis remain poor survival in patients with advanced NPC (Chua et al., 2005). Biomarkers, with dual functions for both disease monitoring and novel molecular targeting, had shed the light on personalized therapy. Recently, many investigations suggested that molecular biomarkers, which are associated with tumor behavior and clinical outcomes, can improve the accuracy of prognosis and provide new directions for novel therapeutic approaches (Ben et al., 2009; Li et al., 2009). However, these biomarkers do not provide targets for therapy. Therefore, identification of novel valuable biomarkers associated with the progression and prognosis of NPC is sorely needed.

NR4A2 is an orphan nuclear receptor and a member of the nerve growth factor I-B subfamily of transcription factors with no known endogenous ligand or stimulator. It is a key regulatory molecule in a number of biological processes, including regulation of proliferation, apoptosis, migration and differentiation in a cell type-specific manner (Ke et al., 2004; Bonta et al., 2010; Lee et al., 2010; Sirin et al., 2010; Maijenburg et al., 2012). Recently, the oncogenic activities of NR4A2 are emerging. NR4A2 may stimulate progression of colorectal cancer by protecting cell survival, and induce suppression of apoptosis in cervical cancer cells, and is an independent prognostic marker for tumor progression and survival in patients with bladder cancer (Ke et al., 2004; Holla et al., 2006; Inamoto et al., 2010). To date, there have been no studies regarding the significance of NR4A2 expression in human NPC.

On the basis of these observations, we hypothesized that the NPC progression is associated with the level of human NR4A2 protein expression. To evaluate this, we

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Table 1. Correlation Between the Clinicopathologic Characteristics and Expression of NR4A2 Protein in NPC

Characteristics	n	Total NR4A2		*P*	Cytoplasmic NR4A2		*P*
		High	Low		High	Low	
Group							
NP	20	11	9	0.277	1	19	<0.001
NPC	84	57	27		44	40	
Gender							
Male	64	46	18	0.158	31	33	0.195
Female	20	11	9		13	7	
Age							
≥48 years	45	31	14	0.828	25	20	0.531
<48 years	39	26	13		19	20	
Smoking							
No	43	27	16	0.309	20	23	0.27
Yes	41	30	11		24	17	
T classfication							
T_1+T_2	57	35	22	0.066	24	33	0.006
T_3+T_4	27	22	5		20	7	
N classfication							
N_0+N_1	38	22	16	0.076	13	25	0.002
N_2+N_3	46	35	11		31	15	
M classfication							
M_0	77	51	26	0.42	39	38	0.437
M_1	7	6	1		5	2	
Clinical stage							
I+II	19	10	9	0.106	5	14	0.017
III+IV	65	47	18		39	26	

assessed the expression of NR4A2 in the primary lesions of 84 NPC patients with different clinicopathologic features, and elucidated its value in clinical prognosis.

Materials and Methods

Clinical specimens

Eighty-four NPC specimens and 20 non-cancerous nasopharyngeal (NP) specimens, both paraffinembedded, were obtained from the People's Hospital of Dongguan City (Dongguan, China). The NP tissue samples were derived from patients with chronic nasopharyngitis. All clinical and clinicopathologic data, including age, gender, and tumor node metastasis (TNM) staging, were obtained from medical records. In the 84 NPC cases, there were 64 male and 20 female with age ranging from 25 to 79 years (median, 48 years). For the use of these clinical materials for research purposes, prior consents from the patients and approval from the Ethics Committees of this hospital were obtained. All specimens had confirmed pathological diagnosis and were staged according to the 1997 NPC staging system of the WHO.

Immunohistochemistry detection

For immunohistochemical studies, 4-μm sections were cut from paraffin blocks form 84 cases of NPC tissues and 20 cases of NP tissues. Slides were dried in an oven (55 - 60 ºC) before being deparaffinized in several changes of xylene and hydrated through a series of graded alcohols to water. For antigen retrieval, slides underwent microwave treatment in 1 mM EDTA (pH 8.0) for 20 min, followed by a 20 min cooldown under running water. After washing in phosphatebuffered saline (PBS), the slides were exposure to 10% normal goat serum for 10 min to reduce non-speciffic binding, this was followed by an overnight incubation at 4 ºC in a humidified chamber with primary rabbit anti-NR4A2 polyclonal antibodies (diluted in the ratio 1: 100, Santa Cruz, CA, USA) at 4 ºC overnight. The sections were then incubated with biotinlabeled goat anti-rabbit secondary antibody (Earthox LLC, San Francisco, USA) and streptavidin-peroxidase for 30 min each. The samples were developed with 3, 3'-diaminobenzidine tetrahydrochloride substrate (Sigma, Steinheim, Germany) and counterstained with hematoxylin (Sigma, Steinheim, Germany). As a negative control, primary antibody was omitted and replaced with PBS.

The immunohistochemically stained tissue sections were scored independently by two pathologists blinded to the clinical parameters. The final score for NR4A2 was the average of the scores obtained by the two observers. Staining was graded for intensity (0 = negative/weak; 1 = moderate; 2 = strong) and percentage of cells stained (0 = 0-10%; 1 = 10-50%; 2 = 50-100%). The overall expression index was determined based on the factor of the previous variables and classified into one of the following groups: low NR4A2 expression (1-2), high NR4A2 expression (3-4). In each case, at least 3 different tumor areas were evaluated, and the mean of the results was taken as the final expression score (Zhou et al., 2012).

Statistical analysis

All statistical analysis was performed using SPSS 17.0 software. The associations between NR4A2 expression and clinicopathologic characteristics were assessed by the Pearson chi-square test or Fisher's exact test. Kaplan–Meier analysis and Log-rank test were used to assess survival rate and to compare the difference of survival rate. Univariate and multivariate regression analyses to identify the independent factors related to prognosis were accomplished by Cox proportional hazards regression model. Statistical significance was accepted as a *P* value of less than 0.05.

Results

BNR4A2 protein expression in NPC tissue and NP tissue

We investigated NR4A2 expression in 84 NPC samples and 20 NP samples by immunohistochemical analysis. The representative results are shown in Figure 1. In NP tissue, NR4A2 protein was mostly localised in the nucleus and very low staining in the cytoplasm was observed; in NPC, the protein was mostly localised both in the cytoplasm and in the nucleus. We observed that in 52.4% of NPC samples, cytoplasmic NR4A2 protein was highly expressed. In comparison, only 5.0% of NP samples had highly expressed cytoplasmic NR4A2 protein, significantly lower than that in the NPC samples ($P < 0.001$) (Table 1). Furthermore, the total NR4A2 expression levels in NPC tissue was not significantly different from NP tissue ($P = 0.277$) (Table 1).

Relationship between clinicopathological characteristics and NR4A2 expression in NPC patients

The association between NR4A2 expression and the

Table 2. Univariate and Multivariate Cox Regression Analysis of Individual Parameters for Correlations with Overall Survival Duration

		Univariate analysis			Multivariate analysis		
		HR	95%CI	*P*	HR	95%CI	*P*
Age	>45 vs. ≤45 years	1.016	0.528-1.955	0.963			
Gender	Male vs. female	1.681	0.826-3.421	0.152			
Smoking	No vs. Yes	0.522	0.264-1.032	0.061			
T classification	T_1-T_2 vs. T_3-T_4	1.796	0.924-3.490	0.084			
N classification	N_0-N_1 vs. N_2-N_3	4.512	1.814-9.503	0.001	3.207	1.318-7.806	0.01
M classification	M_0 vs. M_1	1.269	0.388-4.144	0.694			
Clinical stage	I-II vs. III-IV	3.548	1.087-11.580	0.036	1.178	0.322-4.306	0.805
Total NR4A2	High vs. Low	0.629	0.296-1.338	0.229			
Cytoplasmic NR4A2	High vs. Low	0.313	0.147-0.666	0.003	0.424	0.193-0.933	0.033

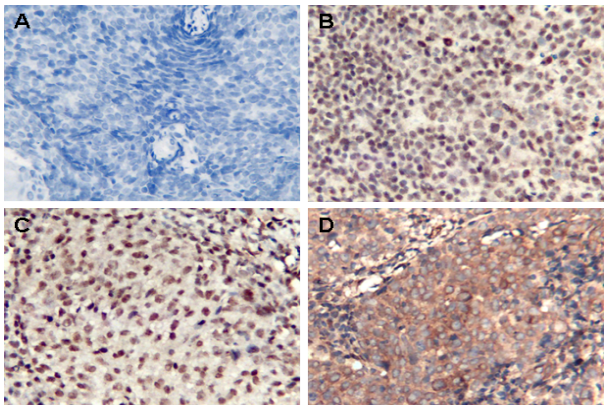

Figure 1. Expression of NR4A2 in Non-cancerous Nasopharyngeal (NP) and Nasopharyngeal Carcinoma (NPC) Samples (magnification 400×) (A-D) (A): Control group of NPC showing no staining when the primary antibody was omitted; (B): Nuclear staining of NR4A2 in NP sample; (C): Nuclear staining of NR4A2 in NPC sample; (D): Cytoplasmic staining of NR4A2 in NPC sample

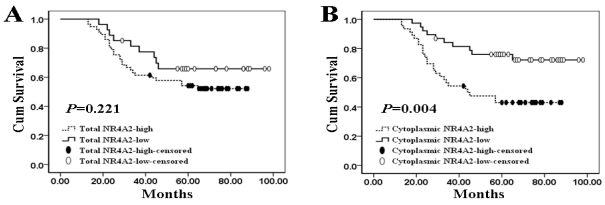

Figure 2. Kaplan-Meier Survival Analysis of the Overall Survival in 84 NPC Patients Based on the Levels of NR4A2 Protein Expression. (A): Correlation between total NR4A2 and survival is shown; (B): Correlation between cytoplasmic NR4A2 and survival is shown

clinicopathological features of NPC was further analyzed, as shown in Table 1. We did not find a significant association between total NR4A2 expression and clinicopathological variables in 84 patients with NPC. However, we observed that cytoplasmic NR4A2 dominance over nuclear NR4A2 was significantly associated with tumor size (T classification) (T_1+T_2 vs. T_3+T_4, $P = 0.006$), lymph node metastasis (N classification) (N_0+N_1 vs. N_2+N_3, $P = 0.002$) and clinical stage (I+II vs. III+IV, $P = 0.017$).

Survival analysis

To examine the correlation between NR4A2 expression and patient survival, we applied a Kaplan-Meier analysis with a logrank test. The Kaplan-Meier analysis showed that the survival rate of patients with NPC was significantly different between the groups with high cytoplasmic NR4A2 expression and low cytoplasmic NR4A2 expression. In patients with NPC, the low cytoplasmic NR4A2 expression group had a higher survival rate compared to the high cytoplasmic NR4A2 expression group ($P = 0.004$) (Figure 2B). However, total NR4A2 expression did not appear to correlate with overall survival ($P = 0.221$) (Figure 2A).

Cox proportional hazard regression analysis was used to evaluate the prognostic significance of NR4A2 expression (Table 2). In the univariate analysis, the cytoplasmic NR4A2 expression was significantly correlated with overall survival (HR 0.313, 95%CI 0.147-0.666, $P = 0.003$). The univariate Cox regression analysis also indicated that clinical variables including N classification (HR 4.512, 95%CI 1.814-9.503, $P = 0.001$) and clinical stage (HR 3.548, 95%CI 1.087-11.580, $P = 0.036$) were significantly associated with overall survival. To determine whether cytoplasmic NR4A2 expression is an independent prognostic factor for NPC, we performed a multivariate survival analysis of the NR4A2 protein expression and factors including N classification and clinical stage in patients with NPC. The results showed that high level of cytoplasmic NR4A2 (HR 0.424, 95%CI 0.193-0.933, $P = 0.033$) and N classification (HR 3.207, 95%CI 1.318-7.806, $P = 0.010$) were identified as independent prognostic factors for overall survival in patients with NPC.

Discussion

In patients with cancer, many factors including stage of disease, depth of invasion, lymph node status, distant metastasis, histopathologic type and tumor volume have been evaluated as potential prognostic indicators. However, these factors have limited value in determining low and high risk groups and the treatment modality. In recent years, the search for novel prognostic factors that more reliably predict the biological behavior of the tumors has focused on the role of the various molecular biomarkers (Acikalin et al., 2012; Kitagawa et al., 2013). In this study, we focused on the expression of NR4A2 in NPC and its relationship with the clinicopathologic parameters and prognosis of the disease. Expression of NR4A2 was first evaluated in 20 NP tissues and 84 NPC tissues by immunohistochemistry. We found that cytoplasmic NR4A2 expression is significantly

associated with the clinicopathologic features including T classification, N classification and clinical stage and the overall survival of patients with NPC.

NR4A2 belongs to the orphan nuclear receptor (NR4A) family which consists of NR4A1 (NGIF-B/Nur77), NR4A2 (NOT/Nurr1), and NR4A3 (MINOR/NOR-1). All three members are transcription factors belonging to the steroid nuclear hormone receptor superfamily. They share high homology in their amino acid sequence with an aminoterminal region encoding activation function 1 (AF-1), followed by the DNA binding domain (DBD), and the ligand binding domain (LBD) (Deutsch et al., 2012). Recently, the oncogenic activities of NR4A2 are emerging. Usually, the NR4A2 as transcription factor which activation up regulates target genes leading to cell proliferation, survival and migration (Li et al., 2009; Zhang et al., 2009; Maijenburg et al., 2012). However, in this study we did not find a significant association of total NR4A2 expression with clinicopathological variables and overall survival in 84 patients with NPC. Interestingly, we found that the cytoplasmic NR4A2 expression in many of the NP tissue specimens was at low level. In many of our NPC tissue specimens, in contrast, a high cytoplasmic expression of NR4A2 was frequently detected, and the frequency of cytoplasmic NR4A2 overexpression increased with ascending of the T classification, N classification and clinical stage and descending of the overall survival of patients with NPC. Furthermore, Cox proportional hazard regression analysis showed that level of cytoplasmic NR4A2 was identified as independent prognostic factor for overall survival. These findings suggest the possibility that upregulated expression of cytoplasmic NR4A2 may provide a selective advantage in the occurrence and progression of NPC.

NR4A1, another NR4A family member, are implicated in cell growth/survival and apoptosis depending on the modulation of its presenting status. Besides transcription factor functions, NR4A1 can translocate to the cytoplasm and targeting mitochondria, thus triggering apoptosis (Li et al., 2000). Nuclear to cytoplasmic shuttling of NR4A1 has been suggested to be a molecular switch that dislodges the Bcl-2 BH4 domain, exposing its BH3 domain, which in turn blocks the activity of antiapoptotic Bcl-X(L) (Lin et al., 2004). Our results may indicate that the cytoplasmic NR4A2 overexpression apparently has an important role in the tumor progression and survival in patients with NPC. It can be hypothesized that cytoplasmic NR4A2 might promote cancer progression because the process of NR4A2 translocation into cytoplasm terminates its role as a nuclear transcription factor, similar to what is reported with NR4A1 (Inamoto et al., 2010). Being differenced with cytoplasmic NR4A1, cytoplasmic NR4A2 might target to non-mitochondria, and serve as a survival factor for the NPC cell. In a word, NR4A2 exerts a transcription-independent function in the occurrence and malignant progression of NPC.

In conclusion, this study shows the value of NR4A2 cytoplasmic expression as a potential marker of poor prognosis in NPC. NR4A2 cytoplasmic overexpression is associated with ascending of the T classification, N classification and clinical stage and descending of the overall survival, and provides independent prognostic information, and may represent a promising therapeutic target for patients with NPC. Further experiments will be required to clarify that the biological functions of NR4A2 and the exact molecular mechanism of NPC pathogenesis mediated by NR4A2.

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References

Acikalin MF, Etiz D, Gurbuz MK, et al (2012). Prognostic significance of galectin-3 and cyclin D1 expression in undifferentiated nasopharyngeal carcinoma. *Med Oncol*, **29**, 742-9.

Brennan B (2006). Nasopharyngeal carcinoma. *Orphanet J Rare Dis*, **1**, 23.

Ben Nasr H, Chahed K, Remadi S, Zakhama A, Chouchane L (2009). Expression and clinical significance of latent membrane protein-1, matrix metalloproteinase-1 and Ets-1 transcription factor in tunisian nasopharyngeal carcinoma patients. *Arch Med Res*, **40**, 196-203.

Bonta PI, Pols TW, van Tiel CM, et al (2010). Nuclear receptor Nurr1 is expressed in and is associated with human restenosis and inhibits vascular lesion formation in mice involving inhibition of smooth muscle cell proliferation and inflammation. *Circulation*, **121**, 2023-32.

Chua DT, Ma J, Sham JS, et al (2005). Long-term survival after cisplatin-based induction chemotherapy and radiotherapy for nasopharyngeal carcinoma: a pooled data analysis of two phase III trials. *J Clin Oncol*, **23**, 1118-24.

Chan AT (2010). Nasopharyngeal carcinoma. *Ann Oncol*, **21**, 308-12.

Cao XJ, Hao JF, Yang XH, et al (2012). Prognostic value of expression of EGFR and nm23 for locoregionally advanced nasopharyngeal carcinoma. *Med Oncol*, **29**, 263-71.

Deutsch AJ, Angerer H, Fuchs TE, Neumeister P (2012). The nuclear orphan receptors NR4A as therapeutic target in cancer therapy. *Anticancer Agents Med Chem*, **12**, 1001-14.

Holla VR, Mann JR, Shi Q, DuBois RN (2006). Prostaglandin E2 regulates the nuclear receptor NR4A2 in colorectal cancer. *J Biol Chem*, **281**, 2676-82.

Inamoto T, Czerniak BA, Dinney CP, Kamat AM (2010). Cytoplasmic mislocalization of the orphan nuclear receptor Nurr1 is a prognostic factor in bladder cancer. *Cancer*, **116**, 340-6.

Ke N, Claassen G, Yu DH, et al (2004). Nuclear hormone receptor NR4A2 is involved in cell transformation and apoptosis. *Cancer Res*, **64**, 8208-12.

Kitagawa N, Kondo S, Wakisaka N, et al (2013). Expression of seven-in-absentia homologue 1 and hypoxia-inducible factor 1 alpha: Novel prognostic factors of nasopharyngeal carcinoma. *Cancer Lett*, **331**, 52-7.

Li H, Kolluri SK, Gu J, et al (2000). Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science*, **289**, 1159-64.

Lin B, Kolluri SK, Lin F, et al (2004). Conversion of Bcl-2 from protector to killer by interaction with nuclear orphan receptor Nur77/TR3. *Cell*, **116**, 527-40.

Li XM, Huang WG, Yi H, Cheng AL, Xiao ZQ (2009). Proteomic analysis to identify cytokeratin 18 as a novel biomarker of nasopharyngeal carcinoma. *J Cancer Res Clin Oncol*, **135**, 1763-75.

Li X, Tai HH (2009). Activation of thromboxane A2 receptors induces orphan nuclear receptor Nurr1 expression and stimulates cell proliferation in human lung cancer cells. *Carcinogenesis*, **30**, 1606-13.

Lee HS, Bae EJ, Yi SH, et al (2010). Foxa2 and Nurr1 synergistically yield A9 nigral dopamine neurons exhibiting improved differentiation, function, and cell survival. *Stem Cells*, **28**, 501-12.

Maijenburg MW, Gilissen C, Melief SM, et al (2012). Nuclear receptors Nur77 and Nurr1 modulate mesenchymal stromal cell migration. *Stem Cells Dev*, **21**, 228-38.

Razak AR, Siu LL, Liu FF, et al (2010). Nasopharyngeal carcinoma: the next challenges. *Eur J Cancer*, **46**, 1967-78.

Sirin O, Lukov GL, Mao R, Conneely OM, Goodell MA (2010). The orphan nuclear receptor Nurr1 restricts the proliferation of haematopoietic stem cells. *Nat Cell Biol*, **12**, 1213-9.

Zhang T, Wang P, Ren H, Fan J, Wang G (2009). NGFI-B nuclear orphan receptor Nurr1 interacts with p53 and suppresses its transcriptional activity. *Mol Cancer Res*, **7**, 1408-15.

Zhou J, Xiao X, Yi H, et al (2012). Upregulation of Gp96 correlates with the radiosensitivity and five-year survival rate of nasopharyngeal carcinoma. *ORL J Otorhinolaryngol Relat Spec*, **74**, 164-71.