RESEARCH ARTICLE

Complement Receptor 1 Expression in Peripheral Blood Mononuclear Cells and the Association with Clinicopathological Features And Prognosis of Nasopharyngeal Carcinoma

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Abstract

Purpose: Complement receptor 1 (CR1) is induced by Epstein-Barr virus (EBV) and may be a potential biomarker of nasopharyngeal carcinoma (NPC). We conducted the present study to evaluate the association of CR1 expression with clinicopathological features and prognosis of NPC. Methods: We enrolled 145 NPC patients and 110 controls. Expression levels of CR1 in peripheral blood mononuclear cells (PBMCs) were detected using quantitative real-time PCR and associations with clinicopathological features and prognosis were examined. Results: CR1 levels in the NPC group [3.54 (3.34, 3.79)] were slightly higher than those in the controls [3.33 (3.20, 3.47)] (P<0.001). Increased CR1 expression was associated with histology classification (type III vs. type II, P=0.002), advanced clinical stage (P=0.003), high T stage (P=0.017), and poor overall survival (HR, 4.89; 95% CI, 1.23-19.42; P=0.024). However, there were no statistically significant differences in CR1 expression among N or M stages. Conclusion: These findings indicate that CR1 expression in PBMCs may be a new biomarker for prognosis of NPC and a potential therapeutic target.

Keywords: Complement receptor 1 - mRNA expression - NPC - PBMCs - prognosis

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Introduction

Complement receptor 1 (CR1, CD35), the receptor for C3b/C4b complement peptides, is presented on different blood cells (Wagner et al., 2006). It controls the proliferation of B-cells and plays roles in T-cell mediated immune regulation (Fingeroth et al., 1989). CR1 also exerts the function of cleaving various immune complexes and particles (Nicholson-Weller et al., 1999). Complement activation with subsequent deposition of complement components on tumor tissues has been demonstrated (Jurianz et al., 1999). Potential of CR1 as a diagnostic and prognostic marker is being increasingly realized (Khera et al., 2009).

Nasopharyngeal carcinoma (NPC) is an epithelial cancer with a high incidence in South China (Cao et al., 2011). This highly invasive and metastatic head and neck cancer is causally associated with Epstein-Barr virus (EBV) infection (Yoshizaki et al., 1999; Young et al., 2004; Li et al., 2007). It was reported that EBV-converted cell line (BL41/B95) had over expression of CR1, whereas no CR1 expression occurred on the corresponding EBV-negative cells (BL41) (Cohen et al., 1987). It was further found that endogenous latent membrane protein 1 in EBV-infected NPC cells induces multiple chemokines including interleukin 8 (Lai et al., 2010), which have been shown to upregulate CR1 in neutrophil leukocytes (Paccaud et al., 1990). Therefore, CR1 expression may represent the extent of EBV infection and associate with the development, progression, and prognosis of NPC. However, the tumor tissue of NPC is hard to collect to some extent and peripheral blood mononuclear cells (PBMCs) are of interest for its greater accessibility. Detection and prognosis value in PBMC gene expression profiles have been found in different types of tumors (Burczynski et al., 2005; Zhou et al., 2006).

In the present study, we evaluated the expression levels of CR1 in PBMCs among NPC patients and healthy subjects, and investigated the correlations between CR1 mRNA expression levels and the relevant clinical features and survival rates of NPC patients.

Materials and Methods

Study subjects

The study method was described in our previous report (He et al., 2011). The study subjects included 146 newly identified NPC patients and 110 healthy controls from the Sun Yat-sen University Cancer Center (SYSUCC); participants were recruited from September 2002 to May

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2006. The tumor stage was defined in accordance with the fifth edition of the International Union Against Cancer (UICC) TNM classification for NPC staging in 1997 (Sobin et al., 1997). All patients with biopsy-proven NPC had undergone routine checkups, including head and neck magnetic resonance imaging, chest X-rays, abdominal ultrasonographies, and bone scans, before treatment. All untreated NPC patients underwent a CT scan or MRI to confirm the initial tumor stage. The controls were subjects undergoing routine health examinations at the SYSUCC during the time when patients were being recruited. All subjects signed an informed consent form approved by the institutional review board of SYSUCC before participating in the study. Blood samples were collected from NPC patients before they received treatment. All of the patients with NPC were followed up until February 2010 by means of outpatient clinical examinations and telephone contacts. Among all of the patients, 93.8% (137/146) successfully completed the follow-up. The date and causes of death were provided by the patients' relatives.

Peripheral blood mononuclear cell preparation

Peripheral blood samples (10 ml) were collected from every subject into a Vacutainer sodium citrate cell purification tube, and PBMCs were isolated according to the manufacturer's protocol (QIAGEN). PBMCs were stored at -70°C before processing. Total RNA was isolated from PBMCs using TRIzol (Invitrogen) and then measured by spectrophotometric analysis. RNA was reverse-transcribed in a final volume of 15 μl; each reaction mixture contained 0.15 μg RNA in 1× RT-PCR buffer, 5.5 mM MgCl2, dNTPs (each at 500 μM), 2.5 μM random hexamers, 0.4 U/μl RNase inhibitor, and 3.125 U/μl MultiScribe Reverse Transcriptase (Applied Biosystems, Foster, CA, USA). The mixture was incubated for 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 95°C.

Quantitative real-time PCR for CR1

The primers and probes used for the CR1 (Hs00167075) and β-actin (Hs99999903) genes were obtained from Applied Biosystems. Quantitative real-time PCR was performed using a 384-well optic plate on an ABI PRISM 7900HT System (Applied Biosystems). The total reaction volume of 5 μl contained 2.2 μl of cDNA template (1:10 dilution), 1 × TaqMan Universal PCR Master Mix (without uracil-N-glycosylase), and 1 × Gene Expression Assay Mix, which included the primers and marked probes from Applied Biosystems Assays-on-Demand. The thermal cycling conditions were as follows: 95°C for 10 minutes to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Every sample was tested in triplicate. Two control samples were used in each plate to monitor inter-plate variations, which were less than 5%. The threshold cycle (Ct) was determined as 0.1 based on the amplification of the linear area for both the CR1 and β-actin genes. The normalized quantity of CR1 was calculated as $2^{-\Delta Ct}$, where ΔCt was obtained directly by subtracting the Ct value for the target gene from the Ct value for the β-actin gene. The final result was expressed as $2^{-\Delta Ct} \times 1{,}000$.

Statistics

Statistical analysis was performed using SPSS 13.0. The data were skewed toward the high value; they were presented as the median (25^{th}, 75^{th} centile) and analyzed by the Wilcoxon rank-sum test. Kaplan-Meier plots were used for survival analysis, with statistical significance measured by the log-rank test. The Cox proportional hazards model was used to calculate hazard ratios. All of the P-values were two-sided, and $P<0.05$ was considered statistically significant.

Results

Difference in CR1 mRNA expression between NPC patients and controls

The NPC group consisted of 110 males and 36 females, aged 23 to 75 years, with a mean age of 49.55±12.58 years. CR1 mRNA expression in one case failed to be detected; thus data of 145 cases were included in further analysis. The control group comprised 71 males and 39 females, with a mean age of 49.70±12.10 years. To determine

Table 1. Comparison of CR1 Expression Between Genders and Ages Among the Control Group

Variable	No. of subjects	Median CR1 mRNA level (25^{th}, 75^{th} centile)	P-value
Gender			
Male	71	3.31 (3.19, 3.47)	0.27
Female	39	3.36 (3.26, 3.50)	
Age (years)			
<50	65	3.32 (3.18, 3.43)	0.13
≥50	45	3.36 (3.26, 3.50)	

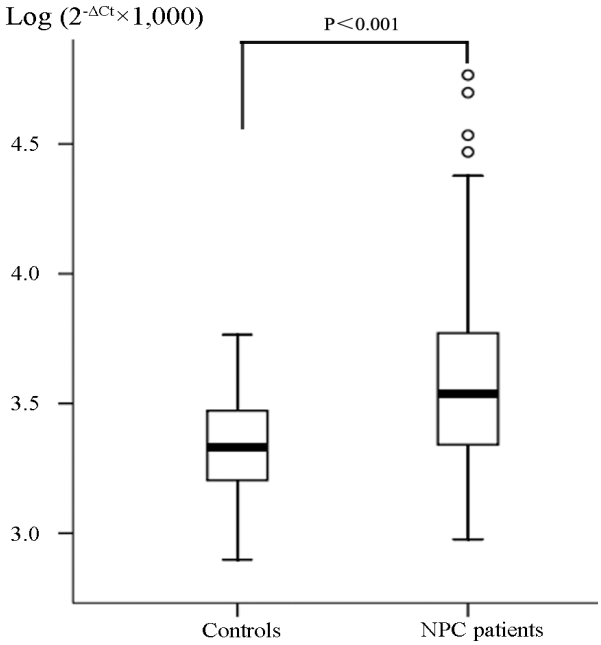

Figure 1. CR1 Expression in PBMCs from the NPC Patients and Controls. Results are shown for differences between NPC patients and healthy controls. P value was calculated with the Wilcoxon rank-sum test. Ct, threshold cycle; CR1, complement receptor 1; PBMCs, peripheral blood mononuclear cells

Table 2. Associations between the CR1 mRNA level in PBMCs and the clinicopathologic characteristics (histology, clinical stage) of NPC patients

Variable	No. of subjects	Median CR1 mRNA level (25^{th}, 75^{th} centile)	P-value
Histology (WHO classification)			
II	29	3.35 (3.23,3.63)	0.002
III	114	3.58 (3.39, 3.86)	
UICC clinical stage			
I-II	44	3.40 (3.21, 3.61)	0.003
III-IV	101	3.60 (3.40, 3.82)	
T stage			
1-2	61	3.42 (3.29, 3.76)	0.017
3-4	84	3.59 (3.40, 3.81)	
N stage			
0-1	96	3.50 (3.34, 3.77)	0.257
2-3	49	3.58 (3.37, 3.85)	
M stage			
0	138	3.54 (3.34, 3.79)	0.572
1	7	3.37 (3.30, 4.26)	

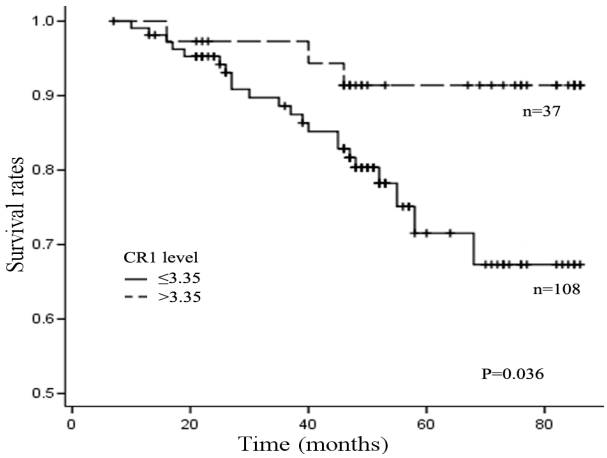

Figure 2. Kaplan-Meier Survival Curve for Overall Survival among NPC Patients by PBMCs CR1 Expression above and below 3.35. P value was calculated with the log-rank test. Solid line presents the lower expression; dash line presents the higher expression; vertical lines present censored patients. CR1: complement receptor 1; PBMCs: peripheral blood mononuclear cells

whether age and gender were associated with CR1 expression in PBMCs, the levels of CR1 were compared by gender and age in the control group (Table 1). Although females and older subjects had higher CR1 levels, these differences did not reach statistical significance, indicating that age and gender may not be confounding factors in this study. Therefore, we directly compared CR1 mRNA expression in PBMCs from NPC patients and controls, as shown in Figure 1. The CR1 level in the NPC group [3.54 (3.34, 3.79)] was significantly higher than that in the control group [3.33 (3.20, 3.47)] (P<0.001).

Correlation between CR1 mRNA level and clinical characteristics of NPC patients

As shown in Table 2, the expression of CR1 differed according to the histological classification; those classified as type III had significantly higher CR1 expression than did those classified as type II (P=0.002). NPC patients in a late clinical stage (III-IV) demonstrated markedly higher CR1 expression than did those in an early clinical stage (I-II) (P=0.003). Patients with a higher T stage (3-4) presented higher CR1 expression than did those with a lower T stage (1-2) (P=0.017). There was no statistically significant difference in CR1 expression between N stages and M stages.

Association of CR1 expression with overall survival

All NPC patients underwent routine radiotherapy. The average follow-up period was 55 months. At the last follow-up, 25 patients had died of NPC. The best cutoff value for the expression of CR1 in PBMCs was 3.35. As shown by the Kaplan-Meier plot (Figure 2), the fatalities for patients with CR1 levels ≤3.35 and >3.35 were 8.1% (3/37) and 20.4% (22/108), respectively, and this difference was statistically significant based on the log-rank test (P=0.036). CR1 expression in PBMCs was found to be an independent prognostic factor for overall survival after adjustment for gender, age, histology, T stage, N stage, and M stage, using a multivariate Cox regression model. Patients demonstrating higher CR1 expression levels (>3.35) had a greater risk of death than did those with lower CR1 expression levels (≤3.35; HR, 4.89; 95% CI, 1.23-19.42; P=0.024).

Discussion

The complement system is a major component of the innate immune system and efficiently protects the host from pathogenic microorganisms and foreign molecules (Walport, 2001a; 2001b; Fishelson et al., 2003). Complement activation elicits recruitment and degranulation of leukocytes, smooth muscle contraction, and increase of vascular permeability and induces proinflammatory conditions that affect cell surface molecules on leukocytes as well as on endothelial cells, which may harm the host by inducing inflammatory tissue destruction (Fishelson et al., 2003). Membrane-bound complement regulatory proteins (mCRPs) act as balanced factors to restrict the action of complement at critical stages of the cascade reaction. Expression of the mCRPs protects normal cells and tissues but also malignant cells from complement attack (Fishelson et al., 2003). CR1, together with other mCRPs, contribute to the control of complement component 3 (C3), a central component in the system (Fishelson et al., 2003). It acts as a cofactor for factor I in cleavage of C3b and binds to C4b to facilitate its degradation (Medicus et al., 1983). CR1 also interferes with complement activation by accelerating the decay of the C3/C5 convertases and functions as a receptor for binding immune complexes or microorganisms (Iida et al., 1983). CR1 is mainly detected on circulating cells, such as erythrocytes and most types of leukocytes (Fischer et al., 1986). The CR1 on leukocytes facilitates phagocytosis of opsonized microorganisms (Ehlenberger et al., 1977). Moreover, CR1 might serve as a barrier to prevent some B cells maturing to plasma cells (Erdei et al., 2009). It has drawn considerable attention for CR1 to be a diagnostic and prognostic marker as well as a therapeutic target (Fishelson et al., 2003; Markiewski et al., 2009). The present study is the first to investigate the association of

CR1 expression levels in PBMCs with NPC and show that CR1 may be related to the development, progression, and unfavorable prognosis of NPC, in accordance with the above findings.

There were other studies on the associations of malignant tumors with soluble CR1 or the derivates. The plasma CR1, which is probably released from the surface of leukocytes, was found to be increased in B cell lymphoma, Hodgkin's lymphoma , and chronic myeloproliferative syndromes (Pascual et al., 1993). SiC3b, the C3b cleavage products by CR1, increased in pancreatic adenocarcinoma patients (Marten et al., 2010). In addition, the CR1 genetic polymorphisms have also been discovered to be associated with gall bladder cancer susceptibility and tumor metastasis (Jiao et al., 2004; Srivastava et al., 2009). All these previous population data support our findings and are in agreement with the results from the experimental studies.

Circulating cells can be regarded as scouts that continuously survey the body for signs of pathogenesis. The gene expression in circulating cells can potentially provide early warnings of pathogenesis. Furthermore, because of their clinical accessibility, circulating blood cells are very useful for assessing disease-related changes in gene expression. Bushel et al. have shown that gene expression data from PBMCs can predict environmental exposure levels (Bushel et al., 2007). Because most diseases, including cancers, result from interactions between genetic and environmental factors, gene expression data for PBMCs can be used as biomarkers for the early detection of diseases and for the identification of clinicopathological features and estimation of prognosis. Recent reports have strongly suggested that gene expression profiling in PBMCs is a valuable tool for investigating pathologic processes that affect cellular components of peripheral blood as well as distant organs (Burczynski et al., 2006; Liew et al., 2006; Grigoryev et al., 2008). In NPC, the tumor tissue is particularly hard to access; consequently, gene expression profiling in PBMCs becomes a useful surrogate. Moreover, gender and age differences of the CR1 expression in PBMCs were not observed in the present study, reflecting that the CR1 expression in PBMCs may be a stable and effective biomarker for the early detection, identification of clinical features, and prognosis estimation of NPC.

We recognize that this is not a prospective study and can not determine the time sequence of elevated CR1 and NPC initiation. It would be ideal to recruit subjects with active EBV infection who are at a high risk for developing NPC and follow the CR1 expression in PBMCs over time. It would also be important and interesting to assess the CR1 levels in tumor tissues and serum/plasma as well as in PBMCs. There are definitely other factors associated with clinical characteristics and prognosis of NPC, and more comprehensive studies including molecular markers other than CR1, such as EBV DNA, cytoeratins, cytokines, are warranted.

In summary, we found that PBMCs from NPC patients had significantly higher CR1 expression than did those from healthy controls; this higher expression correlated with clinicopathologic features and overall survival. These findings increase our understanding of the mechanisms of CR1 in the pathogenesis of NPC and suggest that CR1 expression in PBMCs may be a new biomarker for prognosis of NPC and a potential therapeutic target.

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