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Increased expression of CD55 correlates with tumor progression and poor prognosis in nasopharyngeal carcinoma

Abstract

Purpose: To analyze the expression of complement delay-accelerating factor (CD55) in nasopharyngeal carcinoma and its correlation with clinicopathologic features, including survival rate.

Methods: Eighty-two nasopharyngeal carcinoma tissues were evaluated for CD55 expression using immunohistochemistry. The association between CD55 expression and various clinicopathological characteristics including overall survival was analyzed.

Results: Immunohistochemical analysis revealed that the protein expression of CD55 detected in nasopharyngeal carcinoma tissues was higher than that in the normal nasopharyngeal tissue ($P=0.003$). In addition, high levels of CD55 protein were positively correlated with the status of lymph node metastasis ($P=0.02$) and distant metastasis ($P=0.01$), and clinical stage ($P=0.002$) of nasopharyngeal carcinoma patients. Patients with positive CD55 expression had a significantly shorter overall survival time than did patients with negative CD55 expression ($P=0.001$). Multivariate analysis suggested that the expression pattern of CD55 protein was an independent prognostic indicator ($P=0.009$) for the survival of patients with nasopharyngeal carcinoma.

Conclusion: The data from this study suggest, for the first time, that CD55 is frequently expressed in nasopharyngeal carcinoma and its expression is associated with decreased patient survival; therefore, CD55 expression may be a potential unfavorable prognostic factor for patients with nasopharyngeal carcinoma.

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Nasopharyngeal carcinoma (NPC) is a common Epstein Barr Virus (EBV)-associated cancer with remarkable geographic and racial distributions worldwide [1]. It is highly prevalent in South China where its annual incidence rate is 25-50 cases per 100,000 populations [2]. NPC is a particular type of squamous carcinoma of head and neck. A recent study reported that the etiology of NPC includes viral, genetic, and environmental factors [3]. The primary anatomical site of tumor growth is located in a cryptic area and the disease is usually asymptomatic at early stages. Most NPCs are undifferentiated or poorly differentiated with the following characteristics: fast growth and a great tendency to invade adjacent regions as well as metastasize to regional lymph nodes and distant organs [4]. The molecular mechanism of the development and progression of NPC is still poorly understood; therefore, it is of great interest to find both effective biomarkers for early diagnosis of as well as therapeutic targets for this malignancy.

CD55 (complement decay-accelerating factor; or decay-accelerating factor, DAF) is a glycosylphosphatidylinositol-anchored protein that protects cells from complement-mediated attack [5]. It was described for the first time in 1969 as an erythrocyte surface protein that regulated complement system activation [6]. CD55 is present on all cells that are exposed to complement, including red blood cells, leukocytes, endothelial cells and epithelial cells. Moreover, soluble CD55 is detectable in plasma, tears, saliva, and urine, as well as in synovial and cerebrospinal fluids [7]. Complement attack is a powerful innate mechanism in the protection of host against pathogens, including cancer. In recent years, several studies have demonstrated that CD55 expressed in clinical samples from various kinds of malignant tumors. CD55 expression level is higher in both differentiated and undifferentiated gastric adenocarcinomas in comparison with normal gastric epithelium [8]. The staining intensity in prostatic carcinoma is also higher than in normal prostate epithelium. The knocked-down expression of CD55 reduces tumorigenicity of prostatic adenocarcinoma cell line in severe combined immunodeficient mice [9]. Sakuma *et al.* found that CD55 expression in non-small cell lung cancer cells was closely correlated with histological types, prognosis and neoadjuvant chemotherapy of the disease [10]. These findings suggest that CD55 plays an important role in tumorigenicity of cancer cells.

Despite the importance of CD55 in other malignancies, its expression has not yet been analyzed in NPC. In order to clarify the role of CD55 in the pathogenesis of NPC, our study investigated the correlation of CD55 protein expression with clinicopathologic features, including patient survival.

Materials and Methods

Patients and Tissue Samples

This study was approved by the Research Ethics Committee of Department of Pathology of Southern Hospital, Southern Medical University, Guangzhou, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to the ethical and legal standards.

Paraffin-embedded samples of 82 NPC tissues and 16 non-cancerous nasopharyngeal tissues were obtained under fiberoptic nasopharyngoscopy between 2000 and 2005 from the Department of Pathology of Southern Hospital. The control subjects were individuals presenting with otolaryngologic-related, non-neoplastic diseases. In the 82 NPC cases, there were 58 male and 24 female patients, with ages ranging from 16 to 82 years (median, 49.5 years). The routine staging workup included a detailed clinical examination of the head and neck, fiberoptic nasopharyngoscopy, computed tomography (CT) imaging of the entire neck from the base of the skull, chest radiography, abdominal sonography, a complete blood count and a biochemical profile. All specimens had confirmed pathological diagnosis and were staged according to the 1997 NPC staging system of the WHO. None of the patients recruited in this study had chemotherapy or radiotherapy before the surgery. After the surgery, all patients were treated with definitive radiotherapy (cumulative dose of external beam radiotherapy ≥ 64.8 Gy). Patients whose tumor stages were ≥ 3 received additional cisplatin based concurrent chemoradiotherapy. For all enrolled patients, pathology records were retrieved from pathologic databases and medical records and reviewed for confirmation of the NPC diagnosis. Information on stage, treatment, follow-up and limited information on family history were collected from hospital tumor registries and medical files. The clinical and pathologic parameters were obtained from the pathological reports and presented in Table 1.

Patients were followed-up at three month intervals during the first three years after therapy and at six month intervals thereafter. The median follow-up time for overall survival was 60.8 months for patients, and ranged from 10 to 72 months. Patients who died from diseases other than NPC or from unexpected events were excluded from this study.

Immunohistochemistry analysis

Immunohistochemical study for CD55 was performed on formalin-fixed, paraffin-embedded, 4 μ m-thick tissue sections using the avidin-biotin-peroxidase complex method. In brief, the sections were deparaffinized and dehydrated using a graded

TABLE 1. Correlations of CD55 expressions with the clinicopathological features of nasopharyngeal carcinoma

Clinicopathological features	No. of cases (%)	CD55		P
		Positive (%)	Negative (%)	
Age (years)				
≥50	40 (48.8)	30 (75.0)	10 (25.0)	NS
<50	42 (51.2)	36 (72.3)	6 (27.7)	
Gender				
Male	58 (70.7)	48 (82.8)	10 (17.2)	NS
Female	24 (29.3)	18 (75.0)	6 (25.0)	
Smoking				
Yes	20 (24.4)	15 (75.0)	5 (25.0)	NS
No	62 (75.6)	51 (82.3)	11 (17.7)	
T classification				
T1~2	58 (70.7)	48 (82.8)	10 (17.2)	NS
T3~4	24 (29.3)	18 (75.0)	6 (25.0)	
N classification				
N1~2	37 (45.1)	26 (70.3)	11 (19.7)	0.02
N3	45 (54.9)	40 (88.9)	5 (11.1)	
Distant metastasis				
Yes	4 (5.1)	4 (100.0)	0 (0)	0.01
No	78 (94.9)	62 (79.5)	16 (20.5)	
Clinical stage				
I~II	28 (34.1)	17 (60.7)	11 (39.3)	0.002
III~IV	54 (65.9)	49 (90.7)	5 (9.3)	

series of ethanol solutions. Endogenous peroxidase activity was halted through the administration of 0.3% hydrogen peroxidase and methanol for 20 min. After having been rinsed in phosphate-buffered saline (PBS), the tissue sections were processed in a 0.01 M citrate buffer (pH 6.0) inside a heat-resistant plastic container. Sections were then irradiated in a domestic microwave oven for 20 min. After microwave irradiation, the slides were allowed to cool at room temperature. The following antibody were applied as primary antibodies; rabbit anti-CD55 antibody (1: 35, sc-9156, Santa Cruz Biotechnologies, Santa Cruz, CA, USA). The sections were incubated with these primary antibodies overnight at 4°C followed by the secondary antibody. The results were visualized with diaminobenzidine. In each immunohistochemistry run, negative controls were carried out by omitting the primary antibody, whereas CD55 overexpression confirmed by western blotting was used as positive controls.

Following a hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists, who were blinded to the clinicopathological parameters and clinical

outcomes of the patients. The scores of the two pathologists were compared and any discrepant scores were trained through re-examining the stainings by both pathologists to achieve a consensus score. The number of positive-staining cells showing immunoreactivity on the membrane for CD55 in ten representative microscopic fields was counted and the percentage of positive cells was calculated. The percentage scoring of immunoreactive tumor cells was as follows: 0 (0%), 1 (1-10%), 2 (11-50%) and 3 (>50%). The staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). A final score was obtained for each case by multiplying the percentage and the intensity score. Therefore, tumors with a multiplied score exceeding 4 (i.e., tumors with a moderate or strong staining intensity of >10% of the tumor cells) were deemed to be positive expression of CD55; all other scores were considered to be negative.

Statistical analysis

SPSS version 12.0 for Windows (SPSS Inc, IL, USA) and SAS 9.1 (SAS Institute, Cary, NC) was used for statistical analysis. The association of the CD55 expression with the clinicopathological characteristics was analyzed using χ^2 tests. Univariate survival analysis for the influence of the CD55 expression on the overall survival was estimated according to the Kaplan-Meier method. The significances of various variables in survival were analyzed using multivariate cox proportional hazards model. Differences were considered statistically significant when p was less than 0.05.

Results

Immunohistochemical findings of CD55

The expression levels and subcellular localization of CD55 protein in 82 archived paraffin-embedded NPC samples and 16 non-cancerous nasopharyngeal samples were detected using immunohistochemical staining. Specific CD55 protein staining was found on the membrane of tumor cells (Figure 1A), whereas in the non-cancerous epithelial cells, CD55 staining was absent or only weakly positive (Figure 1B). Furthermore, in 80.5% (66/82) of NPC samples, CD55 protein was observed to be positive expressed. In comparison, only 31.3% (5/16) of non-cancerous nasopharyngeal samples had positively expressed CD55 protein, significantly lower than that in the NPC samples ($p=0.003$).

3.2 Association of the CD55 expressions with the clinicopathological characteristics

The association of CD55 expression patterns with the clinicopathological characteristics of patients with NPC is

FIGURE 1. Immunohistochemical expression of CD55 protein in nasopharyngeal carcinoma and non-cancerous nasopharyngeal samples (original magnification 400×). (A) Strong staining of CD55 in nasopharyngeal carcinoma samples; (B) Absent or only weakly positive expression of CD55 in non-cancerous nasopharyngeal samples.

summarized in Table 1. A significant association of CD55 expression levels with patient's age, sex, smoking and tumor size (T classification) was not observed in 82 NPC cases; however, the expression level of CD55 was positively correlated with the status of lymph node metastasis (N classification) (N0-N1 vs. N2- N3, $P=0.02$) and distant metastasis (No vs. Yes, $P=0.01$), and clinical stage (I-II vs. III-IV, $P=0.002$) in NPC patients (Table 1).

Influence of the CD55 expressions on survival

To investigate the prognostic value of CD55 expression for NPC, the association between the expression patterns of CD55 protein and patients' survival was assessed using Kaplan-Meier analysis using the log-rank test. In 82 NPC cases with prognosis information, the expression of CD55 protein was significantly correlated with the overall survival of NPC patients (Figure 2). Patients with positive CD55 expression had poorer survival than those with negative CD55 expression ($P=0.001$). In addition, T, N, M classifications and clinical stages were also significantly correlated with patients' survival ($P=0.02$, $P<0.001$, $P=0.001$, and $P<0.001$ respectively). To

FIGURE 2. Kaplan-Meier survival curves of patients with nasopharyngeal carcinoma according to the expressions of CD55. The patients with positive CD55 ($P=0.001$) expression tumor showed significantly poorer overall survival rates than those without its expression.

determine whether CD55 is an independent prognostic factor for NPC, multivariate analysis of the levels of CD55 protein expression, adjusted for age, gender, smoking status, T classification, N classification, M classification, and clinical stages of NPC patients, were performed. The results showed that the expression pattern of CD55 protein ($P=0.009$) was an independent prognostic factor for NPC (Table 2).

Discussion

NPC is an endemic carcinoma in Southern China, Taiwan, and Southeast Asia. More than 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 [11]. Although the prognosis of NPC with early clinical

TABLE 2. Univariate and multivariate analysis of prognostic factors in patients with nasopharyngeal carcinoma

Prognostic factors	Univariate analysis		Multivariate analysis	
	Relative risk (95% confidence interval)	P	Relative risk (95% confidence interval)	P
Age	1.8 (0.9-4.0)	0.1	1.6 (0.8-3.6)	0.3
Gender	1.8 (0.8-4.3)	0.1	1.2 (0.5-3.0)	0.4
Smoking	2.0 (1.0-4.8)	0.09	1.8 (0.8-3.8)	0.3
T classification	2.3 (1.2-4.6)	0.02	1.5 (0.7-3.6)	0.1
N classification	4.3 (2.1-9.1)	<0.001	2.9 (1.8-5.6)	0.003
M classification	5.2 (1.7-15.8)	0.001	2.4 (1.6-4.6)	0.01
Clinical stage	5.2 (2.0-14.6)	<0.001	2.8 (1.8-5.2)	0.004
CD55 expression	5.2 (1.6-15.3)	0.001	2.5 (1.6-4.8)	0.009

stage with chemoradiotherapy is relatively good, there are still a fraction of patients who will develop regional relapse or distant metastasis after treatment. Therefore, it is necessary to search for simple and cost-effective prognostic factors to establish risk-adapted treatment for NPC. In this study CD55 was found to be expressed predominantly in the NPC tissues by immunohistochemistry assay, which was consistent with other studies on prostate cancer, breast cancer and colon cancer [8-10,12-14]. CD55 was also found to be overexpressed in NPC tissues compared with nasopharyngeal tissues, suggesting that CD55 is involved in the pathogenesis of NPC.

The complement system provides a powerful means to control both pathogenic organisms and clearance of apoptotic cells. It is an enzymatic cascade consisting of 30 proteins found in plasma and on cell surfaces and three activation pathways, which results in the release of proinflammatory anaphylatoxins, in C3b deposition and in the formation of the membrane attack complex and finally leading to cell lysis [15]. CD55 is one of the complement regulatory proteins that binds to C3 convertases of both the classical and alternative complement pathways and protects cells against bystander attack from complement. It is composed of four external small consensus repeats and is glycosylphosphatidylinositol (GPI)-anchored to the cell membrane [16]. Recently, CD55 has been shown to be deposited in the tumor microenvironment, to contribute to metastasis and to play an important role in tumorigenesis [17-18]. *In vitro*, colorectal tumor cell lines expressing CD55 and endothelial cells deposit CD55 into the extracellular matrix [19]. This has already been detected in many different malignancies, including colorectal cancer, gastric cancer, thyroid cancer, medullary thyroid cancer, malignant glioma, breast cancer, renal cancer, non-small cell lung cancer, ovarian cancer, cervical cancer and prostate cancer [8-10,12-14]. CD55 has also been found in the metastases of colorectal carcinomas. Moreover, studies have shown that it is frequently overexpressed within the stroma of colorectal tumors, suggesting that CD55 comes from the tumor cells and is either released from the cell membrane into the environment by enzymatic cleavage or is secreted by the tumor cells as a soluble peptide [20]. Recently, the expression of CD55 has been demonstrated as an essential requisite for cancer development and progression came from studies on prostate cancer cells *in vitro* and *in vivo* [21]. In this study, CD55 overexpression was significantly associated with N classification (lymph node metastasis), M classification (distant metastasis) and clinical stages of NPC patients. Overexpressed CD55 in NPC may accelerate tumor growth by inducing angiogenesis and enhancing local cell invasion and metastasis. Our results indicate that CD55 plays a significant role in NPC progres-

sion, including tumor invasion and metastasis. Yet, due to our limited sample size, with only four patients with distant metastatic disease, the observed association of CD55 with NPC metastasis should be verified by further studies.

CD55 is expressed by cells to protect them from bystander attack by complement, but it has been shown that the presence of even a small population of cells strongly expressing CD55 is a poor prognostic factor in a series of cancers. For example, Ikeda *et al.* reported that strong CD55 expression is sufficient to predict poor prognosis in patients with breast cancer [22]. In contrast, Madjd *et al.* reported that loss of CD55 expression in breast cancer is associated with a worse prognosis [23]. In colorectal cancer, Durrant *et al.* prospectively analyzed the correlation between CD55 expression and seven year survival in 136 patients, and found that patients with tumors expressing high levels of CD55 had a significantly worse survival than patients with low CD55 levels [24]. In the present study, CD55 protein expression in NPC was found to be inversely correlated with patients' overall survival: the patients with positive expression of CD55 protein had shorter survival times. According to multivariate analyses, increased expression of CD55 protein was a significant predictor of poor prognosis for NPC patients, especially for patients with late-stage cancers. These results were analogous to recent reports on other malignancies.

In conclusion, our data shows that a subset of patients with NPC had upregulation of CD55, which was associated with an aggressive clinical course and poor overall survival, and was consistent with studies on other cancers. This is the first report to suggest a relationship between CD55 expression and prognosis in patients with NPC, and further prospective analysis is warranted.

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