Received: 1 September 2023 | Revised: 26 December 2023 | Accepted: 4 January 2024

DOI: 10.1111/cas.16079

Cancer Science WILEY

ORIGINAL ARTICLE

Soluble CD27 as a predictive biomarker for intra-tumoral CD70/CD27 interaction in nasopharyngeal carcinoma

Toshihiro Nagato[1] | Hiroki Komatsuda[1,2] | Ryusuke Hayashi[2] | Miki Takahara[2,3] | Nanami Ujiie[1,4] | Akemi Kosaka[1] | Takayuki Ohkuri[1] | Kensuke Oikawa[1] | Ryosuke Sato[2] | Risa Wakisaka[2] | Michihisa Kono[2] | Hidekiyo Yamaki[2] | Kenzo Ohara[2] | Takumi Kumai[2,3] | Kan Kishibe[2] | Akihiro Katada[2] | Tatsuya Hayashi[2] | Hiroya Kobayashi[1]

[1]Department of Pathology, Asahikawa Medical University, Asahikawa, Japan

[2]Department of Otolaryngology-Head and Neck Surgery, Asahikawa Medical University, Asahikawa, Japan

[3]Department of Innovative Head and Neck Cancer Research and Treatment, Asahikawa Medical University, Asahikawa, Japan

[4]Department of Thoracic Surgery and Breast Surgery, Asahikawa Medical University Hospital, Asahikawa, Japan

Correspondence
Toshihiro Nagato and Hiroya Kobayashi, Department of Pathology, Asahikawa Medical University, 2-1-1-1 Midorigaoka-Higashi, Asahikawa 078-8510, Japan.
Email: rijun@asahikawa-med.ac.jp and hiroya@asahikawa-med.ac.jp

Funding information
Japan Society for the Promotion of Science, Grant/Award Number: 18K09310, 21K09599 and 22H02838

Abstract

In CD70-expressing tumors, the interaction of CD70 on tumor cells with its lymphocyte receptor, CD27, is thought to play a role in immunosuppression in the tumor microenvironment and elevated serum levels of soluble CD27 (sCD27). Previous studies showed that CD70 is expressed in nasopharyngeal carcinoma (NPC), an Epstein–Barr virus (EBV)-related malignancy. However, the association between intratumoral CD70/CD27 expression and serum levels of sCD27 in NPC remains unclear. In the present study, we show that CD70 is primarily expressed by tumor cells in NPC and that CD27-positive lymphocytes infiltrate around tumor cells. NPC patients with CD27-positive lymphocytes had significantly better prognosis than patients lacking these cells. In addition, high CD70 expression by tumor cells tended to be correlated with shorter survival in NPC patients with CD27-positive lymphocytes. Serum sCD27 levels were significantly increased in patients with NPC and provided good diagnostic accuracy for discriminating patients from healthy individuals. The concentration of serum sCD27 in patients with CD70-positive NPC with CD27-positive lymphocytes was significantly higher than in patients with tumors negative for CD70 and/or CD27, indicating that the intratumoral CD70/CD27 interaction boosts the release of sCD27. Furthermore, positive expression of CD70 by NPC cells was significantly correlated with EBV infection. Our results suggest that CD70/CD27-targeted immunotherapies may be promising treatment options and that sCD27 may become an essential tool for evaluating the applicability of these therapies by predicting the intratumoral CD70/CD27 interaction in NPC.

Abbreviations: Ab, antibody; AUC, area under the curve; EBER, Epstein–Barr virus-encoded small RNA; EBV, Epstein–Barr virus; FBS, fetal bovine serum; FFPE, formalin-fixed paraffin-embedded; LMP1, latent membrane protein 1; NK, natural killer; NPC, nasopharyngeal carcinoma; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; RCC, renal cell carcinoma; ROC, receiver-operating characteristic; sCD27, soluble CD27; SCID, severe combined immunodeficiency; SHO, severe combined immunodeficiency hairless outbred; TIL, tumor-infiltrating lymphocyte; TNF, tumor necrosis factor; Treg, regulatory T cell; WHO, World Health Organization.

Toshihiro Nagato and Hiroki Komatsuda contributed equally to this work.

KEYWORDS
cancer immunotherapy, CD27, CD70, nasopharyngeal carcinoma, soluble form

1 | INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy arising from the nasopharyngeal mucosa, and the disease is more common in East and Southeast Asia than in the Western world.[1] NPC is an Epstein–Barr virus (EBV)-related malignancy, given that clonotypic EBV genomes and EBV oncogenic proteins are detected in most NPC patients in endemic areas such as southern China.[1] Furthermore, many reports to date have shown an important association between persistent EBV infection and EBV gene products and tumor initiation, promotion, and progression in NPC.[1–3] With regard to the histological characteristics of NPC, many immunocompetent cells infiltrate the tumor stroma, including lymphocytes, granulocytes, and macrophages,[4] suggesting that interactions between tumor cells and infiltrating immune cells via cell surface molecules may be an important contributor to malignancy.

CD70 is a member of the tumor necrosis factor (TNF) superfamily and the only known ligand for the CD27 receptor. Normal cells exhibit limited expression of this ligand, but it is transiently upregulated on activated T and B cells and mature dendritic cells.[5,6] By contrast, CD27, which is a TNF receptor superfamily member and a costimulatory molecule, is expressed on several lymphocyte subsets, including naïve and activated T cells, memory B cells, and natural killer (NK) cells.[5,6] Interaction between CD70 and CD27, at the correct time and intensity, induces the activation and proliferation of T and B cells, although the signaling pathway is tightly regulated under normal physiological conditions through strictly controlled expression of CD70.[7,8] In contrast to physiological conditions, CD70 is abundantly and persistently expressed by tumor cells in some malignancies, such as renal cell carcinoma (RCC), glioblastoma, lung carcinoma, and various leukemias and lymphomas.[5,6] Constitutive expression of CD70 on tumor cells may elicit the induction of apoptosis and/or exhaustion of CD27-expressing tumor-infiltrating lymphocytes (TILs) by suppressing the proper immune response via continuous CD70/CD27 ligation,[5,6,9,10] thereby enabling tumor cells to evade the antitumor immune response. Thus, blockade of the CD70/CD27 axis, as well as the use of direct killing agents capable of targeting CD70 on tumor cells, may be attractive therapeutic options for treating CD70-positive malignancies.

Another important aspect of the CD70/CD27 interaction is that soluble CD27 (sCD27) is cleaved from the extracellular domain of CD27 upon ligation of CD27 to CD70.[5,6] Indeed, high levels of sCD27 have been found in the serum of patients with some CD70-expressing tumors,[9–13] suggesting that measurement of serum sCD27 levels may be useful for predicting the interaction between CD70-positive tumor cells and CD27-positive TILs in order to determine the indication of CD70/CD27-targeted immunotherapies. Considering the importance of the CD70/CD27 interaction, detailed analyses of the expression of these molecules in malignant diseases would be of great interest to elucidate the tumor immune escape mechanism and develop immunological therapeutic strategies. To date, only a few studies of NPC have addressed the expression of CD70 and CD27.[14–17] However, the association between the expression of these molecules in NPC and patient clinicopathological characteristics and prognosis remains unclear. Furthermore, little is known about the expression of sCD27 in the serum of patients with NPC.

In the present study, we examined the expression of CD70 and CD27 in NPC patients using immunohistochemistry. We also assessed the correlation between CD70/CD27 expression and the major clinical features and prognosis of patients with NPC. Additionally, we investigated whether sCD27 can be detected in the serum of patients with NPC, and if so, whether serum sCD27 levels are associated with intratumoral CD70 and CD27 expression. Finally, we assessed the relationship between EBV infection and EBV oncoprotein latent membrane protein 1 (LMP1) and the induction of CD70 expression in NPC cells.

2 | MATERIALS AND METHODS

2.1 | Patients

Forty-one patients with NPC were analyzed in this study, all of whom were newly diagnosed at Asahikawa Medical University between 1999 and 2016. Patient clinicopathological characteristics are summarized in Table 1. TNM classification was determined according to the 2009 Union Internationale Contre Le Cancer classification.[18] The patients were classified into 3 histologic subtypes defined by the World Health Organization (WHO): keratinizing squamous cell carcinoma (type I), non-keratinizing carcinoma (type II), and undifferentiated carcinoma (type III). As the primary treatment, 27 patients received alternating chemoradiotherapy, as described elsewhere.[19] Seven patients received platinum-based concurrent chemoradiotherapy. Seven patients received radiotherapy due to increased age (>70 years) and/or renal dysfunction. We also analyzed 23 healthy volunteers as controls. Informed consent was obtained using the opt-out method on the Asahikawa Medical University website. This study was conducted with the approval of the Institutional Review Board at Asahikawa Medical University (#19222).

2.2 | Cell lines

C666-1 EBV-positive NPC cells were kindly provided by Drs. Tomokazu Yoshizaki and Satoru Kondo (Kanazawa University).

TABLE 1 Clinicopathological characteristics of patients with nasopharyngeal carcinoma.

Characteristics	Cases ($n=41$)
Age (year)	
Median (range)	63 (22–88)
Gender	
Female	9
Male	32
WHO histologic type	
I	4
II	21
III	16
EBER expression	
Negative	12
Positive	29
Tumor classification	
T1	12
T2	9
T3	5
T4	15
Node classification	
N0	8
N1	12
N2	17
N3	4
Metastasis classification	
M0	39
M1	2
Stage	
I	1
II	11
III	10
IV	19
Treatment	
ACRT	27
CCRT	7
RT	7

Abbreviations: ACRT, alternating chemoradiotherapy; CCRT, concurrent chemoradiotherapy; EBER, Epstein–Barr virus-encoded small RNA; RT, radiotherapy; WHO, World Health Organization.

TW03 EBV-negative NPC cells were kindly provided by Dr. Eva Klein (Karolinska Institute). C666-1 and TW03 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. SNK-6 extranodal NK/T-cell lymphoma, nasal-type cells were kindly provided by Dr. Norio Shimizu (Tokyo Medical and Dental University) and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 700 U/mL recombinant human interleukin-2.

2.3 | Immunohistochemistry and in situ hybridization

Formalin-fixed, paraffin-embedded (FFPE) samples were prepared from pretreatment biopsy tissues of patients with NPC or from subcutaneous tumors of mice transplanted with C666-1 cells and then cut into 4-μm-thick sections. The primary antibodies (Abs) included anti-CD70 (301731, 1:50; R&D Systems), anti-CD27 (137B4, ready-to-use; abcam plc), and anti-LMP1 (CS1-4, 1:400; Dako) monoclonal Abs. The Envision HRP System (Dako) was used for visualization of signals. For antigen retrieval, slides were treated with Target Retrieval Solution (pH 9; Dako) using a Decloaking Chamber (Biocare Medical) for 10 min at 110°C. Serial sections were used for CD70 and CD27 or CD70 and LMP1 staining. A case was considered CD70 positive if >10% of the tumor cells were positive for CD70, as described previously by other researchers and us.[11,13] In addition, the degree of CD70 staining intensity was classified as follows: no, weak, moderate, and strong. In the analysis to evaluate the relevance of CD70 expression to survival, moderate and strong staining was defined as high CD70 expression, and no or low staining was defined as low CD70 expression. When >5% of tumor-infiltrating mononuclear immune cells were positive for CD27, the specimen was defined as CD27 positive.[13] The presence of EBV-encoded small RNA (EBER) in FFPE tissue sections was detected using in situ hybridization, as described previously.[20,21]

2.4 | Coculture of peripheral blood mononuclear cells (PBMCs) with NPC cell lines

NPC cells were seeded into a 24-well plate at a density of 5×10^4 cells/well in 1 mL of RPMI 1640 medium supplemented with 10% FBS 24 h prior to adding PBMCs. PBMCs from a healthy individual were isolated by density gradient centrifugation using Lymphoprep (Serumwerk Bernburg AG). A total of 1×10^6 PBMCs were added in 1 mL of RPMI 1640 medium supplemented with 10% FBS. PBMCs and NPC cells were maintained in monoculture in parallel. After 48 h, the supernatants were collected for sCD27 measurement.

2.5 | Measurement of sCD27 using ELISA

sCD27 levels in serum and cell culture supernatant were quantified using the PeliKine Compact™ human soluble CD27 ELISA kit (Sanquin) and Human CD27/TNFRSF7 DuoSet ELISA (R&D Systems), respectively. Blood was collected from patients at diagnosis and processed to serum; the resulting samples were frozen at −80°C until analysis. For ELISA, the capture Ab was coated onto the bottom of 96-well ELISA plates by overnight incubation at room

temperature. A serum sample (50 μL) or sample of cell culture supernatant (100 μL) was added to each well. A standard curve was generated by serial dilution of recombinant sCD27. The plates were washed after 1 h of incubation at room temperature, and biotinylated detection Ab was added to each well. After 1 h, the plates were washed again, streptavidin-labeled horseradish peroxidase was added, and the plates were incubated for 30 min. Following another round of washing, substrate solution was added to the plates, which then were incubated for 30 min in the dark, after which the reaction was quenched by the addition of 2 N H_2SO_4. The absorbance of the contents of each well was determined at 450 nm using a microplate reader. The sCD27 concentration in each sample was calculated using the standard curve.

2.6 | Flow cytometric analysis

Flow cytometric analysis was performed using anti-CD70 monoclonal Ab conjugated to phycoerythrin (PE; 113–16; BioLegend) as described previously.[22] PE-conjugated mouse IgG1 kappa (BioLegend) was used as an isotype control. The fluorescence of samples was measured using a BD Accuri C6 flow cytometer, and the data were analyzed using the supplied software (BD Biosciences).

2.7 | Western blot analysis

Western blot analysis was performed using anti-LMP1 (CS1-4, 1:3000; Dako) and control anti–β-actin (AC-15, 1:10000; Sigma-Aldrich) monoclonal Abs as described previously.[23]

2.8 | Transplantation of C666-1 cells into severe combined immunodeficiency (SCID) hairless outbred (SHO) mice

Six- to eight-week-old female SHO mice, which are hairless SCID mice (Crl:SHO-*Prkdc*^{scid}*Hr*^{hr}), were obtained from Jackson Laboratory Japan, Inc. and maintained under specific pathogen-free conditions in the Animal Laboratory of the Center for Advanced Research and Education, Asahikawa Medical University. SHO mice were injected subcutaneously in the flank with 5×10^6 C666-1 cells. The mice were euthanatized, and subcutaneous tumors were collected when the average tumor area reached approximately 300 mm^2. All animal experiments were approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University.

2.9 | Statistical analysis

Correlations between immunohistochemical CD70 or CD27 expression and various clinicopathological characteristics were examined using Fisher's exact test or the chi-square test. The Kaplan–Meier method was used to estimate survival curves; the statistical significance of differences in survival curves was examined using the log-rank test. Two-group comparisons were conducted using the Mann–Whitney *U* test or unpaired Student's *t*-test. Receiver-operating characteristic (ROC) curve and area under the curve (AUC) were used to evaluate the diagnostic utility of serum sCD27 in NPC. Correlations between CD70 and LMP1 expression were examined using Fisher's exact test. Differences were considered significant at $p<0.05$. All analyses were run as two-tailed tests. All graphics and analyses used Prism 9 software (GraphPad Software).

2.10 | Additional materials and methods

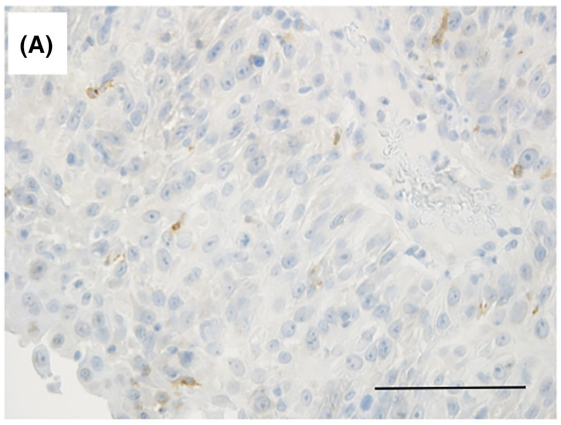
Other information regarding the materials and methods used in this study is provided in the supporting information (Data S1).

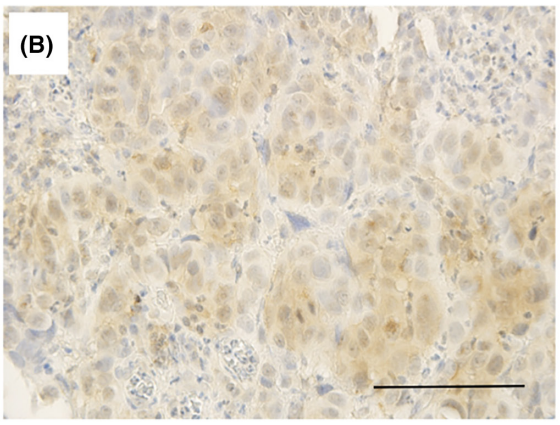
3 | RESULTS

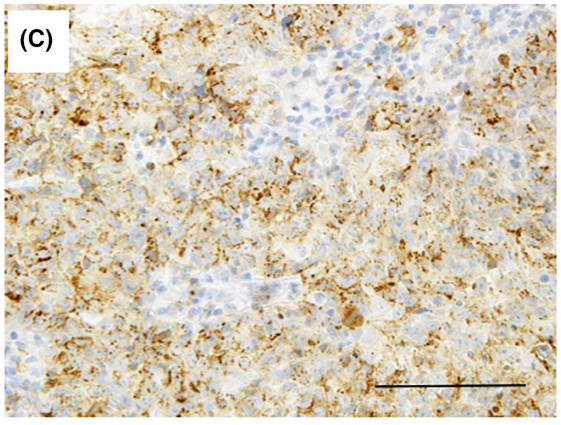
3.1 | Expression of CD70 and CD27 in NPC and their correlation with clinicopathological features and prognosis

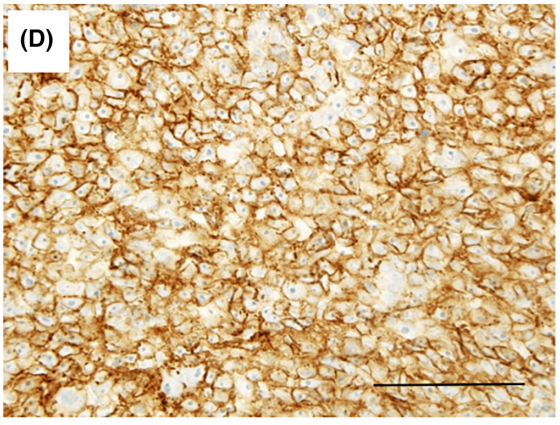
We initially assessed the expression of CD70 in tissues from 41 NPC patients using immunohistochemical staining. CD70 was primarily expressed in tumor cells, and expression was classified into four levels (no, weak, moderate, or strong) based on staining intensity (Figure 1A–D). Twelve patients (29.3%) exhibited no staining, four (9.7%) weak staining, twelve (29.3%) moderate staining, and thirteen (31.7%) strong staining (Figure 1F). Twenty-nine of forty-one samples (70.7%) were positive for CD70 expression when positivity was defined as weak, moderate, or strong staining. We also investigated the expression of CD27 in NPC specimens using serial sections of slides stained with CD70. As shown in Figures 1E and 3C, mononuclear immune cells (but

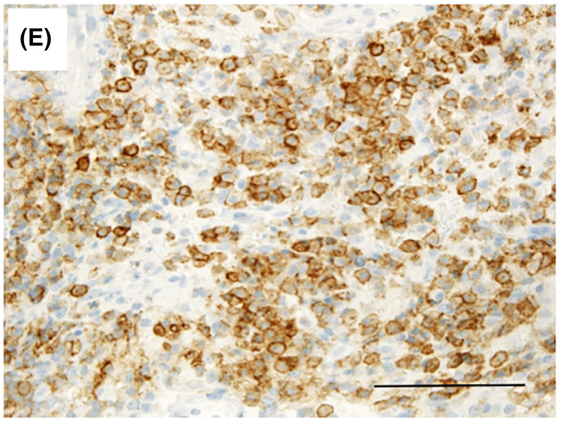
FIGURE 1 Expression of CD70 and CD27 in nasopharyngeal carcinoma (NPC) specimens and correlation with overall survival. (A–D) Representative immunohistochemical features of CD70. Expression level as measured by immunohistochemical staining intensity of tumor cells was classified as (A) no staining, (B) weak staining, (C) moderate staining, and (D) strong staining. (E) Representative immunohistochemical features of CD27. Scale bars in (A–E) are 100 μm. (F) Distribution of CD70 and CD27 expression based on immunohistochemical staining. When >5% of tumor-infiltrating mononuclear immune cells were positive for CD27, the specimen was defined as CD27 positive. (G) Kaplan–Meier overall survival curve relative to CD27 expression in NPC specimens. (H) Kaplan–Meier overall survival curve relative to CD70 expression in CD27-positive NPC specimens. Patients with CD27-positive NPC were separated into two groups: CD70 low (no and weak staining) and CD70 high (moderate and strong staining).





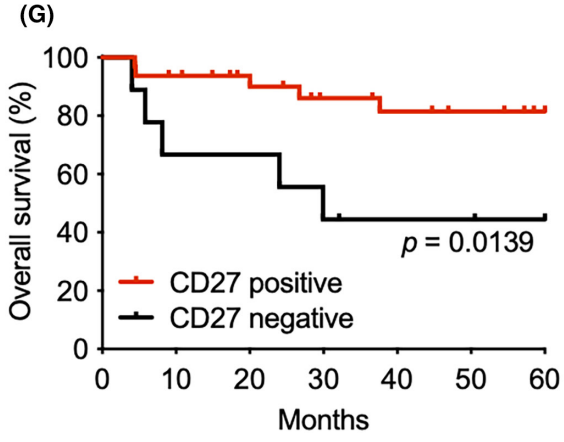


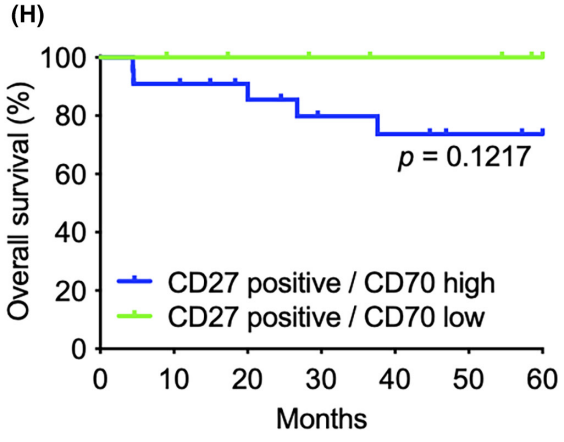




(F)

	CD70				
	Negative	Weak	Moderate	Strong	
CD27 Negative	6	0	2	1	9 22.0%
CD27 Positive	6	4	10	12	32 78.0%
	12 29.3%	4 9.7%	12 29.3%	13 31.7%	41





not tumor cells) expressed CD27, and CD27-positive cells primarily infiltrated around tumor cells. Thirty-two of forty-one patients (78.0%) were positive for CD27 (Figure 1F). These results indicate that NPC tumor cells primarily express CD70, whereas tumor-infiltrating immune cells express CD27.

To identify the cellular source of CD27-positive cells, double immunofluorescence staining for CD27 and CD4, CD8, or CD20 was performed using tissues from immunohistochemical CD27-positive NPC patients. As shown in Figure S1A–C, CD27-positive CD4 T cells, CD8 T cells, and CD20 B cells were present in NPC stroma. With regard to the proportions of these three subsets among all CD27-positive cells, high percentages of CD4 and CD8 T cells were detected as compared with the percentage of CD20 B cells (Figure S1D). These findings demonstrate that CD27-positive cells in NPC tissues comprise a heterogeneous population that includes T and B lymphocytes.

We then examined the relationships between CD70 and CD27 expression and various clinicopathological features of NPC patients. Positive CD70 expression was significantly correlated with EBER expression ($p=0.0016$) and WHO histologic type ($p=0.0411$, Table 2). A significant correlation was also observed between positive expression of CD27 and EBER ($p=0.0106$, Table 3). Furthermore, a significant relationship was observed between the expression of CD70 and CD27 ($p=0.0106$, Table 3). These results suggest that EBV promotes both the expression of CD70 in tumor cells and the infiltration of CD27-expressing immune cells into the tumor microenvironment.

We also estimated the survival rate of patients with NPC using Kaplan–Meier analysis with log-rank comparison to evaluate the survival relevance of CD70 and CD27 expression. As shown in Figure 1G, the CD27-positive NPC group ($n=32$) had significantly better prognosis than the CD27-negative NPC group ($n=9$, $p=0.0139$), although CD70 expression alone had no effect on prognosis (data not shown). We also investigated whether the prognosis of patients with CD27-positive NPC is affected by CD70 expression in tumor cells. For this analysis, patients with CD27-positive NPC were separated into two groups: CD70 low (no and weak staining) and CD70 high (moderate and strong staining). As shown in Figure 1H, patients in the CD27-positive group with low CD70 expression ($n=10$) demonstrated a favorable clinical course, with an overall survival rate of 100%. By contrast, patients in the CD27-positive group with high CD70 expression ($n=22$) had an unfavorable course, with an overall survival rate of 74%, although there was no statistically significant difference between these two groups. These results suggest that expression of CD70 on tumor cells inhibits the antitumor immune response of CD27-expressing lymphocytes via the interaction of CD70 with CD27.

TABLE 2 Relationship between the clinicopathological characteristics of nasopharyngeal carcinoma patients and CD70 expression.

	CD70		
Characteristics	**Negative**	**Positive**	***p***
Age (years)			0.4410
<50	4	6	
≧50	8	23	
Gender			>0.9999
Female	3	6	
Male	9	23	
WHO histologic type			0.0411
I	3	1	
II	7	14	
III	2	14	
EBER expression			0.0016
Negative	8	4	
Positive	4	25	
T classification			0.5055
T1, 2 (early)	5	16	
T3, 4 (advanced)	7	13	
N classification			0.2024
N0 (negative)	4	4	
N1, 2, 3 (positive)	8	25	
M classification			>0.9999
M0	12	27	
M1	0	2	
Stage			0.0726
I, II (early)	1	11	
III, IV (advanced)	11	18	

Abbreviation: EBER, Epstein–Barr virus-encoded small RNA; WHO, World Health Organization.

3.2 | Serum of NPC patients contains elevated levels of sCD27

Other studies have reported the detection of sCD27 in the serum of patients with some CD70-positive tumors, reflecting the interaction between CD70 and CD27 in the tumor microenvironment.[9–13] Therefore, we measured the concentration of sCD27 in the serum of 29 of 41 NPC patients and 23 healthy volunteers. As shown in Figure 2A, serum sCD27 levels were significantly higher in NPC patients (mean ± SD = 145.8 ± 81.4 U/mL; median = 138.2 U/mL; range = 15.3–333.9 U/mL) than healthy individuals (mean ± SD = 52.3 ± 29.8 U/mL; median = 41.5 U/mL; range = 10.2–126.7 U/mL, $p<0.0001$). To characterize sCD27 as a possible biomarker of NPC, ROC curve analysis was performed. sCD27 exhibited an AUC of 0.8606, with a sensitivity of 75.9% and specificity of 87.0% at a cutoff value of 84.9 U/mL for discriminating patients with NPC from healthy controls (Figure 2B). We also examined the correlation between serum sCD27 levels and the survival rate of patients with NPC; however, no significant association was found (Figure S2). These results suggest that increased levels of sCD27 in the serum may have diagnostic value for NPC.

TABLE 3 Relationship between the clinicopathological characteristics of nasopharyngeal carcinoma patients and CD27 expression.

Characteristics	CD27		*p*
	Negative	Positive	
Age (years)			>0.9999
<50	2	8	
≧50	7	24	
Gender			>0.9999
Female	2	7	
Male	7	25	
WHO histologic type			0.0965
I	2	2	
II	6	15	
III	1	15	
EBER expression			0.0106
Negative	6	6	
Positive	3	26	
T classification			0.7186
T1, 2 (early)	4	17	
T3, 4 (advanced)	5	15	
N classification			0.3415
N0 (negative)	3	5	
N1, 2, 3 (positive)	6	27	
M classification			>0.9999
M0	9	30	
M1	0	2	
Stage			0.7017
I, II (early)	2	10	
III, IV (advanced)	7	22	
CD70 expression			0.0106
Negative	6	6	
Positive	3	26	

Abbreviation: EBER, Epstein–Barr virus-encoded small RNA; WHO, World Health Organization.

3.3 | Expression of CD70 and CD27 in NPC tissues is correlated with elevated serum sCD27 levels

We further assessed the relationship between serum levels of sCD27 and the expression of CD70 and CD27 in specimens in the subset of patients ($n=29$) with NPC for whom both serum and tissue samples were available. As shown in Figure 3A, serum levels of sCD27 in patients with CD70-positive NPC were significantly higher than those in patients with CD70-negative NPC ($p=0.0025$). Similarly, serum levels of sCD27 in patients with CD27-positive NPC were significantly higher than in patients with CD27-negative NPC ($p=0.0241$, Figure 3B). Moreover, serum sCD27 levels were compared with the combination of CD70 and CD27 expression status in serial sections of the corresponding patients (Figure 3C). Serum sCD27 levels were significantly higher in patients with CD70-expressing tumor cells and CD27-positive infiltrating lymphocytes than in patients with either CD70-positive tumor cells but no CD27-positive infiltrating lymphocytes or CD70-negative tumor cells and CD27-positive infiltrating lymphocytes or negativity for both ($p<0.0001$, Figure 3D), suggesting that the intratumoral CD70/CD27 interaction upregulates the release of sCD27 into the serum of patients with NPC.

3.4 | Expression of CD70 in NPC cell lines and association between CD70 and LMP1 expression in EBV-positive NPC

Similar to our abovementioned finding of an association between EBER and CD70 expression in NPC (Table 2), it has been shown that EBV infection might promote the induction of CD70 expression on tumor cells in some malignancies.[24] In addition, previous studies have reported that the EBV oncoprotein LMP1 can upregulate CD70 expression in epithelial and hematological cells.[13,15,25] To determine whether the expression of CD70 in tumor cells of NPC is associated with EBV infection and LMP1 expression, we examined the expression of CD70 using two NPC cell lines, C666-1 and TW03. CD70 was highly expressed on EBV-positive C666-1 cells, whereas a low level of CD70 expression was observed on EBV-negative TW03 cells (Figure 4A). To determine whether differences in CD70 expression between NPC cell lines affect the release of sCD27 from lymphocytes, we cocultured NPC cells with freshly isolated PBMCs and measured sCD27 in the supernatant by ELISA (Figure 4B). Coculture of PBMCs with CD70 high-expressing C666-1 cells significantly increased the sCD27 concentration compared with monoculture of PBMCs ($p=0.0174$). In contrast, there was no significant difference in sCD27 concentration between PBMCs cocultured with CD70 low-expressing TW03 cells and monoculture of PBMCs. sCD27 was not detectable in the supernatant of C666-1 cells or TW03 cells in monoculture.

Western blot analysis was performed to investigate whether EBV-positive C666-1 cells express LMP1 protein. As shown in Figure 4C, LMP1 was not detected in C666-1 cells or EBV-negative TW03 cells (negative control), but LMP1 was expressed by positive control SNK-6 cells, an LMP1-positive NK/T-cell lymphoma cell line.[26,27]

We next assessed the expression of CD70 using a murine xenograft model of SHO mice inoculated subcutaneously with C666-1 to ascertain whether not only cultured C666-1 cells but also C666-1 tumor cells in the in vivo xenograft model express CD70. Immunohistochemical staining was performed to analyze CD70 expression in FFPE tissue sections prepared from subcutaneous tumors. Histologically, the tumors were formed by atypical cells positive for CD70 and EBER but not LMP1 (Figure 4D). These characteristics were consistent with those of in vitro cultured C666-1 cells.

Using immunohistochemical staining, we also investigated the correlation between CD70 and LMP1 expression in a subset of

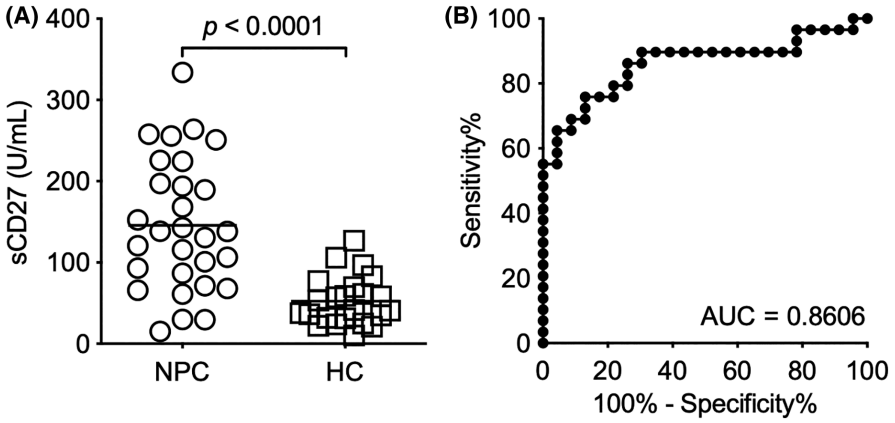


FIGURE 2 Soluble CD27 (sCD27) levels in serum of patients with nasopharyngeal carcinoma (NPC). (A) sCD27 levels in serum from patients with NPC and healthy controls (HC) as measured using ELISA. Horizontal lines indicate mean values. (B) Receiver-operating characteristic curve of sCD27 for discrimination between NPC patients and HC. The area under the curve (AUC) value was 0.8606 (95% confidence interval 0.7563–0.9649).

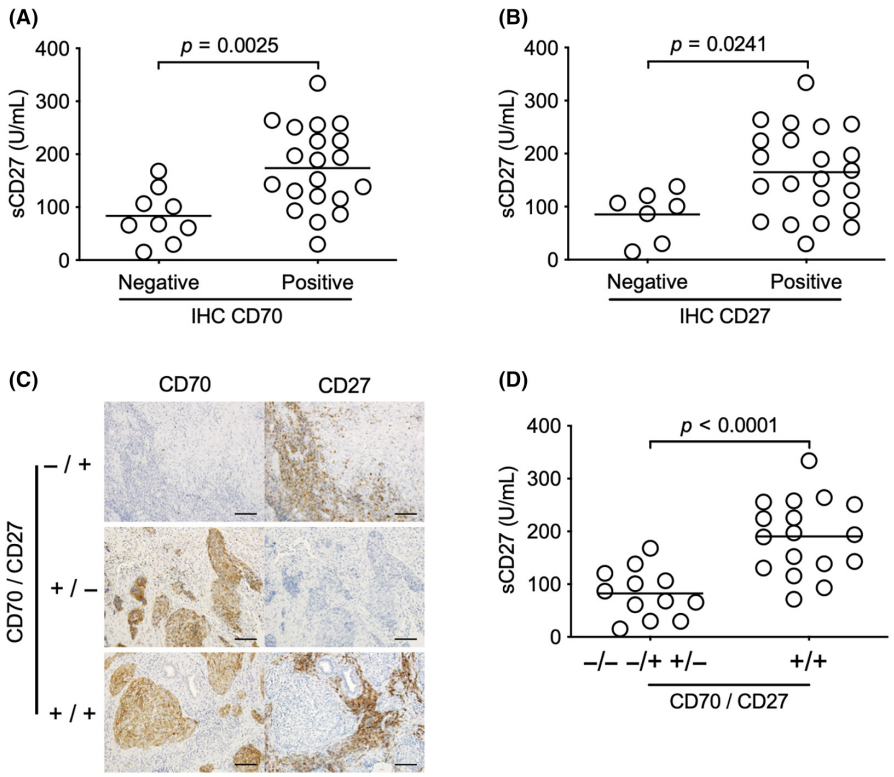
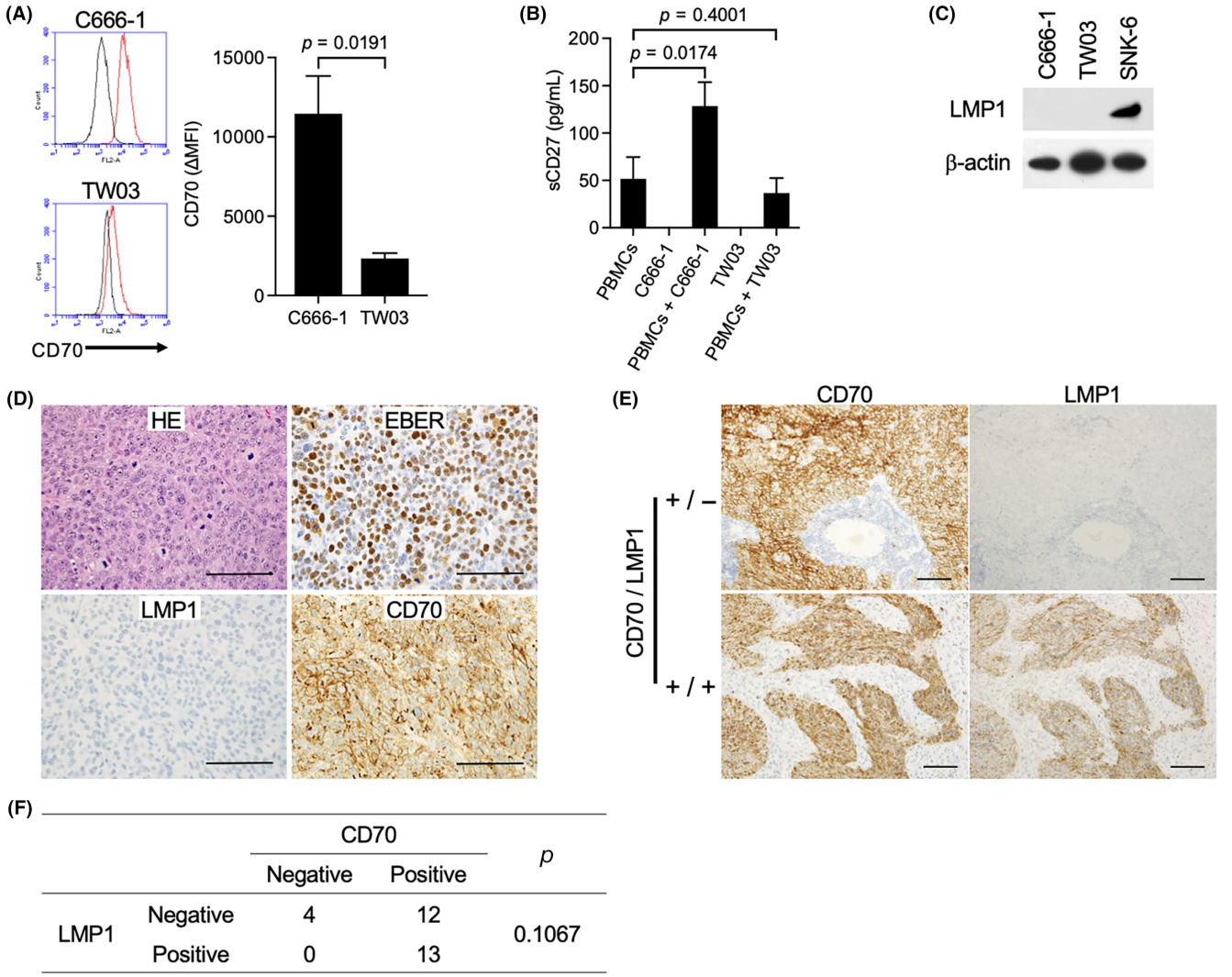


FIGURE 3 Correlations between serum soluble CD27 (sCD27) level and the expression of CD70 and CD27 in tumor tissues of nasopharyngeal carcinoma (NPC) patients. (A) Association between serum sCD27 level and immunohistochemical (IHC) CD70 expression in tissue tumor cells. Weak, moderate, and strong staining were defined as positive for CD70. (B) Association between serum sCD27 level and IHC CD27 expression in tumor-infiltrating immune cells. When >5% of tumor-infiltrating mononuclear immune cells were positive for CD27, the specimen was defined as CD27 positive. (C) Representative IHC features of NPC tissues stained for CD70 and CD27 using serial slides. The presence or absence of CD70-positive tumor cells and CD27-positive immune cells infiltrating around tumor cells (CD70/CD27) is indicated by (+) or (–), respectively. Scale bars are 100 μm. (D) Association between serum sCD27 level and combined IHC CD70 and CD27 expression patterns. Horizontal lines in (A), (B), and (D) indicate mean values.

patients ($n = 29$) with EBER-positive NPC. Figure 4E shows representative immunohistochemical features of serial slides of NPC tissues stained for CD70 and LMP1. Thirteen of twenty-nine patients (44.8%) were positive for LMP1; however, no significant correlation between positive staining for CD70 and LMP1 was observed (Figure 4F). Collectively, these results suggest that EBV infection in NPC tumor cells plays an important role in CD70 expression, whereas persistent LMP1 expression in EBV-positive NPC tumor cells is not necessarily required for CD70 expression.

4 | DISCUSSION

It is generally assumed that CD70 expressed on tumor cells interacts with CD27 on TILs in the tumor microenvironment. However, determining whether this interaction actually occurs can be technically challenging. Measurement of serum levels of sCD27 could potentially be used to detect this interaction because sCD27 is cleaved from the cell surface upon ligation of CD27 to CD70 and therefore enters body fluids.[5,6] In the present study, we clearly



(F)

		CD70		p
		Negative	Positive	
LMP1	Negative	4	12	0.1067
	Positive	0	13	

FIGURE 4 Expression of CD70 and latent membrane protein 1 (LMP1) in nasopharyngeal carcinoma (NPC) cell lines and tissues from Epstein-Barr virus (EBV)-positive NPC patients. (A) Flow cytometric analysis of the surface expression of CD70 on NPC cells. Left histograms show the CD70 level as measured by flow cytometry. Cells were stained with a PE-conjugated anti-CD70 monoclonal Ab (red lines). Black lines: cells stained with isotype-control Ab. Right graph shows delta mean fluorescence intensity (ΔMFI) of CD70 calculated by subtracting the isotype control staining value. Values are plotted as mean + SEM ($n = 3$). (B) Release of soluble CD27 (sCD27) from peripheral blood mononuclear cells (PBMCs) cocultured with NPC cell lines. Concentrations of sCD27 in supernatants of PBMC and NPC cell line cocultures or monocultures were determined by ELISA. Values are plotted as mean + SD ($n = 3$). (C) LMP1 expression in NPC cell lines as assessed by Western blotting. β-actin was used as a loading control. SNK-6 cells were used as a positive control for LMP1 expression. (D) Representative immunohistological features of tumor samples from a xenograft model mouse inoculated with C666-1 cells. (E) Representative immunohistochemical features of serial slides of EBV-positive NPC tissues stained for CD70 and LMP1. Expression of CD70 and LMP1 in tumor cells (CD70/LMP1) is indicated as negative (–) or positive (+). Scale bars in (D) and (E) are 100 μm. (F) Correlation between CD70 and LMP1 expression in EBV-positive NPC tissues.

demonstrated that serum sCD27 levels are significantly elevated in NPC patients, as demonstrated by the good diagnostic accuracy for discriminating between patients and healthy subjects. More importantly, the levels of serum sCD27 in NPC patients with both CD70-positive tumor cells and CD27-positive TILs were significantly higher than in other patients. Furthermore, coculturing PBMCs with CD70 high-expressing NPC cells led to increased levels of sCD27 in the supernatant. These results indicate that the intratumoral CD70/CD27 interaction boosts the release of sCD27 into the serum of NPC patients. Our present findings are consistent with previous results reported by Ruf et al.,[9] who demonstrated significantly higher sCD27 serum levels in RCC patients with CD70-expressing tumor cells and CD27-positive TILs compared with healthy individuals and patients that either had CD70-expressing tumor cells but no CD27-positive TILs or had CD27-positive TILs and CD70-negative tumor cells or were negative for both. These data thus suggest that the intratumoral CD70/CD27 interaction is accompanied by the presence of high

levels of sCD27 in the serum of NPC patients, similar to findings in RCC patients, although further studies are required to validate our findings.

We also found that NPC patients with CD27-positive TILs exhibited better survival than patients without such cells. Consistent with our findings, recent studies by other researchers showed a correlation between the presence of CD27-positive TILs and a favorable prognosis in some solid tumors,[28–30] suggesting that CD27-positive immune cells may include lymphocytes capable of eliciting antitumor immune responses, such as tumor-specific cytotoxic and helper T cells.

By contrast, higher CD70 expression in CD27-positive NPC patients tended to correspond with poor prognosis. Kashima et al.[29] reported similar findings in thymic squamous cell carcinoma, as patients with CD27-positive TIL-high tumors exhibited longer survival than patients with CD27-positive TIL-low tumors, and this tendency was weaker in the CD70-high subset. Thus, our results suggest that the presence of CD70 in tumor tissues imparts a survival advantage to NPC cells by facilitating evasion of the host immune response through the interaction with CD27. Other studies have reported that CD70-positive tumor cells evade elimination by antitumor immune surveillance through two distinct primary mechanisms.[5,6] The first mechanism involves induction of exhaustion or apoptosis in CD27-positive lymphocytes.[31–34] A recent single-cell RNA sequencing study found that CD27-positive CD8 T cells sorted from fresh RCC samples were more apoptotic and displayed a dysfunction/exhaustion phenotype.[10] The second mechanism of immune escape involves increased accumulation of regulatory T cells (Tregs) in the tumor microenvironment. Several studies have demonstrated that CD27 is also expressed on Tregs and that CD70-expressing tumors may favor the generation of Tregs through CD27 costimulation, thereby leading to the suppression of antitumor immune responses within the tumor microenvironment and the subsequent acceleration of tumor growth.[5,6] Gong et al.[17] recently revealed that NPC cells can enhance the development and suppressive activity of Tregs via the CD70/CD27 interaction. Therefore, it is possible that CD70-expressing NPC cells induce a state of immune suppression via these distinct mechanisms involving the CD70/CD27 pathway.

With regard to the analysis of CD70 expression in NPC using immunohistochemistry, a few studies demonstrated that most EBV-positive NPC cells express CD70, although these studies included only patients with EBV-positive NPC and not patients with EBV-negative NPC.[14–16] The immunohistochemical experiments performed in the present study used tissue samples from 41 NPC patients, including 12 EBV-negative patients. In the case of patients with EBV-positive NPC, 25 of 29 (86.2%) samples were positive for CD70, similar to the high positive percentages reported in the previous studies mentioned above, whereas tumor cells were positive for CD70 in only 4 of 12 (33.3%) patients with EBV-negative NPC. In addition, a significant correlation was observed between positive staining for CD70 and EBER. Furthermore, the expression of CD70 in the EBV-positive NPC cell line was significantly higher than that in the EBV-negative NPC cell line. A recent single-cell analysis showed higher expression of the *CD70* gene in tumor cells from EBV-positive NPC patients compared with tumor cells from EBV-negative patients.[17] Our results and the data reported by other researchers indicate that EBV infection plays a crucial role in the induction of CD70 expression in NPC. However, the effect of LMP1 expression on CD70 expression in NPC remains unclear. A previous study reported that transfection of epithelial cells with *LMP1* induced the expression of CD70.[15] However, we could not find any relationship between the expression of CD70 and LMP1 in the NPC cell lines and tissue samples examined, consistent with the results of previous immunohistochemical studies by other researchers.[14,16] This discrepancy may indicate that continuous LMP1 expression is not required to maintain CD70 expression once it is established. In any case, additional studies will be required to clarify details of the mechanism by which EBV induces CD70 expression in NPC.

Our abovementioned results and those of other researchers suggest that immunotherapeutic strategies targeting the CD70/CD27 axis hold great potential for eliminating NPC cells. A recent preclinical study showed that blockade of the CD70/CD27 axis using an anti-CD70 Ab significantly suppressed the growth of established NPC in patient-derived xenograft-bearing humanized mice,[17] suggesting that agents capable of inhibiting the CD70/CD27 interaction could become attractive therapeutic options for treating NPC in the future. In addition, cytotoxic anti-CD70 Abs or CD70-specific chimeric antigen receptor T cells may also be effective for treating NPC by directly inducing the killing of tumor cells. Moreover, we believe that serum sCD27 could be useful as a predictive tool for CD70/CD27-targeted anti-NPC therapies, given that high levels of sCD27 are correlated with increased CD70 expression in tumor cells and detrimental excessive interaction between CD70-positive tumor cells and CD27-positive lymphocytes.

AUTHOR CONTRIBUTIONS

Toshihiro Nagato: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing – original draft; writing – review and editing. **Hiroki Komatsuda:** Data curation; formal analysis; investigation; resources; writing – review and editing. **Ryusuke Hayashi:** Data curation; formal analysis; resources; writing – review and editing. **Miki Takahara:** Resources; writing – review and editing. **Nanami Ujiie:** Formal analysis; writing – review and editing. **Akemi Kosaka:** Formal analysis; writing – review and editing. **Takayuki Ohkuri:** Formal analysis; writing – review and editing. **Kensuke Oikawa:** Formal analysis; writing – review and editing. **Ryosuke Sato:** Resources; writing – review and editing. **Risa Wakisaka:** Resources; writing – review and editing. **Michihisa Kono:** Resources; writing – review and editing. **Hidekiyo Yamaki:** Resources; writing – review and editing. **Kenzo Ohara:** Resources; writing – review and editing. **Takumi Kumai:** Resources; writing – review and editing. **Kan Kishibe:** Resources; writing – review and editing. **Akihiro Katada:** Resources; writing – review and editing. **Tatsuya Hayashi:** Resources; writing – review and editing. **Hiroya**

Kobayashi: Conceptualization; data curation; formal analysis; funding acquisition; methodology; project administration; supervision; validation; writing – review and editing.

ACKNOWLEDGMENTS

The authors thank Dr. Tomokazu Yoshizaki (Kanazawa University), Dr. Satoru Kondo (Kanazawa University), Dr. Eva Klein (Karolinska Institute), and Dr. Norio Shimizu (Tokyo Medical and Dental University) for generously providing cell lines. The authors also thank Ms. Rie Matsumoto (Department of Pathology, Asahikawa Medical University) and Ms. Keiko Nishikura (Department of Dermatology, Asahikawa Medical University) for technical assistance.

FUNDING INFORMATION

This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI (grant numbers 18K09310 [Nagato T], 21K09599 [Nagato T], and 22H02838 [Kobayashi H]).

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: This study was approved by the Institutional Review Board of the Asahikawa Medical University and performed in accordance with the Declaration of Helsinki.

Informed Consent: Informed consent was obtained using the opt-out method on the Asahikawa Medical University website.

Registry and Registration No. of the study/trial: N/A.

Animal Studies: The protocols for animal studies were approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University.

ORCID

Toshihiro Nagato https://orcid.org/0000-0001-8326-8296
Hiroki Komatsuda https://orcid.org/0000-0002-3783-2746
Takayuki Ohkuri https://orcid.org/0000-0002-6889-7809
Takumi Kumai https://orcid.org/0000-0002-3411-671X

REFERENCES

1. Chen YP, Chan ATC, Le QT, Blanchard P, Sun Y, Ma J. Nasopharyngeal carcinoma. *Lancet*. 2019;394:64-80.
2. Su ZY, Siak PY, Leong CO, Cheah SC. The role of Epstein-Barr virus in nasopharyngeal carcinoma. *Front Microbiol*. 2023;14:1116143.
3. Yoshizaki T, Kondo S, Endo K, et al. Modulation of the tumor microenvironment by Epstein-Barr virus latent membrane protein 1 in nasopharyngeal carcinoma. *Cancer Sci*. 2018;109:272-278.
4. Gong L, Kwong DL, Dai W, et al. The stromal and immune landscape of nasopharyngeal carcinoma and its implications for precision medicine targeting the tumor microenvironment. *Front Oncol*. 2021;11:744889.
5. Jacobs J, Deschoolmeester V, Zwaenepoel K, et al. CD70: an emerging target in cancer immunotherapy. *Pharmacol Ther*. 2015;155:1-10.
6. Flieswasser T, Van den Eynde A, Van Audenaerde J, et al. The CD70-CD27 axis in oncology: the new kids on the block. *J Exp Clin Cancer Res*. 2022;41:12.
7. Croft M. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol*. 2009;9:271-285.
8. Nolte MA, van Olffen RW, van Gisbergen KP, van Lier RA. Timing and tuning of CD27-CD70 interactions: the impact of signal strength in setting the balance between adaptive responses and immunopathology. *Immunol Rev*. 2009;229:216-231.
9. Ruf M, Mittmann C, Nowicka AM, et al. pVHL/HIF-regulated CD70 expression is associated with infiltration of CD27+ lymphocytes and increased serum levels of soluble CD27 in clear cell renal cell carcinoma. *Clin Cancer Res*. 2015;21:889-898.
10. Benhamouda N, Sam I, Epaillard N, et al. Plasma CD27, a surrogate of the Intratumoral CD27-CD70 interaction, correlates with immunotherapy resistance in renal cell carcinoma. *Clin Cancer Res*. 2022;28:4983-4994.
11. Jacobs J, Zwaenepoel K, Rolfo C, et al. Unlocking the potential of CD70 as a novel immunotherapeutic target for non-small cell lung cancer. *Oncotarget*. 2015;6:13462-13475.
12. Dong MP, Thuy LTT, Hoang DV, et al. Soluble immune checkpoint protein CD27 is a novel prognostic biomarker of hepatocellular carcinoma development in hepatitis C virus-sustained virological response patients. *Am J Pathol*. 2022;192:1379-1396.
13. Nagato T, Komatsuda H, Hayashi R, et al. Expression of soluble CD27 in extranodal natural killer/T-cell lymphoma, nasal type: potential as a biomarker for diagnosis and CD27/CD70-targeted therapy. *Cancer Immunol Immunother*. 2023;72:2087-2098.
14. Niedobitek G, Young LS, Sam CK, Brooks L, Prasad U, Rickinson AB. Expression of Epstein-Barr virus genes and of lymphocyte activation molecules in undifferentiated nasopharyngeal carcinomas. *Am J Pathol*. 1992;140:879-887.
15. Niedobitek G, Fahraeus R, Herbst H, et al. The Epstein-Barr virus encoded membrane protein (LMP) induces phenotypic changes in epithelial cells. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1992;62:55-59.
16. Agathanggelou A, Niedobitek G, Chen R, Nicholls J, Yin W, Young LS. Expression of immune regulatory molecules in Epstein-Barr virus-associated nasopharyngeal carcinomas with prominent lymphoid stroma. Evidence for a functional interaction between epithelial tumor cells and infiltrating lymphoid cells. *Am J Pathol*. 1995;147:1152-1160.
17. Gong L, Luo J, Zhang Y, et al. Nasopharyngeal carcinoma cells promote regulatory T cell development and suppressive activity via CD70-CD27 interaction. *Nat Commun*. 2023;14:1912.
18. Sobin LH, Gospodarowicz MK, Wittekind C. *International Union against C. TNM Classification of Malignant Tumours*. 7th ed. Wiley-Blackwell; 2009.
19. Ohara K, Takahara M, Kumai T, et al. Treatment outcomes of alternating chemoradiotherapy for nasopharyngeal carcinoma: a single-center safety and efficacy study. *Braz J Otorhinolaryngol*. 2023;89:440-446.
20. Harabuchi Y, Imai S, Wakashima J, et al. Nasal T-cell lymphoma causally associated with Epstein-Barr virus: clinicopathologic, phenotypic, and genotypic studies. *Cancer*. 1996;77:2137-2149.
21. Nagato T, Kobayashi H, Kishibe K, et al. Expression of interleukin-9 in nasal natural killer/T-cell lymphoma cell lines and patients. *Clin Cancer Res*. 2005;11:8250-8257.
22. Kumai T, Nagato T, Kobayashi H, et al. CCL17 and CCL22/CCR4 signaling is a strong candidate for novel targeted therapy against nasal natural killer/T-cell lymphoma. *Cancer Immunol Immunother*. 2015;64:697-705.
23. Hayashi R, Nagato T, Kumai T, et al. Expression of placenta-specific 1 and its potential for eliciting anti-tumor helper T-cell responses in head and neck squamous cell carcinoma. *Oncoimmunology*. 2020;10:1856545.
24. Israel BF, Gulley M, Elmore S, Ferrini S, Feng WH, Kenney SC. Anti-CD70 antibodies: a potential treatment for EBV+ CD70-expressing lymphomas. *Mol Cancer Ther*. 2005;4:2037-2044.

25. Choi IK, Wang Z, Ke Q, et al. Signaling by the Epstein-Barr virus LMP1 protein induces potent cytotoxic CD4(+) and CD8(+) T cell responses. *Proc Natl Acad Sci U S A*. 2018;115:E686-E695.
26. Nagato T, Ueda S, Takahara M, et al. Cyclin-dependent kinase 1 and survivin as potential therapeutic targets against nasal natural killer/T-cell lymphoma. *Lab Invest*. 2019;99:612-624.
27. Yoshino K, Kishibe K, Nagato T, et al. Expression of CD70 in nasal natural killer/T cell lymphoma cell lines and patients; its role for cell proliferation through binding to soluble CD27. *Br J Haematol*. 2013;160:331-342.
28. Doescher J, Minkenberg P, Laban S, et al. Immune checkpoint expression in HNSCC patients before and after definitive chemoradiotherapy. *Head Neck*. 2021;43:778-787.
29. Kashima J, Hishima T, Okuma Y, et al. CD70 in thymic squamous cell carcinoma: potential diagnostic markers and immunotherapeutic targets. *Front Oncol*. 2021;11:808396.
30. Ding J, Wang H, Hou R, et al. Total T cell density and expression of T memory stem cell markers are associated with better prognosis in colon cancer. *Int J Gen Med*. 2023;16:2285-2294.
31. Wischhusen J, Jung G, Radovanovic I, et al. Identification of CD70-mediated apoptosis of immune effector cells as a novel immune escape pathway of human glioblastoma. *Cancer Res*. 2002;62:2592-2599.
32. Chahlavi A, Rayman P, Richmond AL, et al. Glioblastomas induce T-lymphocyte death by two distinct pathways involving gangliosides and CD70. *Cancer Res*. 2005;65:5428-5438.
33. Diegmann J, Junker K, Loncarevic IF, Michel S, Schimmel B, von Eggeling F. Immune escape for renal cell carcinoma: CD70 mediates apoptosis in lymphocytes. *Neoplasia*. 2006;8:933-938.
34. Wang QJ, Hanada K, Robbins PF, Li YF, Yang JC. Distinctive features of the differentiated phenotype and infiltration of tumor-reactive lymphocytes in clear cell renal cell carcinoma. *Cancer Res*. 2012;72:6119-6129.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Nagato T, Komatsuda H, Hayashi R, et al. Soluble CD27 as a predictive biomarker for intra-tumoral CD70/CD27 interaction in nasopharyngeal carcinoma. *Cancer Sci*. 2024;115:1073-1084. doi:10.1111/cas.16079