Association between Carbonic anhydrase 9 expression and poor prognosis in sinonasal squamous cell carcinoma

Chunyan Hu
Fudan University Eye Ear Nose and Throat Hospital
Huan Wang
Fudan University Eye Ear Nose and Throat Hospital
Lan Lin (l1891778@163.com)
Fudan University Eye Ear Nose and Throat Hospital https://orcid.org/0000-0001-7187-5048
Xicai Sun
Fudan University Eye Ear Nose and Throat Hospital
Dehui Wang
Fudan University Eye Ear Nose and Throat Hospital

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Abstract

Background: Carbonic anhydrase 9(CA9), as a member of the carbonic anhydrase enzyme family, was an endogenous marker of hypoxia. Previous studies suggested CA9 expression was correlated with poor prognosis in multiple types of malignancies. The purpose of current study was to evaluate the role of CA9 as a prognostic marker in sinonasal squamous cell carcinoma(SNSCC).

Patients and methods: We assessed the immunohistochemical expression of CA9 in 63 tumor specimens of patients who underwent curative surgery and evaluated the relationship between the expression levels and clinicopathological factors as well as outcome.

Results: We observed positive expression of CA9 in 21(33.3%) patients. Positive expression of CA9 in patients with SNSCC was significantly correlated with local recurrence (p=0.016) and both overall survival (OS) (p=0.003) and disease-free survival (DFS) (p=0.002). In Cox's multivariate analysis, CA9 expression was an independent negative prognostic factor for OS (p=0.027) and DFS (p=0.018).

Conclusions: Our findings demonstrated that CA9 overexpression could be used as an independent prognostic biomarker and therapeutic target in SNSCC.

Background

Malignant tumors of the nasal cavity and paranasal sinuses are very rare, representing approximately 3%-6% of head and neck cancers[1]. The most frequent malignant types of the sinonasal region are squamous cell carcinomas (SCCs). Although early diagnosis and treatment methods have been improved in the past few years, local recurrence remains the major factors influencing the prognosis of patients with SNSCC, and the 5-year survival rate has not significantly improved over the past several decades ranging from 30.2% to 59.5%[2-4]. Hence, it is crucial to identify novel biomarkers that could provide a better understanding of the molecular mechanisms implicated in tumor development and progression, in order to identify better diagnostic methods and more effective prognostic indicators for SNSCC patients.

The survival and proliferation of cancer cells under hypoxic conditions mainly depends on the energy produced by glycolysis, which leads to the production of a large amount of lactic acid and protons. Carbonic anhydrase 9 (CA9), as a member of the carbonic anhydrase enzyme family, plays a pivotal role in stabilizing the extracellular pH value by catalyzing the reversible conversion of carbon dioxide into bicarbonate ions and protons and thus promoting intracellular proton carboxylation and excretion[5]. Previous studies have confirmed that the acidification of tumor microenvironment could promote tumor cell migration and invasion[6]. Physiologically, CA9 is only presented with low expression in a small set of normal tissues such as gastric mucosa epithelium, small intestine epithelium, and gallbladder. Whereas, overexpression of CA9 has been reported in multiple types of malignancies including lung cancer, colon cancer, breast cancer, cervical cancer, renal cell carcinoma, and bladder cancer[7]. Moreover, increased expression of CA9 has been suggested to be a poor prognostic factor in different types of cancer (e.g. breast, head and neck, stomach, liver, pancreas, colorectal carcinomas)[5]. To the best of our knowledge,

there have not been any studies focusing on CA9 expression in SNSCC to date. In the current study, we aimed to investigate the immunohistochemical expression pattern of CA9 in SNSCC, and then determine its potential as a prognostic biomarker and for future therapeutic stratification.

Patients And Methods

Patient cohort

This study was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Research and Ethical Committee of Fudan University Affiliated Eye, Ear, Nose, and Throat Hospital (EENT). Sixty-three patients with histologically diagnosed SNSCC who were surgically treated at Fudan University Affiliated EENT Hospital between January 2014 and December 2017 were retrospectively reviewed. All clinical data including age, sex, smoking status, surgical history, tumor location and pathological grade were obtained from patients' medical records. Disease in all patients was staged using the 7th edition of the American Joint Committee on Cancer (AJCC) Staging Manual. The normal control group consisted of 10 patients (7 male patients; mean age, 42 years; range, 19–68 years), and uncinate tissue (UT) was obtained from these patients during endoscopic optic nerve decompression or endonasal repair of cerebrospinal fluid rhinorrhea. The median age of patients with SNSCC was 57 years (range, 22–85 years), and the mean follow-up time was 25.7 months (range, 3–57 months). Table 1 shows the patient details and their clinicopathological characteristics.

Immunohistochemical (IHC) staining

All tumor samples were fixed in 10% neutral formalin solution, embedded in paraffin, and cut into 4-mm-thick sections. The sections were deparaffinized in xylene and rehydrated, and then incubated in 3% hydrogen peroxide for 15 minutes to reduce nonspecific background staining caused by endogenous peroxidases. Subsequently, the sections were placed in 10 mM citrate buffer and heated in a microwave oven for 20 minutes for antigen epitope retrieval. Then the sections were incubated in 3% bovine serum albumin to block nonspecific staining. The serial sections were incubated with the primary antibody specific for CA9(D47G3, Cell Signaling Technology, Danvers, US) at a dilution of 1:100 in a humidified chamber at 4°C overnight. After washing with PBS, the sections were incubated with a horseradish peroxidase-conjugated secondary antibody (Gene Tech, HK) for 30 minutes at room temperature. Color development was performed with diaminobenzidine and the slides were counterstained with hematoxylin.

Quantitative scoring of IHC staining

Immunohistochemically stained sections were reviewed by two independent authors who was blinded to the clinical data. The expression of CA9 was evaluated under high-power (×100) microscopy in five randomly selected fields. The samples were scored according to the percentage of positively stained tumor cells. Samples were considered positive for CA9 when at least 10% of cancer cells showed a reaction for CA9, as previously reported[8].

Statistical analysis

All data analysis was performed with IBM SPSS 22.0 statistical software (SPSS, Chicago, IL, USA). The $\chi 2$ test or independent t-test was used to evaluate the associations between CA9 expression and clinical features. OS was calculated from the date of initial surgery in our hospital to the date of death or the date of the last follow-up. Disease-free survival (DFS) was defined as the period between the initial surgery in our hospital and the detection of residual or recurrent disease or death. Survival curves were constructed using the Kaplan–Meier method and survival rates were compared using the log-rank test. Multivariate analysis of prognostic factors related to survival was performed using Cox proportional hazards analysis. A p-value of<0.05 indicated statistical significance.

Results

CA9 expression in SNSCC and normal control tissue

From the immunohistochemistry experiments, positive staining for CA9 was detected in cancer cells of patients with SNSCC (Figure 1A-C). However, no CA9 was detected in the stromal cells of these patients (Figure 1A-D). CA9 expression was exclusively localized on the plasma membrane of SNSCC tumor cells. Positive immunoreactivity of CA9 in cancer cells was observed in 21 (33.3%) patients. In contrast, the normal mucosa of uncinate process from ten control patients showed absence of positive staining. Statistical analysis revealed a significant difference between CA9 expression in SNSCC and normal control tissues ($p < 0.05$).

Correlation between CA9 expression and clinical features

To explore whether CA9 expression was associated with clinical features of SNSCC, we divided the SNSCC patients into two groups according to the presence of CA9 expression in SNSCC samples and compared CA9 expression with clinicopathological data. Table 2 summarizes the relationship between CA9 expression and the clinicopathological characteristics as well as clinical outcome of the 63 sinonasal SCC patients. Notably, the chi-square test showed that patients with positive CA9 expression had significantly higher local recurrence rates than those with negative CA9 expression. However, no other parameters of clinicopathological features and treatment outcomes were significantly associated with CA9 expression.

Survival analysis

To evaluate the potential role of CA9 for prognosis in SNSCC patients, OS and DFS rate was determined by Kaplan-Meier survival analysis. Log rank analysis revealed that OS and DFS in SNSCC patients with positive CA9 expression was significantly shorter than that in patients with negative CA9 expression ($p = 0.003$ and $p=0.002$, Figure2). The Cox proportional hazard model was applied for univariate and multivariate analyses to assess the effects of CA9 and clinicopathological factors on OS as well as DFS in SNSCC patients. Univariate analysis demonstrated that age, pT stage, clinical stage and CA9

expression were significantly correlated with overall and disease-free survival rates. Multivariate analysis for OS and DFS was performed using a multivariate Cox hazards model and included age, higher pT stage and CA9 expression. In multivariate analysis, only the level of CA9 expression was found to be independent risk factor predicting poor overall survival. As for disease-free survival, high CA9 expression and older age was independent risk factors.

Discussion

Hypoxia often occurs in some regions of solid tumors and has also been suggested to be associated with aggressive tumor behavior, treatment resistance to chemotherapy and radiotherapy, and poor outcome. CA9, an endogenous marker for hypoxia, is induced by HIF-1a transcriptionally, but more stable than HIF-1a as the marker of hypoxia. CA9 is upregulated and has been described with prognostic and predictive value as well as a compelling therapeutic target in diverse tumor types due to its role in promoting tumor cell survival, invasiveness and metastasis. In the current study, we first observed CA9 expression in SNSCC and demonstrated that CA9 overexpression in cancer cells was associated with tumor recurrence postoperatively. Furthermore, we found CA9 could serve as an independent adverse prognostic factor for SNSCC.

In a meta-analysis published in 2016, 147 studies and more than 24 thousand patients with solid tumors were included to evaluate the prognostic value of CA9 expression[9]. The authors demonstrated strong significant associations between CA9 expression in cancer cells assessed by immunohistochemistry and all endpoints including overall survival, disease-free, locoregional control, disease-specific, metastasis-free survival, and progression-free survival. In the subgroup analyses, similar associations in the majority of tumor sites and types including breast, bladder, lung, liver and head and neck cancer were observed. Additionally, previous studies shown that overexpression of CA9 was associated with more advanced T stage and lymphatic spread to the cervical region[10-12]. In line with previous meta-analysis, we also observed a significant correlation between CA9 overexpression and local recurrence, shorter OS as well as DFS of patients with SNSCC. However, we did not observe a correlation between CAXI overexpression and advanced T stage or lymph node metastases. This may be related to the occult growth pattern of SNSCC which leads to high percentage of patients with advanced T stage and low incidence of neck nodal metastases. In combination with previous reports, our findings demonstrated that CA9 may serve as a universal prognostic marker which indicates higher risk of disease progression in patients with higher expression of CA9 independent of tumor type or site. Furthermore, numerous studies showed that CA9 positivity could predict the treatment resistance to chemotherapy and radiotherapy[13-15]. Therefore, the prognostic value of CA9 in treatment response of SNSCC patients after chemotherapy or radiotherapy is deserved to further evaluated.

In our analysis, advanced pT stage or advanced age was significantly associated with worse OS and DFS. This is in agreement with a recent study by Jain et al[4] who demonstrated increased age at diagnosis and higher stage as prognostic factors associated with poor survival by analyzing data from the Surveillance, Epidemiology and End Results (SEER) database. Similarly, Michel et al[16] reported that

patients with stage T3 or T4 disease had significantly worse OS than patients with stage T1 or T2 disease. However, in the multivariate regression analysis, we only found that advanced age was an independent prognostic factor for worse OS and DFS.

Accumulating experimental evidences suggested that increased expression of CA9 under hypoxic conditions play pivotal roles in tumor development. A majority of studies reported the expression of CA9 was significantly increased in head and neck carcinoma, breast carcinoma, brain tumors, lung, and colorectal carcinoma. CA9 was functionally implicated in adaptation to metabolism generating excess of acidic products, thus allowing for cancer cell survival and proliferation. Moreover, CA IX may promote the tumor growth and progression by exacerbating extracellular acidosis that can activate proteases to degrade extracellular matrix, affect cell adhesion, facilitate epithelial-mesenchymal transition and invasion, and support angiogenesis. Overexpression of CA9 was shown to be a potential hypoxic biomarker for head and neck squamous cell carcinoma, and correlated with advanced stage and poor survival[9, 17]. Hoogsteen further et al[18] reported that CA9 positive expression in cancer cells of HNSCC was also indicative of proliferative capacity. In the present study, we observed that CA9 expression in the SNSCC group was significantly higher than that in the normal control group. In spite of only 33.3% (21/63) of patients with positive CA9 expression in our cohort, the mechanisms that upregulate CA9 expression in SNSCC are unclear. We also found that patients with positive CA9 expression was more likely to have postoperative recurrence and poor survival than patients without CA9 expression. Our findings implied that CA9 was associated with tumor progression of SNSCC, but further studies are required to investigate the molecular mechanism controlling CA9 expression as well as its role in regulating the survival and growth of SNSCC cancer cells.

In recent years, new treatment options such as tumor immunotherapy and targeted therapy have made great breakthroughs for various malignancies including lung cancer, breast cancer, liver cancer and HNSCC. However, these new treatment strategies were rarely explored in SNSCC and the entity is always not incorporated into the research of HNSCC due to the extremely low incidence of sinonasal SCC. CA9 has been investigated as a potential therapeutic target by utilization of specific monoclonal antibodies to detect and cause selective killing of CA9-positive cancer cells or synthetic compounds to inhibit CA9 enzymatic activity in preclinical and early clinical stages of various cancers[19, 20]. Our study firstly showed the positive expression of CA9 in a subset of SNSCC patients and its predictive value for patient survival. Therefore, CA9 expression is a potentially useful biomarker for therapeutic targeting using monoclonal antibodies or synthetic inhibitors for SNSCC.

In conclusion, our findings showed that CA9 is an independent prognostic marker for SNSCC patients who have undergone surgical treatment. To our knowledge, this is the first study demonstrating CA9 expression as a strong predictor for tumor recurrence and poor prognosis in patients with SNSCC after curative resection. These findings suggest that CA9 may serve as a novel therapeutic target for SNSCC.

Abbreviations

CA9: carbonic anhydrase 9; SNSCC: sinonasal squamous cell carcinoma; SCCs: squamous cell carcinomas; OS: overall survival; DFS: disease-free survival; AJCC: American Joint Committee on Cancer; UT: uncinate tissue; SEER: Surveillance, Epidemiology and End Results

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of The Science and Technology Commission of Shanghai Municipality, and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

Study concepts: Xicai Sun, Lan Lin, Dehui Wang

Study design: Chunyan Hu, Huan Wang

Data acquisition: Huan Wang, Chunyan Hu

Data analysis and interpretating: Huan Wang, Chunyan Hu

Statistical analysis: Huan Wang, Chunyan Hu

Manuscript preparation: Chunyan Hu, Huan Wang

Manuscript editing: Huan Wang, Chunyan Hu

Manuscript review: Xicai Sun, Lan Lin, Dehui Wang

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References

1. Lund VJ, Stammberger H, Nicolai P et al. European position paper on endoscopic management of tumours of the nose, paranasal sinuses and skull base. Rhinol Suppl 2010; 22: 1-143.
2. Ansa B, Goodman M, Ward K et al. Paranasal sinus squamous cell carcinoma incidence and survival based on Surveillance, Epidemiology, and End Results data, 1973 to 2009. Cancer 2013; 119: 2602-2610.
3. Sanghvi S, Khan MN, Patel NR et al. Epidemiology of sinonasal squamous cell carcinoma: a comprehensive analysis of 4994 patients. Laryngoscope 2014; 124: 76-83.
4. Jain S, Li Y, Kuan EC et al. Prognostic Factors in Paranasal Sinus Squamous Cell Carcinoma and Adenocarcinoma: A SEER Database Analysis. J Neurol Surg B Skull Base 2019; 80: 258-263.
5. Zamanova S, Shabana AM, Mondal UK, Ilies MA. Carbonic anhydrases as disease markers. Expert Opin Ther Pat 2019; 29: 509-533.
6. Jing X, Yang F, Shao C et al. Role of hypoxia in cancer therapy by regulating the tumor microenvironment. Mol Cancer 2019; 18: 157.
7. Pastorekova S, Gillies RJ. The role of carbonic anhydrase IX in cancer development: links to hypoxia, acidosis, and beyond. Cancer metastasis reviews 2019; 38: 65-77.
8. Kwon OJ, Park JJ, Ko GH et al. HIF-1alpha and CA-IX as predictors of locoregional control for determining the optimal treatment modality for early-stage laryngeal carcinoma. Head Neck 2015; 37: 505-510.
9. van Kuijk SJ, Yaromina A, Houben R et al. Prognostic Significance of Carbonic Anhydrase IX Expression in Cancer Patients: A Meta-Analysis. Front Oncol 2016; 6: 69.
10. Kim JY, Lee SH, An S et al. Carbonic anhydrase 9 expression in well-differentiated pancreatic neuroendocrine neoplasms might be associated with aggressive behavior and poor survival. Virchows Arch 2018; 472: 739-748.
11. Yang J-S, Lin C-W, Chuang C-Y et al. Carbonic anhydrase IX overexpression regulates the migration and progression in oral squamous cell carcinoma. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 2015; 36: 9517-9524.
12. Liu Z, Yang Z, Jiang S et al. Paxillin and carbonic anhydrase IX are prognostic markers in gallbladder squamous cell/adenosquamous carcinomas and adenocarcinomas. Histopathology 2014; 64: 921-934.
13. Ohtaki Y, Shimizu K, Kawabata-Iwakawa R et al. Carbonic anhydrase 9 expression is associated with poor prognosis, tumor proliferation, and radiosensitivity of thymic carcinomas. Oncotarget 2019; 10: 1306-1319.

14. Vitoratou DI, Tolia M, Liakos P et al. Clinical value of significance of Hypoxia Inducible Factor-1alpha, Glucose Transporter-1 and Carbonic Anhydrase IX in rectal cancer after preoperative chemoradiotherapy. J buon 2019; 24: 456-463.
15. Koukourakis MI, Bentzen SM, Giatromanolaki A et al. Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. J Clin Oncol 2006; 24: 727-735.
16. Michel J, Fakhry N, Mancini J et al. Sinonasal squamous cell carcinomas: clinical outcomes and predictive factors. Int J Oral Maxillofac Surg 2014; 43: 1-6.
17. Beasley NJ, Wykoff CC, Watson PH et al. Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. Cancer research 2001; 61: 5262-5267.
18. Hoogsteen IJ, Marres HAM, Wijffels KIEM et al. Colocalization of carbonic anhydrase 9 expression and cell proliferation in human head and neck squamous cell carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research 2005; 11: 97-106.
19. Benej M, Pastorekova S, Pastorek J. Carbonic anhydrase IX: regulation and role in cancer. Sub-cellular biochemistry 2014; 75: 199-219.
20. Pastorek J, Pastorekova S. Hypoxia-induced carbonic anhydrase IX as a target for cancer therapy: from biology to clinical use. Semin Cancer Biol 2015; 31: 52-64.

Tables

Table 1. Clinicopathological characteristics of 63 patients with sinonasal squamous cell carcinoma.

Variable	Number (%)
Sex	
Male	47 (74.6%)
Female	16 (25.4%)
Age (years), median age at diagnosis (range)	57(22-85)
Smoking history	
Ever-smoker	19 (30.2%)
Never-smoker	44 (69.8%)
Surgical history	
Yes	9 (14.3%)
No	54 (85.7%)
Tumor differentiation	
Well+Moderate	34 (54%)
Poor	29 (46%)
Primary site	
Nasal cavity	14 (22.2%)
Maxillary sinus	33 (52.4%)
Ethmoid sinus	16 (25.4%)
T stage	
T1	3 (4.8%)
T2	8 (12.7%)
T3	16(25.4%)
T4	36 (57.1%)
N stage	
N0	61(96.8%)
N+	2(3.2%)
Clinical stage	
I	3 (4.8%)
II	8 (12.7%)

III	16(25.4%)
IV	36 (57.1%)
Treatment	
Surgery plus adjuvant radiotherapy	18
Surgery plus adjuvant chemoradiotherapy	21
Neoadjuvant therapy plus surgery	24

Table 2. Association between clinicopathological characteristics and CA9 expression.

	CA9 expression (no. of patients)		
Variables	Positive (n=21)	Negative (n=42)	P-value
Sex			0.222
Male	18	29	
Female	3	13	
Age (years, mean±SD)	60±12.5	54.5±14.7	0.152
Smoking history			0.332
Ever-smoker	8	11	
Never-smoker	13	31	
Surgical history			0.466
Yes	4	5	
No	17	37	
Tumor differentiation			0.063
Well+Moderate	15	19	
Poor	6	23	
Primary site			0.789
Ethmoid sinus+Nasal cavity	9	21	
Maxillary sinus	12	21	
T stage			0.310
T1+T2	2	9	
T3+T4	19	33	
Local recurrence			0.016*
Yes	16	18	
No	5	24	
Regional recurrence			0.595
Yes	2	2	
No	19	40	
Distant metastasis			0.595
Yes	2	2	

No	19	40

Table 3. Univariate analysis of various prognostic factors in patients with sinonasal squamous cell carcinoma.

Prognostic factor	Overall survival			Disease-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (years) (≥56 vs<56)	2.263	0.989-5.177	0.042*	2.504	1.185-5.291	0.012*
Sex (male vs female)	0.943	0.399-2.231	0.892	1.29	0.56-2.964	0.544
Smoking history (ever vs never)	0.908	0.397-2.076	0.815	1.077	0.524-2.212	0.838
Surgical history (yes vs no)	1.633	0.618-4.32	0.307	2.178	0.938-5.057	0.059
Tumor differentiation (well+moderate vs poor)	0.44	0.192-1.006	0.04*	0.712	0.359-1.413	0.322
Primary site (nasal cavity+ ethmoid sinus vs maxillary sinus)	0.603	0.276-1.317	0.189	0.797	0.4-1.587	0.512
T stage (T3+T4 vs T1+T2)	7.072	0.959-52.176	0.022*	4.358	1.041-18.239	0.026*
CA9 expression in cancer cells (positive vs negative)	2.978	1.382-6.416	0.003*	2.732	1.376-5.424	0.002*

HR, hazard ratio; CI, confidence interval at the 95% level; CAFs, cancer-associated fibroblasts

*The P value is significant

Table 4. Multivariate Cox regression analyses of prognostic factors associated with overall survival and disease-free survival in patients with sinonasal squamous cell carcinoma.

Prognostic factor	Overall survival			Disease-free survival		
	HR	95% CI	P-value	HR	95% CI	P-value
Age (years) (≥56 vs<56)	2.07	0.898-4.768	0.084	2.441	1.151-5.177	0.02*
T stage (T3+T4 vs T1+T2)	5.885	0.789-43.889	0.088	3.882	0.919-4.655	0.065
CA9 expression in cancer cells (positive vs negative)	2.401	1.103-5.226	0.027*	2.32	1.157-4.655	0.018*

HR, hazard ratio; CI, confidence interval at the 95% level

* The P value is significant

Figures

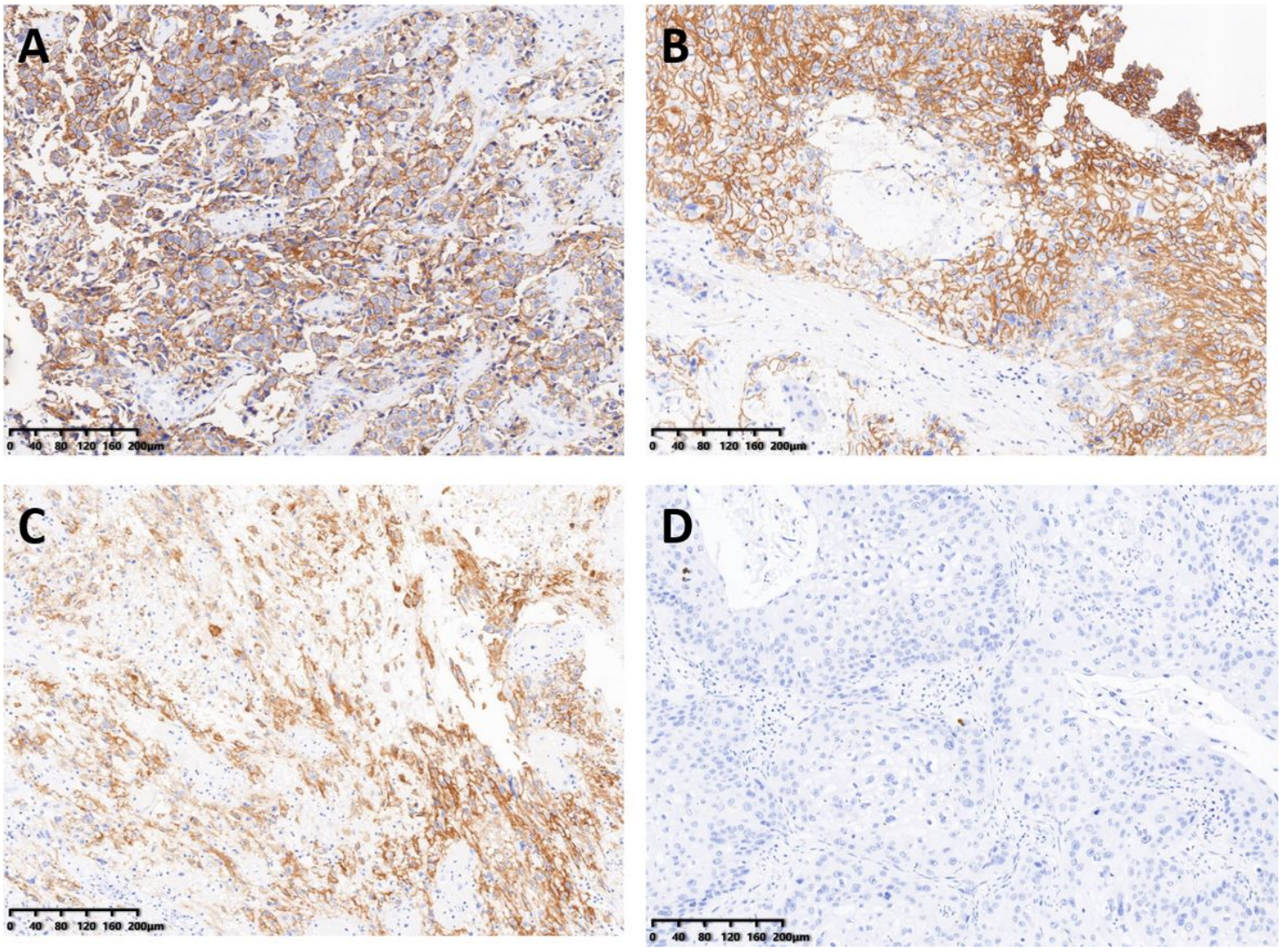

Figure 1

Representative images of immunohistochemical staining for CA9 in sinonasal squamous cell carcinoma tissues. (original magnification ×200). (A-C) Representative positive staining. (D) Representative negative staining.

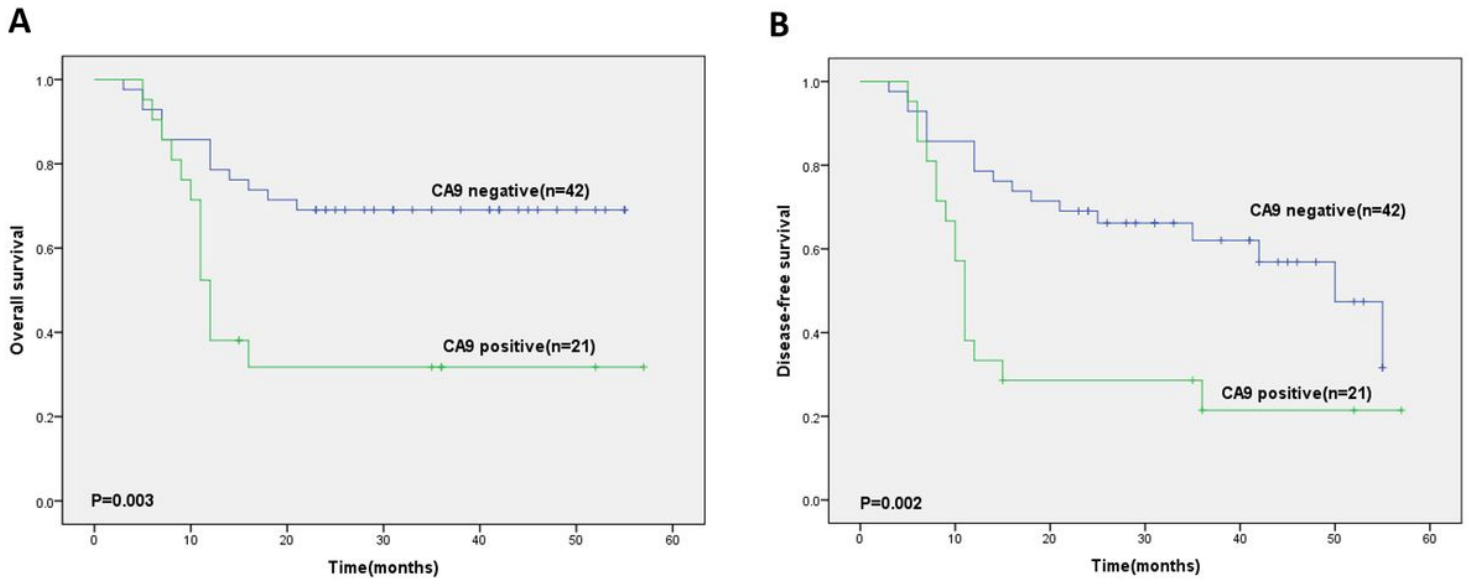

Figure 2

Kaplan–Meier survival curves for the overall (A) and disease-free (B) survival of 63 patients with sinonasal squamous cell carcinoma according to the CA9 expression status in sinonasal squamous cell carcinoma cells. P values were calculated by the logrank test.