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ORIGINAL ARTICLE

CD44 Variant 6 Expression and Tumor Budding in the Medullary Invasion Front of Mandibular Gingival Squamous Cell Carcinoma Are Predictive Factors for Cervical Lymph Node Metastasis

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Abstract

Oral squamous cell carcinoma (OSCC) with invasion into the mandibular medullary space has been reported to be a predictive factor for cervical lymph node metastasis (CLNM). As CLNM has been associated with the stemness of cancer cells, we aimed to evaluate the relationship between clinical characteristics and immunohistochemical findings on the invasion front of the medullary invasive OSCC and CLNM. The medical records of 25 patients with the mandibular medullary invasive OSCC who were performed mandibulectomy and neck dissection in our department from 2010 to 2016 were examined. Serial sections were stained with antibodies against CD44 variant 6 (CD44v6) to examine cancer stemness and to evaluate the number of tumor buds in the medullary invasion front of the mandibular invasive OSCC. Categorical data were analyzed by Fisher's exact test. The expression of CD44v6 and the number of tumor buds between the groups with and without pathological CLNM (CLNM+ and CLNM-, respectively) were analyzed using the Mann-Whitney U test. Of the 25 patients, 11 patients were CLNM+. Of the several measured variables, histologic differentiation of the mandibular invasive OSCC was a significant factor for CLNM+. CD44v6 expression and tumor bud formation in the medullary invasion front of the mandibular invasive OSCC were significantly higher in the CLNM+ group, suggests that both CD44v6 and tumor budding in the medullary invasion front are predictive factors for CLNM.

Keywords CD44 variant 6 · Tumor budding · Mandibular gingival squamous cell carcinoma · Medullary invasion · Cervical lymph node metastasis

Abbreviations

CLNM	cervical lymph node metastasis
DSS	disease-specific survival
EMT	epithelial-mesenchymal transition
MGSCC	mandibular gingival squamous cell carcinoma
OS	overall survival

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Introduction

The mandibular invasive oral squamous cell carcinoma (OSCC) has been reported to account for up to one quarter of cases of oral squamous cell carcinomas, although estimates range from less than 10% to as high as 30%, depending on the study [1–4]. The treatment outcomes of the mandibular invasive OSCC are deemed to be poor, because of the frequent occurrence of early bony invasion into underlying alveolar bone [4–8]. Moreover, relationship between cervical lymph node metastasis (CLNM) and the mandibular invasive OSCC have also been reported [9–11]. Although some reports have indicated that medullary invasion of the mandibular invasive OSCC is not an independent prognosticator for survival [5, 12–14], some studies have shown that medullary invasion of the mandibular invasive OSCC is a predictive factor for lymph node and/or distant metastasis [10, 11]. The control of CLNM is important for improving the prognosis of the

mandibular invasive OSCC patients. To prevent neck failure, it is essential to have a more comprehensive understanding of the predictive factors that contribute to CLNM. Already, National Comprehensive Cancer Network (NCCN) Clinical Practice Guideline 2017 recommends that the cases with mandibular bone invasion should get postoperative adjuvant treatment [15]. However, the basic evidence has never been reported to data.

CD44 is a widely expressed polymorphic integral membrane adhesion molecule that binds hyaluronic acid and contributes to cell-cell and cell-matrix adhesion, cell growth and trafficking, the epithelial-mesenchymal transition (EMT), and tumor progression [16]. Expression of CD44 variant isoforms has been associated with deregulation of CD44 expression and tumor progression and prognosis; this has been described in several cancers including hepatocarcinoma, melanoma, lung, breast, gastric cancers and head and neck cancers, particularly in patients expressing the CD44v6 isoform [17, 18]. Another factor often associated with EMT is the presence of tumor budding, which is regarded as an indicator of poor prognosis [19–21]. Although tumor budding in soft tissue was reported as a prognosticator [19–21], no immunohistochemical analysis on tumor budding invaded in hard tissue has been reported to data. Moreover, the presence of tumor budding is also a poor prognosticator and related the expression of CD44 in colorectal region [22].

As there have been no reports to date evaluating immunohistochemical (IHC) staining and presence of tumor budding in the medullary invasion front in the mandibular invasive OSCC, we aimed to characterize the clinical significance of CD44v6 expression and the presence of tumor buds in the medullary invasive OSCC specimens with the aim of determining whether these features are significant predictors for CLNM.

Materials and Methods

Patients

We retrospectively reviewed the medical records of 25 mandibular invasive OSCC patients who underwent marginal or segmental or hemi-mandibulectomy without neoadjuvant chemotherapy or radiotherapy and who were pathologically diagnosed with medullary invasion between January 2010 and December 2016. All patients were followed up over 1 year after surgery. Neck dissection was performed concurrently in all cases. Tumors were clinically classified according to the Union for International Cancer Control [23]. Tumor histologic differentiation was defined according to the World Health Organization classification (4th edition) [24]. In addition to these features, tumor location, vascular/perineural invasion, and invasion pattern were statistically analyzed to determine whether there was an association with CLNM. Tumor location was defined as anterior (tumor located only near anterior teeth), posterior (tumor located only near posterior teeth, not including canines), or both (tumor located near both anterior and posterior teeth, including canines) [25]. Details of clinical evaluation in the present patients were summarized in Table 1.

IHC Staining and Evaluation

Post-surgical tissue specimens from 25 mandibular invasive OSCC patients with medullary invasion were formalin-fixed and paraffin-embedded. Sections were deparaffinized in xylene, soaked in 10 mmol/L citrate buffer (pH 6.0) and placed in a hot water bath at 95 °C for 30 min for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min. IHC staining was performed using the EnVision method (EnVision+; Dako, Glostrup, Denmark). As the primary antibodies, CD44v6 [VFF-18] (dilution 1:100; ab78960, Abcam, Cambridge, UK) was used to evaluate the stemness of the tumor epithelium (Fig. 1). The sections were then washed in Dulbecco's phosphate buffered saline, followed by incubation with the primary antibodies at 4 °C overnight. Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) solution, and the samples were counterstained with Meyer's hematoxylin and mounted. CD44v6 expression in each case was analyzed by calculating the total immunostaining score which ranged from 0 to 7; this was the sum of the positive area score and the intensity score in the invasion front of the tumor. The highest score among each of the 3 randomly selected observation fields was adopted as the total score. The CD44v6 positive area scores were based on the estimated fraction of tumor cells (0, none; 1, <10%; 2, 10–50%; 3, 50–80%; 4, >80%) (Fig. 2, upper). The intensity score represents the staining intensity of CD44v6 (0, no staining; 1, weak; 2, moderate; 3, strong) (Fig. 2, lower). The Mann-Whitney U test was used to determine statistical significance of the total immunostaining score in patients with and without CLNM.

Tumor budding was defined as the presence of isolated small clusters (<5 cancer cells) ahead of the invasive front [19]. Tumor specimens stained by CD44v6 were initially scanned using a × 4 objective lens and a × 10 ocular lens to select the areas with the highest density of tumor budding. Tumor buds in the selected fields were then counted using a × 10 objective lens and a × 10 ocular lens, and the highest count per field was used as the number of tumor buds (Fig. 3a and b).

All IHC assessments, scoring, and analyses were performed by 2 examiners in a blinded fashion. Tumor characterization and comparison of responses were based on the results of these assessments.

Statistical Analysis

All statistical analyses were conducted using SPSS Version 24.0 for Windows (SPSS Inc., Chicago, IL, USA). Fisher's

Table 1 Patients characteristics

Variable	Category	Value*
Age		60-93 (median: 75 ± 8.7) years60–93 (median: 75 ± 8.7) years
Sex	male	13 (52%)
	female	12 (48%)
Clinical T classification	cT1–3	3 (12%)
	cT4	22 (88%)
Clinical N classification	cN0	12 (48%)
	cN1–3	13 (52%)
Clinical stage	stage I-IIstage I-II	3 (12%)
	stage III-IVstage III-IV	22 (88%)
CLNM	No	14 (56%)
	Yes	11 (44%)
Tumor location	Anterior	2 (8%)
	Posterior	14 (56%)
	Both	9 (36%)
Surgery	Marginal resection	6 (24%)
	Segmental resection	18 (72%)
	Hemimandibulectomy	1 (4%)
Local recurrence	No	18 (72%)
	Yes	7 (28%)
Death cause	T	2 (8%)
	N	1 (4%)
	M	3 (12%)
	Other	2 (8%)

*Values are expressed as the mean ± standard deviation in a parametric data, minimal-maximum (median) in a nonparametric data, or number (%) in a categorized data

CLNM, cervical lymph node metastasis

exact test was used to assess correlation between clinicopathological parameters in the groups that were positive for pathological CLNM (CLNM+) and the group that were negative for pathological CLNM (CLNM-). The distributions of the expression of CD44v6 and the number of tumor buds were statistically analyzed using the Mann-Whitney U test. For all statistical analyses, *P*-values <0.05 were considered statistically significant.

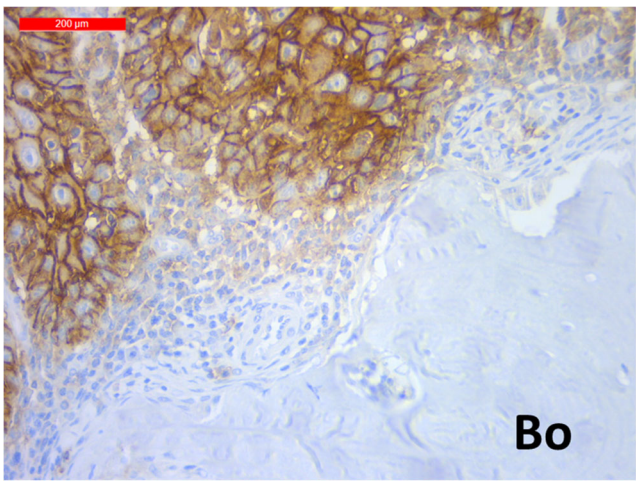

Fig. 1 Representative image of the immunoexpression of CD44 variant 6 (CD44v6) in the invasion front of mandibular invasive squamous cell carcinoma. CD44v6 was expressed in cancer cell membranes, but it was not expressed in cancer cell cytoplasm. Bo, bone. Scale bar = 200 μm

Results

Patient Characteristics and Treatment

The study included 25 specimens of mandibular invasive OSCC from 13 men (52%) and 12 women (48%), whose mean ± standard deviation (SD) age was 75 ± 8.7 years (range: 60–93 years). With regard to clinical tumor classification using the TNM (tumor, node, metastasis) system, 3 (12%) and 22 lesions (88%) were T1–3 and T4, respectively; 12 (48%) and 13 (52%) cases were N0 and N1–3, respectively. Six (24%), 18 (72%), and 1 (4%) patients were performed marginal, segmental, and hemi-mandibulectomy, respectively. Of the 25 specimens, 21 (84%) were well-differentiated and 4 (16%) were moderately-differentiated. With regard to the

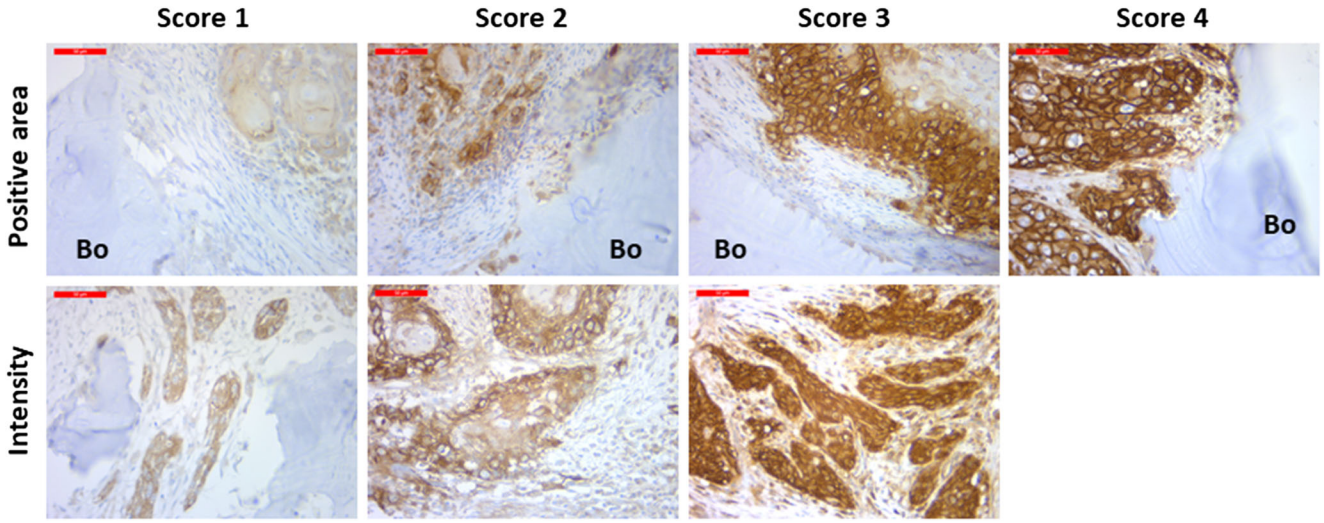

Fig. 2 CD44 variant 6 (CD44v6) expression was analyzed by calculating the total immunostaining score which ranged from 0 to 7; this was the sum of the positive area score and the intensity score in the invasion front of the tumor. The highest score among each of the 3 randomly selected observation fields was adopted as the total score. The CD44v6 positive area scores were based on the estimated fraction of tumor cells (0, none; 1, <10%; 2, 10–50%; 3, 50–80%; 4, >80%). The intensity score represents the staining intensity of CD44v6 (0, no staining; 1, weak; 2, moderate; 3, strong). Bo, bone. Scale bar = 50 μm

mandibular invasive OSCC location, 2 (8%) were anterior, 14 (56%) were posterior, and 9 were both (36%). Seventeen (68%) demonstrated expansive type patterns and 8 (32%) invasive type. CLNM was pathologically detected in 11 cases (44%). Of such cases, 5 cases (20%) had extranodal extension. Then, 8 cases (32%) were detected local recurrence. Eight patients (32%) in the present study died. The death causes were 2 in T (8%), 1 in N (4%), and 3 in M (12%), respectively. In three patients with distant metastasis, two were positive for CLNM, but in one patient CLNM was not pathologically detected. The correlation between clinicopathological parameters for the CLNM+ and CLNM- groups are summarized in Table 2. Statistic examination revealed that sex and histologic differentiation were significant risk factors for the development of CLNM ($P = 0.047$ and 0.026).

Correlation between CD44v6 Expression and Tumor Budding Formation

Immunoexpression of CD44v6 were found in all samples. CD44v6 was strongly expressed in cancer cell membranes, but it was not expressed in cancer cell cytoplasm, and was positive in the invasion front. Cancer nests were negative in almost cases. Significantly high expression of CD44v6 in the mandibular invasive front in the CLNM+ group was detected ($P = 0.009$) (Fig. 4a). The CLNM+ group also had

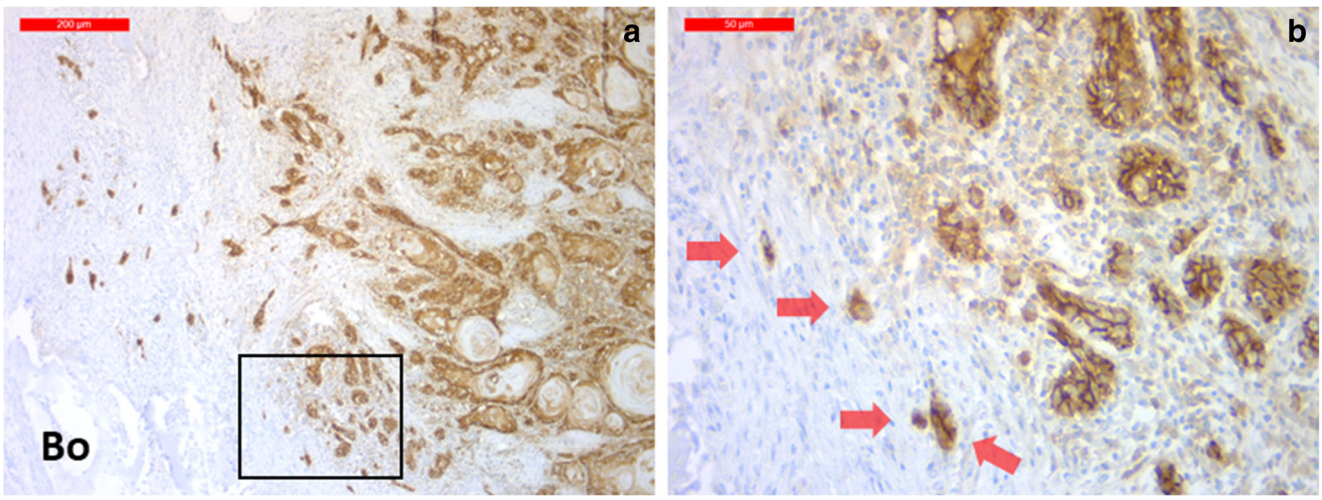

Fig. 3 Immunohistochemical analysis of tumor budding in bony invasion front of mandibular gingival squamous cell carcinoma in a CD44 variant 6 staining section. **a** Tumor budding was observed in the invasion front, distant from the main tumor tissue (40×). Scale bar = 200 μm. **b** Magnification image of the window in part **(a)**, showing tumor buds (red arrows) in greater detail (100×). Bo, bone. Scale bar = 50 μm

Table 2 Clinicopathological characteristics and association with cervical lymph node metastasis

Characteristics	Number of cases (%)	CLNM+	CLNM-	*p*
Age, y				
≤65 ≤65	2 (8%)	1	1	1
> 65> 65	23 (92%)	10	13	
Sex				
Male	13 (52%)	3	10	**0.047**
Female	12 (48%)	8	4	
T Classification				
cT1–3	3 (12%)	2	1	0.565
cT4	22 (88%)	9	13	
N Classification				
cN0	12 (48%)	4	8	0.428
cN1–3	13 (52%)	7	6	
Tumor location				
Anterior	2 (8%)	1	1	0.701
Posterior	14 (56%)	5	9	
Both	9 (36%)	5	4	
Invasion pattern				
Expansive	17 (68%)	7	10	1
Invasive	8 (32%)	4	4	
Histologic differentiation				
Well	21 (84%)	7	14	**0.026**
Moderate, Poor	4 (16%)	4	0	
Vascular invasion				
No	15 (60%)	7	8	1
Yes	10 (40%)	4	6	
Perineural invasion				
No	17 (68%)	7	10	1
Yes	8 (32%)	4	4	

P-values were calculated using Fisher's exact test. Tumors were classified according to the TNM (tumor, node, metastasis) system

CLNM, cervical lymph node metastasis

significantly more tumor buds than CLNM- patients in the medullary invasion front ($P = 0.042$) (Fig. 4b). The significant higher expression of CD44v6 and significant more numbers of tumor buds in the CLNM+ group support the assertion by Wang et al. that tumor budding is associated with cancer stemness [19].

Discussion

The mandibular invasive OSCC has unique clinical and pathological characteristics. The mandibular invasive OSCC can invade into the thin gingival mucosa, penetrate the underlying periosteum, and easily reach mandibular bone. National Comprehensive Cancer Network Clinical Practice Guideline 2017 suggested that OSCC which has invaded the cortical bone should be considered adjuvant radiotherapy [15]. The results of the present study, the mandibular medullary invasion of OSCC can significantly increase the risk of CLNM [9–11], also support the recommendation of the guideline and exhibits basic evidence.

Several reports have revealed that CD44v6 expression can be an effective prognostic marker. Yanamoto et al. reported that the high immunoexpression pattern and intensity of CD44v6 in the tongue squamous cell carcinoma were related to the local recurrence in the cases with neoadjuvant chemotherapy [18]. More, Fonseca et al. reported that the pattern and intensity of immunoexpression of the CD44 isoforms in the deep invasive front of OSCC of the tongue were related to CLNM [26]. In the field of colorectal cancer, up-regulation of CD44v6 has been shown to contribute to tumor bud forming, and is useful for identifying those at high risk for locoregional failure in early-staged tumor [27]. Their results agreed with those of the present study in which higher expression of CD44v6 was linked to CLNM and the formation of tumor

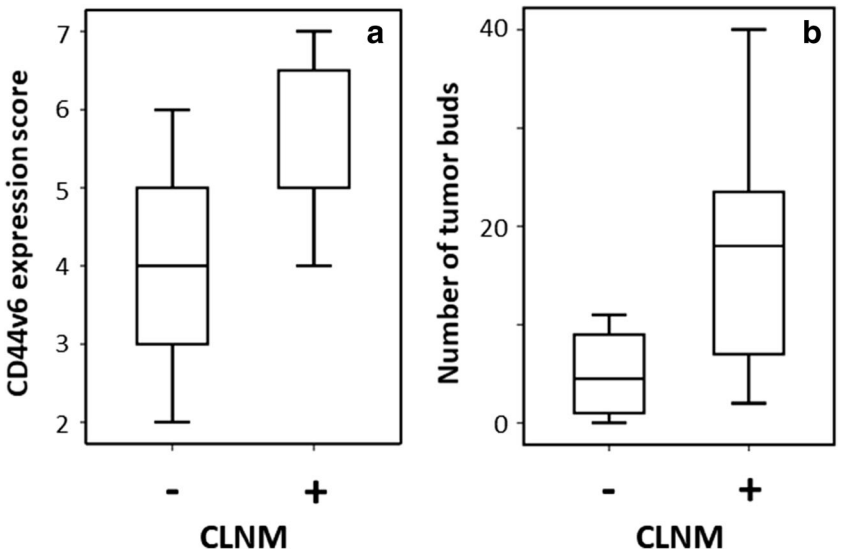

Fig. 4 Box-and-whisker plots showing **a** CD44 variant 6 (CD44v6) expression scores and **b** number of tumor buds in patients with and without cervical lymph node metastasis (CLNM). Mann-Whitney U tests revealed that there was significantly greater CD44v6 and tumor bud formation in patients with CLNM ($P =$ 0.009 and 0.042). As the median score of CD44v6 in CLNM negative group was 5, the median line of the box plots was piled on quartile line

buds. However, since the study was retrospective investigation and only included a small number of the mandibular invasive OSCC patients, it was hard to calculate strict cut-off points for these factors; consequently, accumulation of future cases of the mandibular invasive OSCC will enable us to determine whether these can be used as predictive factors for CLNM. Considering the fact that IHC and tumor bud analysis can be time-consuming and quick diagnosis during operation cannot be demonstrated, those factors are not useful whether additional resection should be performed or not, but are supportable to determine whether adjuvant treatment should be performed or not.

There is no clear consensus on how tumor buds in the medullary invasion front migrate to neck lymph nodes. Tumor budding is a form of cancerous growth involving the differentiation and a dissociation of cancer cells; thus, it can be regarded as the initial phase of vascular invasion [28]. Nomura et al. reported that micro metastases via Haversian canals or Volkmann's canals of mandibular cortical bone cannot be ruled out as a route of tumor migration [29]. Then, cell clusters reach the periosteum via vessels in the aforementioned canals. Then, along with periosteum, OSCC cells go down to cervical lymph-vascular network, and finally achieve lymph node metastasis. However, this occult migration cannot be diagnosed by currently available imaging techniques. Therefore, we suggest that the number of tumor buds can be useful as an indirect marker of CLNM via these canals. In addition, 11 of 25 (44%) cases with medullary invasion were pathologically diagnosed with CLNM. As there were also OSCC cases without medullary invasion which were pathologically diagnosed with CLNM (date not shown), it is hard to exclude the possibility that CLNM was derived from the soft tissue margin of the tumor. In such cases, it is considered that the occult migration from the primary tumor on gingiva also invaded into periosteum and achieved to lymph-vascular network.

In conclusion, the present study first revealed that the CD44v6 expression and the amount of tumor budding can be used as prognostic markers of CLNM. The results basically supported the recommendation of adjuvant treatment by NCCN Guideline. After adjuvant treatment, such cases continuously need frequent and strict follow-up using several modalities over a long period of time. To evaluate further applications of those factors and to determine definite cut-off points of IHC and the number of tumor buds, it will be necessary to conduct further studies of the mandibular invasive OSCC in the future.

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Compliance with Ethical Standards

Conflict of Interest The authors do not have any conflicts of interest to declare.

Ethics Statement/Confirmation of Patients' Permission This retrospective study followed the Declaration of Helsinki and ethics and the regional Ethical Review Board of Nagasaki University approved the study (Registration number; 17,091,115).

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