BMC Oral Health

RESEARCH

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NKG2D (Natural Killer Group 2, Member D) ligand expression and ameloblastoma recurrence: a retrospective immunohistological pilot study

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Abstract

Background/Purpose This retrospective immunohistological pilot study aimed to investigate the influence of natural killer group 2, member D (NKG2D) ligand expression on ameloblastoma recurrence after surgical resection. It also aimed to elucidate additional clinical factors that could serve as predictors of ameloblastoma recurrence.

Materials and methods This study included 96 patients who were histologically diagnosed with ameloblastoma after surgical resection. The expression of NKG2D ligands, including UL16-binding proteins (ULBPs) 1–3 and major histocompatibility complex class I chain-related molecule (MIC) A/B, was evaluated in formalin-fixed paraffin-embedded tumor tissues via immunohistochemistry assays. Furthermore, the patients' electronic medical records were reviewed. Multivariate Cox regression analysis was conducted, and data were expressed as adjusted hazard ratios [HRs] with 95% confidence intervals [95% CIs].

Results Multivariate analysis revealed that recurrent tumors (ref.: primary; adjusted HR [95% CI]: 2.780 [1.136, 6.803], $p=0.025$) and positive MICA/B expression (ref.: negative; adjusted HR [95% CI]: 0.223 [0.050, 0.989], $p=0.048$) independently affected recurrence-free survival in ameloblastoma.

Conclusion This study identified recurrent cases and loss of MICA/B expression as independent predictors of early ameloblastoma recurrence following surgical resection. The findings suggest that decreased MICA/B expression might undermine NKG2D-mediated tumor immunosurveillance, thereby influencing early recurrence.

Keywords Ameloblastoma, Odontogenic tumors, Killer cells, natural, Cytotoxicity, immunologic

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Introduction

Ameloblastoma, though rare, is one of the most frequently occurring odontogenic tumors, predominantly located in the mandible and maxilla. Despite being classified as "benign," ameloblastoma poses a significant challenge due to its marked local invasiveness and high potential for recurrence [1]. The primary therapeutic approach for ameloblastoma is surgical resection [2, 3]. Nevertheless, recurrence remains a significant concern despite meticulous surgical intervention [2, 3]. The known risk factors for ameloblastoma recurrence include maxillary location, large tumor size, nonunicystic type, conservative surgical procedures, and recurrent tumors [2–5]. However, there is a paucity of literature investigating the impact of immunosurveillance-related factors on ameloblastoma recurrence.

Natural killer group 2, member D (NKG2D) and its ligands play a pivotal role in natural killer (NK) cell-mediated tumor immune surveillance [6–8]. NKG2D ligands, including UL16-binding proteins (ULBPs) 1–3 and major histocompatibility complex class I chain-related molecule (MIC) A/B, are prominently expressed on tumor cell surfaces [9]. Upon binding to their receptors on NK cells, NKG2D ligands transmit activating signals that enable NK cells to identify and destroy tumor cells [9]. NKG2D is universally present in all NK cells, and its activating signal can override other inhibitory signals [8, 9]. The correlation between NKG2D ligand expression and disease prognosis has predominantly been explored in the context of malignant tumors, with findings indicating that positive expression of NKG2D ligands is correlated with a more favorable prognostic outcome [10–12]. Numerous recent investigations imply that the degree of NKG2D ligand expression in tumors could function as an indicator for forecasting patient prognosis [10–12]. However, as far as we are aware, there has been no documented research to date on the association between NKG2D ligand expression and the prognosis of ameloblastoma.

Hence, this retrospective immunohistological pilot study aimed to explore the effect of NKG2D ligand expression on the recurrence of ameloblastoma after surgical resection. It also aimed to elucidate additional clinical factors that could serve as predictors of ameloblastoma recurrence.

Materials and methods

Study design, subjects, and ethical considerations

This retrospective study was approved by the Institutional Review Board (IRB; IRB no: 2023-05-010). The subjects of this study include paraffin tissue blocks and medical records of patients histologically diagnosed with jaw ameloblastoma after surgical treatment at the Department of Oral and Maxillofacial Surgery of our hospital, between January 2017 and August 2023. For recurrent ameloblastoma cases, previous paraffin tissue blocks were also examined (from November 2006 to August 2016). All case slides underwent independent review by two oral pathologists, M.S.K and S.Y.P, adhering to the protocols delineated in the WHO classification of Head and Neck Tumors (5th edition). Cases presenting controversies based on histological and radiological findings were excluded from the study. Furthermore, exclusion criteria encompassed patients with paraffin tissue blocks deemed unsuitable for immunohistochemistry staining, individuals diagnosed with ameloblastic carcinoma, those who solely underwent diagnostic incisional biopsy or marsupialization without subsequent therapeutic resection, individuals with a history of other neoplasms, and those afflicted with hepatic, renal, neuromuscular, or autoimmune diseases. All samples were not sourced from executed prisoners or prisoners of conscience.

Immunohistochemistry and pathologic findings

All specimens were fixed for 18–24 h using 10% neutral buffered formalin (NBF) in accordance with the routine processing protocol and the formalin-fixed, paraffin-embedded blocks were stored at temperatures ranging from 17 °C to 24 °C and humidity levels between 20% and 60% at Kyungpook National University Hospital. One representative slide and a formalin-fixed, paraffin-embedded block were chosen from each case for immunohistochemistry analysis, conducted by an oral pathologist (M.S.K). The representative blocks were sectioned into 4-μm slices, floated on a 40 °C water bath containing distilled water, and then transferred onto slides for immunohistochemistry. The slides were deparaffinized and washed three times with xylene, followed by rehydration with a graded series of alcohols, sequentially from 100%, 100%, 80%, to 70%. The slides were immersed in a preheated retrieval solution (ethylenediamine-tetraacetic acid [EDTA], pH = 8.0) for 20 min and then treated with H_2O_2 to inhibit endogenous peroxidase activity. Protein blocking buffer (TA-125-PBQ; Thermo Fisher Scientific Inc., Middlesex County, MA, USA) was applied to each slide for 7 min. The primary antibodies included ULBP1 (1:400 dilution, MBS719240; MyBioSource, San Diego, CA, USA), ULBP2 (1:500 dilution, MBS8106208; MyBioSource, San Diego, CA, USA), ULBP3 (1:200 dilution, ab89931; Abcam, Cambridge, UK), and MICA/B (1:50 dilution, sc-271535, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Diluted primary antibodies were applied to the slides and incubated overnight at 4 °C. Subsequently, the slides were washed with phosphate-buffered saline (PBS) immunohistochemistry wash buffer and then treated with secondary antibodies (ULBP1 and ULBP2: EnVision + Single Reagent [K4003, Agilent, Santa Clara, CA, USA]; ULBP3 and MICA/B:

EnVision+Single Reagent, [K4001, Agilent, Santa Clara, CA, USA]). Next, the slides were reacted with 3,3′-diaminobenzidine (DAB) solution (K3468; Dako, Carpinteria, CA, USA) and counterstained with Mayer's hematoxylin (S3309; Dako, Carpinteria, CA, USA). The controls for ULBP1 and ULBP2 were hepatocellular carcinoma tissues, as indicated by previous studies [13, 14]. The control for ULBP3 was placental tissue, as mentioned in the data sheet for the primary antibody provided by the manufacturer (ab89931; Abcam). The control for MICA/B was colon cancer tissue, as indicated by a previous study [15].

The immunohistochemistry slides were independently examined by two oral pathologist (M.S.K and S.Y.C) using light microscope (Olympus BX53, Olympus Corporation, Tokyo, Japan) at 200x magnification. Any disparities were thoroughly reviewed to reach a consensus. The Immunoreactive Score (IRS) is calculated as the product of the positive cell Proportion Score (0=0%, 1=<10%, 2=10–50%, 3=51–80%, 4=>80%) and the Intensity Score (0=no reaction, 1=mild reaction, 2=moderate reaction, 3=intense reaction). The resulting score ranges from 0 to 12, where 0–1 is considered negative, 2–3 is classified as mild, 4–8 is classified as moderate, and 9–12 is classified as strong stain (Fig. 1) [16].

Medical data acquisition

The following data were retrieved from the patients' electronic medical records: (1) demographics—age, sex, height, weight, American Society of Anesthesiologists (ASA) physical status, and comorbidities; (2) type of surgery—classified as conservative surgery (surgical excision and enucleation with or without bone curettage) or radical surgery (wide resection with a bone margin [>1 cm], *en bloc* resection, mandibulectomy, or maxillectomy) [3, 4]; (3) preoperative radiological findings—tumor location (mandible or maxilla), largest tumor diameter, and cortical bone perforation; (4) tumor characteristics—stages (I: ≤6-cm tumor diameter; II: >6-cm tumor diameter or maxillary sinus/orbital floor invasion; III: skull-base invasion or regional lymph node metastasis) [3, 17], tumor recurrence (primary or recurrent), and type of ameloblastoma (unicystic vs. other types); (5) intraoperative data—anesthetic technique (general anesthesia vs. local anesthesia), maintenance agents (inhalation agent vs. total intravenous anesthesia), total anesthesia time, and blood transfusion; (6) nonsurgical

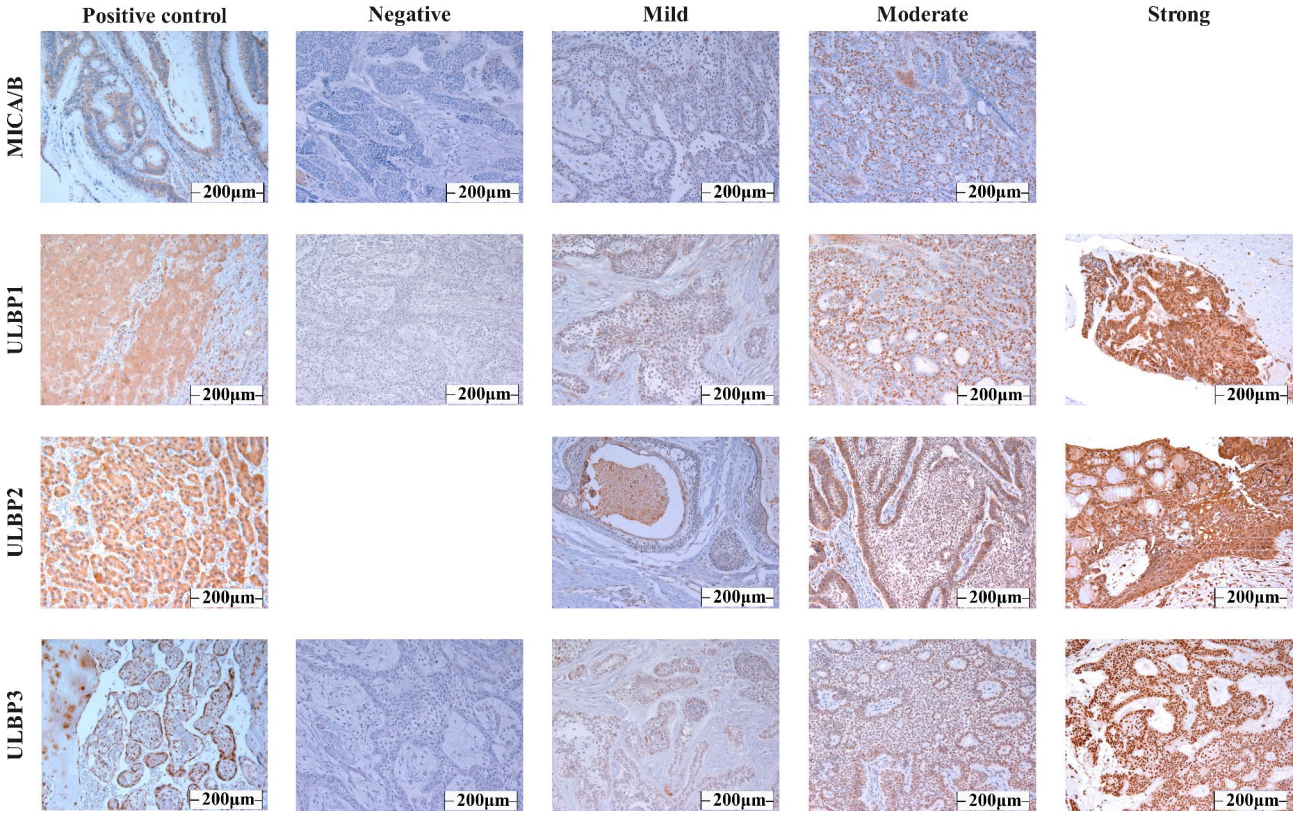

Fig. 1 Immunohistochemical analysis of NKG2D ligand expression in ameloblastoma. Positive controls were as follows: colon cancer tissue for MICA/B, hepatocellular carcinoma tissues for ULBP1 and ULBP2, and placental tissue for ULBP3. There were no cases with strong expression of MICA/B and negative expression of ULBP2 (original magnificaiton×200). Major histocompatibility complex class I chain-related molecules A/B (MICA/B) and UL16-binding proteins (ULBP)

treatment—chemotherapy or radiation therapy; (7) follow-up data—recurrence status (recurrence-free or recurred), total follow-up duration (from histological diagnosis to the last follow-up), and recurrence-free survival (RFS; from histological diagnosis to histologically confirmed relapse).

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics ver. 22 (IBM Corporation, Armonk, NY) and MedCalc ver. 18.11.6 (MedCalc Software bvba, Ostend, Belgium). Continuous variables were expressed as mean ± standard deviation (SD) or as median with first and third quartiles (Q1, Q3), whereas categorical data were expressed as absolute numbers and corresponding percentages. The results of Cox regression analyses were reported as hazard ratios (HRs) or adjusted HRs, along with their corresponding 95% confidence intervals (95% CIs).

The patients were categorized into the recurrence-free group and recurrent groups. Based on the normality test results, parametric data were analyzed using independent *t*-tests, nonparametric data using the Mann–Whitney U test, and categorical data using either the chi-squared test (with Yates' correction for the 2×2 contingency table) or Fisher's exact test.

At the outset, univariate Cox regression analysis was conducted to evaluate the association between prognostic variables and RFS. The prognostic variables included in the univariate analysis were patients' demographics, type of surgery (conservative vs. radical surgery), preoperative radiological findings, tumor characteristics, intraoperative data, clinicopathological types (unicystic type vs. conventional), and expression of NKG2D ligands (negative vs. positive [mild to strong]) determined via immunohistochemistry. In the multivariate Cox regression analysis, we included candidate prognostic variables that had p values < 0.2 in the univariate analysis. Variable selection was performed using backward elimination, with a removal criterion of $p \geq 0.1$ based on the likelihood-ratio statistic probability. Patients with missing data for one or more predictors were excluded from the Cox regression analysis. The proportional hazards assumption, overall model fit, and predictive performance of the final Cox regression model were evaluated using log-minus-log survival plots, the −2 log likelihood (comparing the null model with the final model), and Harrell's C-index, respectively. Subsequently, the Kaplan–Meier method was used along with the log-rank tests to investigate differences in RFS associated with each prognostic factor. A two-tailed p-value < 0.05 was considered statistically significant.

Results

Of the initial cohort of 128 patients, 32 were excluded due to the following reasons: inappropriate paraffin tissue blocks for immunohistochemistry staining ($n=11$), underwent diagnostic incisional biopsy or marsupialization without therapeutic resection ($n=7$), presence of ameloblastic carcinoma ($n=5$), history of other neoplasms ($n=3$), presence of hepatic or renal disease ($n=1$), presence of neuromuscular diseases ($n=3$), and presence of autoimmune diseases ($n=2$) (Fig. 2).

The immunoreactivity for NKG2D ligands was observed in the membrane, cytoplasm and appeared to be present in the nuclear area. The immunohistochemical staining occurs in both the tumor epithelium and stroma. In the epithelium, the staining is predominantly observed in the peripheral cells of the islands, nests, and cellular cords, compared to the stellate reticulum.

In our final sample of 96 patients, 73 (76.0%) remained recurrence-free whereas the remaining 23 (24.0%) had recurrence during the follow-up period. The median follow-up duration was 617.5 days (interquartile range: 314.0–1106.0 days), with a 95% CI for the median ranging from 510.9 to 756.9 days.

Table 1 presents the patients' demographics, tumor characteristics, NKG2D ligand expression, and intraoperative variables. The two patient groups were comparable in terms of patient demographics and intraoperative variables. Regarding tumor characteristics, recurrent tumors were more frequent in the recurrence group (number [%]; primary: 13 [56.5], recurrent: 10 [43.5]) than in the recurrence-free group (primary: 60 [82.2], recurrent: 13 [17.8], $p=0.025$). However, no significant differences were observed between the groups in terms of tumor stage, location, and size, frequency of unicystic type, and cortical bone perforation. Furthermore, no patients received nonsurgical treatments such as radiotherapy or chemotherapy in conjunction with surgical treatment.

In terms of NKG2D ligand expression (Table 1), MICA/B positive expression was more prevalent in the recurrence-free group than in the recurrence group (number [%]: recurrence-free: 33 [45.2], recurrence: 2 [8.7], $p=0.003$). In addition, a significant difference was observed in the intensity of MICA/B expression between the groups (number [%]; no recurrence group: none/mild/moderate/strong: 40/27/6/0 [54.8/37.0/8.2/0.0]; recurrence group: 21/0/2/0 [91.3/0.0/8.7/0.0], $p=0.003$). However, there were no differences in the expressions of ULBP1, ULBP2, and ULBP3 between the groups.

Prognostic factors for ameloblastoma recurrence

Table 2 presents the results of the univariate Cox regression analysis for the association between RFS and prognostic factors. The univariate analysis revealed seven potential prognostic factors for RFS: age (crude HR [95%

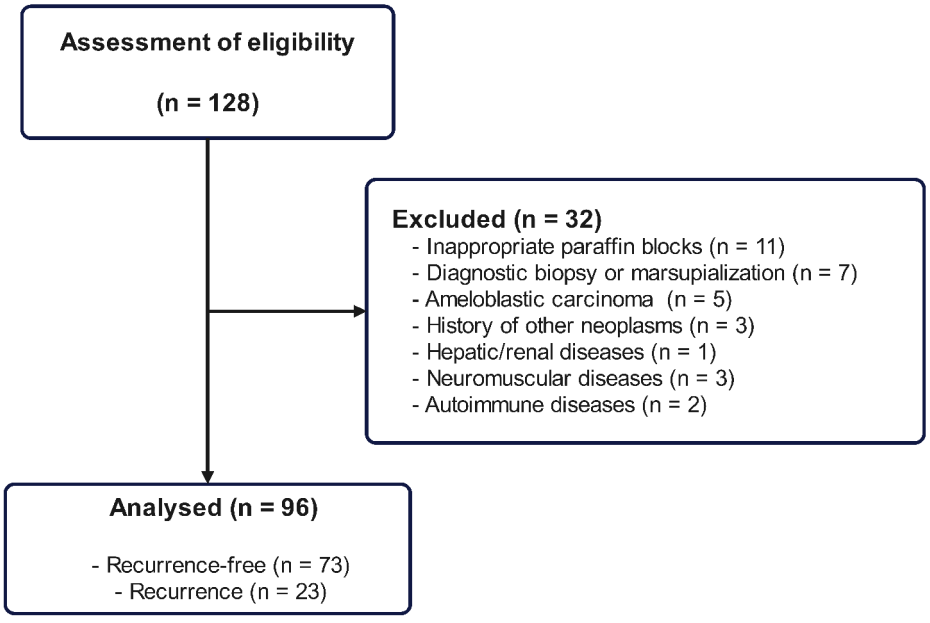

Fig. 2 Study flow chart

CI]; 1.017 [0.997, 1.038], $p=0.103$), ASA classification (1.604 [0.858, 2.998], $p=0.139$), recurrent tumor (ref.: primary; 3.671 [1.516, 8.887], $p=0.004$), size (0.790 [0.596, 1.046], $p=0.100$), cortical bone perforation (ref.: nonperforation; 2.098 [0.798, 5.512], $p=0.133$), MICA/B positive (ref.: negative; 0.176 [0.041, 0.757], $p=0.020$), and anesthesia time (0.662 [0.384, 1.142], $p=0.139$).

All seven candidate prognostic factors were entered in the multivariate Cox regression analysis. Multivariate analysis with variable selection using backward elimination revealed that recurrent tumors (ref.: primary; adjusted HR [95% CI], 2.780 [1.136, 6.803], $p=0.025$) and positive MICA/B expression (ref.: negative; 0.223 [0.050, 0.989], $p=0.048$) independently influenced the RFS of ameloblastoma (Table 3). Figure 3 presents log-minus-log survival plots for the assessment of the proportional hazard assumption of the final model. The −2 log likelihood values for the null and final models were 160.0 and 146.7, respectively ($p=0.001$). In our final model, Harrell's C-index, the predictive performance metric, was 0.704 (95% CI: 0.610, 0.797).

The Kaplan–Meier estimate also revealed RFS differences based on the presence or absence of independent predictors (Fig. 4). Primary tumors had longer RFS than recurrent tumors (log-rank test: $p=0.002$). Furthermore, tumors with positive MICA/B expression had longer RFS than those with negative expression (log-rank test: $p=0.008$).

Sample size

Traditionally, the sample size for Cox regression analysis has been evaluated using the 1-in-10 rule, which recommends a minimum of 10 events per predictor variable to prevent overfitting and ensure adequate statistical power [18]. However, in recent years, this rule has been suggested to be overly conservative as a general guideline [18]. Notably, Vittinghoff et al. demonstrated that the rule of 1 in 10 or more in logistic analysis is not a well-defined bright line and that having 5–9 events per predictor variable could be adequate for Cox analysis [18]. In the final model, two predictor variables were included: tumor recurrence (primary vs. recurrent) and MICA/B expression (positive vs. negative). Given the occurrence of 23 events, the sample size for our study could be sufficient, regardless of the specific criteria employed.

Discussion

In this retrospective immunohistological pilot study, ameloblastoma recurrence was observed in 23 (24.0%) out of 96 patients during the follow-up period (median [Q1, Q3]: 617.5 [314.0, 1106.0] days). The independent prognostic factors associated with ameloblastoma recurrence were recurrent tumors (vs. primary tumor) and MICA/B expression.

Ameloblastoma, benign odontogenic epithelial tumor mainly originating from undifferentiated enamel tissue, ranks among the most common benign tumors affecting the mandible and maxilla, accounting for approximately 1% of all oral area tumors [3, 19]. Ameloblastoma presents clinical challenges owing to its dual nature.

Table 1 Patient demographics, tumor characteristics, NKG2D ligand expression, and intraoperative variables

Variables	Total (*n* = 96)	Recurrence-free (*n* = 73)	Recurrence (*n* = 23)	*P* value
Demographics				
Age (yr)	39.9 (21.6)	38.2 (20.9)	45.4 (23.4)	0.163
Sex (Male), *n* (%)	44 (45.8)	35 (47.9)	9 (39.1)	0.617
ASA classification, *n* (%) I / II / III	45 / 47 / 4 (46.9 / 49.0 / 4.2)	38 / 33 / 2 (52.1 / 45.2 / 2.7)	7 / 14 / 2 (30.4 / 60.9 / 8.7)	0.109
Height (cm)	165.4 (9.3)	165.8 (9.0)	164.2 (10.4)	0.473
Weight (kg)	64.4 (12.6)	64.7 (12.8)	63.3 (12.3)	0.653
BMI (kg/m^2)	23.5 (3.7)	23.5 (3.8)	23.5 (3.6)	0.992
Tumor characteristics				
Tumor recurrence Primary / Recurrent tumor	73 / 23 (76.0 / 24.0)	60 / 13 (82.2 / 17.8)	13 / 10 (56.5 / 43.5)	0.025
Stage, *n* (%) I / II / III	82 / 14 / 0 (85.4 / 14.6 / 0.0)	62 / 11 / 0 (84.9 / 15.1 / 0.0)	20 / 3 / 0 (87.0 / 13.0 / 0.0)	> 0.999
Location, *n* (%) Mandible / Maxilla	84 / 12 (87.5 / 12.5)	64 / 9 (87.7 / 12.3)	20 / 3 (87.0 / 13.0)	> 0.999
Size (cm)	3.0 (2.0, 4.4)	3.2 (2.2, 4.4)	2.3 (1.7, 3.5)	0.123
Unicystic type, *n* (%)	26 (27.1)	19 (26.0)	7 (30.4)	0.884
Cortical bone perforation, *n* (%)	13 (13.5)	7 (9.6)	6 (26.1)	0.075
NKG2D ligand expression				
MICA/B expression, *n* (%) None / mild / mod / strong	61 / 27 / 8 / 0 (63.5 / 28.1 / 8.3 / 0.0)	40 / 27 / 6 / 0 (54.8 / 37.0 / 8.2 / 0.0)	21 / 0 / 2 / 0 (91.3 / 0.0 / 8.7 / 0.0)	0.003
ULBP1 expression, *n* (%) None / mild / mod / strong	13 / 41 / 33 / 9 (13.5 / 42.7 / 34.4 / 9.4)	9 / 32 / 26 / 6 (12.3 / 43.8 / 35.6 / 8.2)	4 / 9 / 7 / 3 (17.4 / 39.1 / 30.4 /13.0)	0.750
ULBP2 expression, *n* (%) None / mild / mod / strong	0 / 2 / 76 / 18 (0.0 / 2.1 / 79.2 / 18.8)	0 / 2 / 58 / 13 (0.0 / 2.7 / 79.5 / 17.8)	0 / 0 / 18 / 5 (0.0 / 0.0 / 78.3 / 21.7)	0.864
ULBP3 expression, *n* (%) None / mild / mod / strong	1/ 12 / 25 / 58 (1.0 / 12.5 / 26.0 / 60.4)	0 / 10 / 20 / 43 (0.0 / 13.7 / 27.4 / 58.9)	1 / 2 / 5 / 15 (4.3 / 8.7 / 21.7 / 65.2)	0.388
Intraoperative variables				
Type of surgery Conservative / Radical surgery	93 / 3 (96.9 / 3.1)	70 / 3 (95.9 / 4.1)	23 / 0 (100.0 / 0.0)	> 0.999
General anesthesia, *n* (%)	75 (78.1)	60 (82.2)	15 (65.2)	0.153
Anesthesia time (h)	1.2 (0.8, 1.5)	1.3 (1.0, 1.7)	1.0 (0.0, 1.3)	0.052
Anesthetic agent, *n* (%) Local / inhalation / total intravenous	21 / 61 / 14 (21.9 / 63.5 / 14.6)	13 / 48 / 12 (17.8 / 65.8 / 16.4)	8 / 13 / 2 (34.8 / 56.5 / 8.7)	0.214
Transfusion, *n* (%)	1 (1.0)	1 (1.4)	0 (0.0)	> 0.999

Data were presented as mean (SD), median (IQR), and absolute numbers (%). American society of anesthesiologists (ASA), body mass index (BMI), natural killer group 2, member D (NKG2D), UL16-binding proteins (ULBP), and major histocompatibility complex class I chain-related molecules A/B (MICA/B)

Classified as "benign" tumor with a slow growth pattern, it exhibits "malignant tumor-like" behavior, including local invasiveness, frequent recurrence rate, and potential for metastasis [3].

The overall recurrence rate for ameloblastoma was 22–31% [1, 4, 20]. Our study's overall recurrence rate of 24% is consistent with this range. The factors known to affect recurrence rate are the type of surgery, tumor recurrence (primary vs. recurrent), tumor size, clinicopathological type, and tumor location [2–5]. Surgical factors are considered to strongly influence ameloblastoma recurrence rates [2–5]. A recent systematic review reported that the recurrence rates for ameloblastoma following conservative surgery ranged from 33 to 93%, whereas those following radical surgery ranged from 7 to 22% [4]. Despite the advantages of radical surgery in terms of recurrence control, radical surgery necessitates a margin of over 1 cm [3, 4]. This led to a recent preference for conservative surgery, which preserves anatomical form and provides cosmetic and functional benefits [1, 21]. A recent meta-analysis failed to demonstrate the superiority of radical surgery over conservative surgery for ameloblastoma recurrence prevention [3, 21]. Moreover, the changing preferences of recent patients, who prioritize their quality of life, have added to the complexity of the decision-making process for surgeons. In the present study, a higher preference for conservative surgery (96.9%) over radical surgery (3.1%) was observed, which is presumed to be a reflection of patient choices. Due to the low incidence of radical surgery in this study, the impact of this factor was not assessed, which represents a limitation of our study.

Table 2 Univariate cox regression analyses of predicting recurrence-free survival in ameloblastoma

Variable	Crude HR (95% CI)	*P* value
Age (yr)	**1.017 (0.997, 1.038)**	**0.103**
Female gender (Ref. Male)	0.704 (0.279, 1.779)	0.458
ASA classification	**1.604 (0.858, 2.998)**	**0.139**
BMI (kg/m^2)	1.003 (0.889, 1.131)	0.960
Recurrent tumor (ref. Primary)	**3.671 (1.516, 8.887)**	**0.004**
Stage	0.765 (0.221, 2.643)	0.671
Maxilla (Ref. Mandible)	1.393 (0.403, 4.814)	0.601
Size (cm)	**0.790 (0.596, 1.046)**	**0.100**
Unicystic type (Ref. non-unicystic)	0.779 (0.303, 2.001)	0.604
Cortical bone perforation (Ref. nonperforation)	**2.098 (0.798, 5.512)**	**0.133**
MICA/B positive (ref. negative)	**0.176 (0.041, 0.757)**	**0.020**
ULBP1 positive (Ref. negative)	0.694 (0.228, 2.119)	0.522
ULBP2 positive (Ref. negative)	-	N/A
ULBP3 positive (Ref. negative)	1.211 (0.129, 11.363)	0.867
Radical surgery (Ref. Conservative surgery)	0.047 (0.000, 70131.489)	0.674
Anesthesia time (h)	**0.662 (0.384, 1.142)**	**0.139**
General anesthesia (ref. local)	0.647 (0.261, 1.605)	0.347

Reference value (Ref.), hazard ratio (HR) American society of anesthesiologists (ASA), body mass index (BMI), UL16-binding proteins (ULBP), major histocompatibility complex class I chain-related molecules A/B (MICA/B), and not applicable (N/A)

Table 3 Multivariate cox regression analyses of predicting recurrence-free survival in ameloblastoma

Variable	Adjusted HR* (95% CI)	*P* value
Age (yr)	—	—
ASA classification	—	—
Recurrent tumor (ref. Primary)	2.780 (1.136, 6.803)	0.025
Size (cm)	—	—
Cortical bone perforation (Ref. nonperforation)	—	—
MICA/B positive (ref. negative)	0.223 (0.050, 0.989)	0.048
Anesthesia time (h)	—	—

Reference value (Ref.), hazard ratio (HR) American society of anesthesiologists (ASA), and major histocompatibility complex class I chain-related molecules A/B (MICA/B). * Each HR is adjusted for the other variable included in the final model

Recurrent tumors, as demonstrated in our final model, are an independent risk factor for ameloblastoma recurrence, suggesting that primary tumors are associated with a lower risk of ameloblastoma recurrence. Hresko et al. conducted a retrospective analysis on patients with ameloblastoma who had a clinical follow-up period exceeding 3 years [22]. The study found that of the 69 patients with primary ameloblastoma, 24 experienced recurrences. Notably, these 24 patients further experienced a cumulative total of 35 recurrence episodes, underscoring an increased recurrence risk in patients that had previously experienced recurrence.

Tumor immunotherapy has emerged as a cornerstone in the oncological arsenal, complementing traditional modalities like surgery, chemotherapy, and radiation [23]. NK cells play a pivotal role in tumor immunosurveillance due to their unique ability to recognize and eliminate transformed cells without the need for prior sensitization [23, 24]. Within this realm, the NKG2D ligand-mediated pathway is gaining prominence as a potential target for immunotherapy, attributed to its selective expression of "stress-induced ligands" on tumor cells and the potent activation of NK cells by NKG2D [23]. To distinguish transformed tumor cells from healthy normal ones, NK cells recognize specific ligands on the surface of target tumor cells [25]. Cells that have sustained damage or undergone tumorigenesis express NKG2D ligands on their surface, making them more susceptible to detection and eradication by NK cells [9, 25]. Although human NKG2D ligands such as MICA/B and ULBP1-6 have been recognized, understanding of the roles of ULBP4-6 is limited [25–27]. In this study, NKG2D ligands, including MICA/B (36.5%), ULBP1 (86.5%), ULBP2 (100%), and ULBP3 (99.0%), were found to be frequently expressed in ameloblastoma cases. Furthermore, the expression of MICA/B was found to be lower in the recurrence group (8.7%) than in the recurrence-free group (45.2%, $p=0.003$), whereas there was no significant difference in the expression of ULBPs (ULBP1, $p=0.504$; ULBP2, not applicable; ULBP3, $p=0.240$). The intensity of NKG2D ligand expression differed in MICA/B between the recurrent and recurrence-free groups, whereas no significant difference was observed in ULBPs. Our final model also demonstrated that positive MICA/B expression is an independent prognostic factor for ameloblastoma recurrence, suggesting that the expression of MICA/B is associated with a reduced risk of ameloblastoma recurrence. The association between NKG2D ligands and disease prognosis has mainly been investigated in malignant tumors, demonstrating that positive NKG2D ligand expression is associated with a favorable outcome, as observed in our study [10–12]. In an experiment with tissue samples from patients with hepatocellular carcinoma, Kamimura et al. showed that while the expression of ULBP1 did not influence overall survival, loss of ULBP1 expression was associated with reduced RFS (95% CI of adjusted HR: 1.537–16.261, $p=0.008$) [10]. In a large-scale study involving 574 breast cancer tissue samples, high expression of NKG2D ligands was associated with advantageous clinicopathological parameters, and the presence of MICA/B (HR [95% CI]: 0.60 [0.448, 0.810], $p=0.001$) and ULBP2 expression (HR [95% CI]: 0.63 [0.454, 0.869], $p=0.005$) was indicative of extended recurrence-free periods in breast cancer [11]. In a study involving 462 patients with colorectal cancer, reduced MICA/B expression was associated with higher tumor

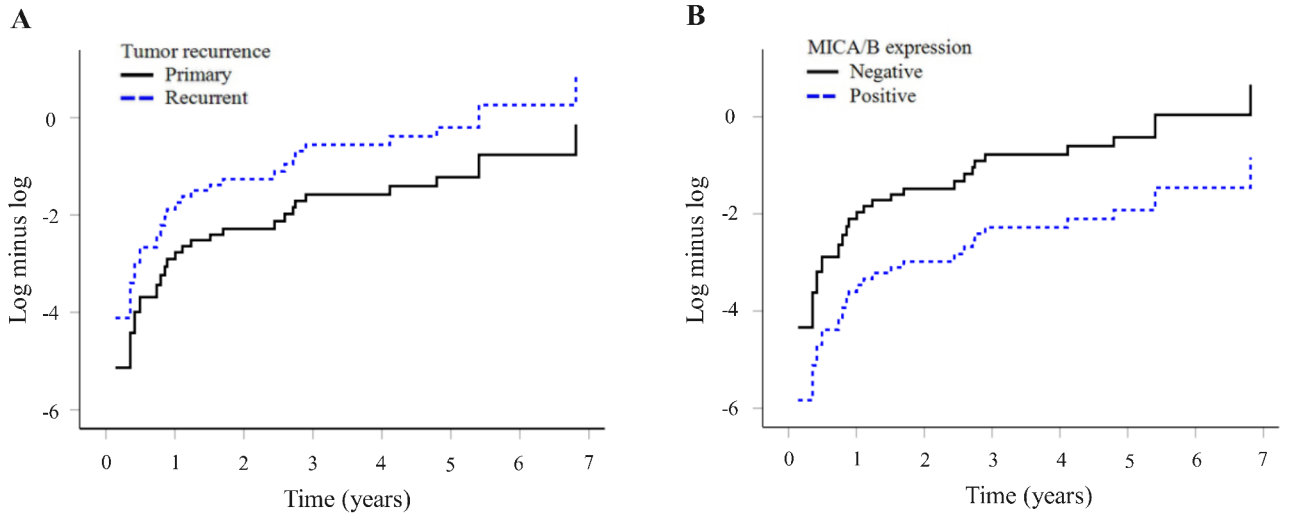

Fig. 3 Log-minus-log survival plots. The log-minus-log survival plots demonstrate that each variable, **(A)** tumor recurrence and **(B)** MICA/B expression, satisfies the proportional hazards assumption in the final model. Major histocompatibility complex class I chain-related molecules A/B (MICA/B). This figure was generated with IBM SPSS Statistics version 22 (IBM Corporation, Armonk, NY)

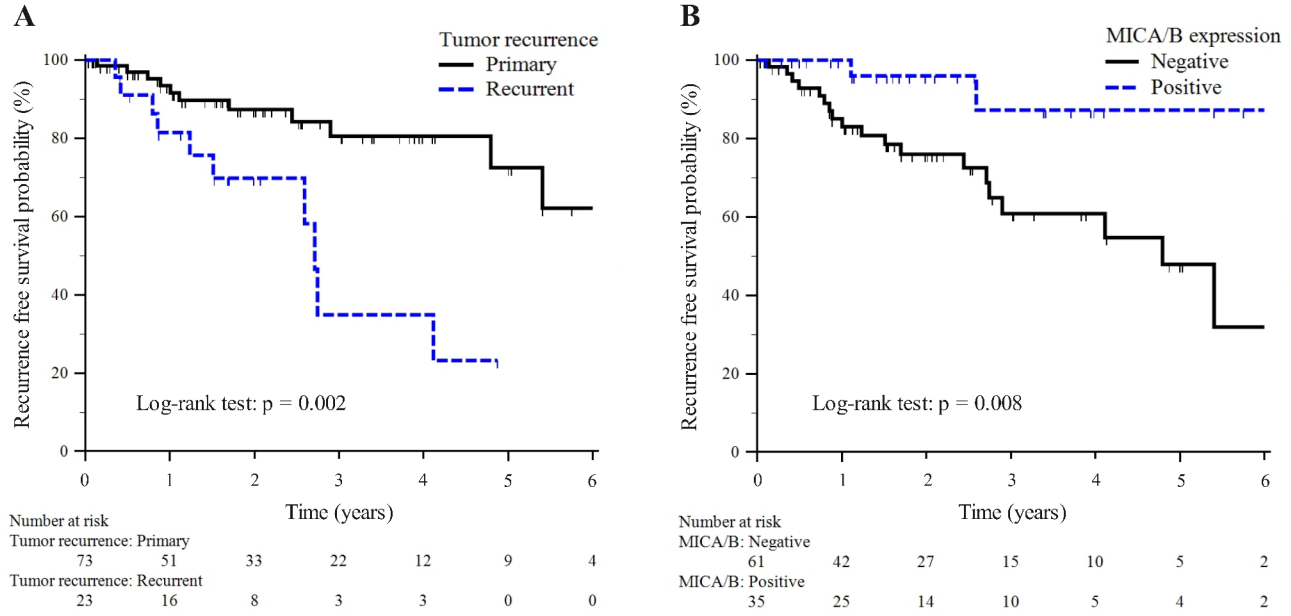

Fig. 4 Kaplan-Meier analysis. Recurrence-free survival based on independent predictors: **(A)** tumor recurrence and **(B)** MICA/B expression. Major histocompatibility complex class I chain-related molecules A/B (MICA/B). This figure was created using MedCalc version 18.11.6 (MedCalc Software bvba, Ostend, Belgium)

grade (p=0.037) and MICA/B expression emerged as an independent predictor associated with improved overall survival (p=0.012) [12].

Although NKG2D ligands are typically cell surface proteins, our study observed several cases with nuclear or cytoplasmic reactivity (Fig. 1). Previous research suggests that the subcellular localization of NKG2D ligands is regulated to prevent autoimmunity (e.g., cell membrane versus cytoplasm) [28]. Another study also found that viral infection can induce the translocation of NKG2D ligands from the cell surface to other areas, such as the endoplasmic reticulum (ER) or cis-Golgi, as an immune evasion mechanism [29]. These findings indicate that NKG2D ligands can also be present in the cytoplasm as well as on the cell surface [28, 29]. We considered that if the protein is present in the rough endoplasmic reticulum (RER), it could appear as nuclear staining under the light microscope. The intracellular localization of NKG2D ligands is not yet fully understood. Consequently, the simultaneous staining patterns (cell membrane, cytoplasm, and

nuclear) observed in several of our cases highlight the need for further research in this area to better understand these observations and their implications.

Obesity has been associated with unfavorable outcomes in some cancers, although the exact mechanism is unclear [30]. Specifically, higher recurrence rates have been observed in patients with breast, colorectal, prostate, and gastroesophageal cancers [30]. In this study, we analyzed the association between the recurrence of ameloblastoma and body mass index; however, we did not find any significant correlation between them.

Recent meta-analyses suggest a potential association between anesthesia and cancer recurrence and prognosis [31, 32]. Furthermore, in our previous preclinical in vitro studies, we observed that sevoflurane, a widely used inhalation anesthetic, could influence NKG2D-mediated immunosurveillance in non-small-cell lung cancer and breast cancer cell lines [25, 33]. However, in this study involving ameloblastoma patients, no significant relationship was found between the type of anesthetic agent and the recurrence rate of ameloblastoma. This may be due to the relatively short anesthetic duration of approximately one hour in the included cases or because the impact of the anesthetic agent on ameloblastoma recurrence is limited. Further evaluation is necessary to accurately assess this relationship.

The present study has several limitations. First, given the low incidence of ameloblastoma, at 0.92 per million person-years [34], this investigation was structured as a retrospective pilot study rather than full-scale prospective research. Recognizing the limitations of pilot studies, including risks of bias and over-interpretation of results, we have highlighted the need for more comprehensive and advanced full-scale research in this area. Second, due to the low preference for radical surgery in the study, surgical factors impact on ameloblastoma recurrence was not assessed. Third, the recruitment period for the study spanned over 7 years due to the low prevalence rate of ameloblastoma. This extended period may introduce bias due to the evolving surgical and anesthetic skills during this time. Fourth, as NKG2D ligand-mediated cancer immunotherapies are still in the pre-clinical stage [23], in vitro diagnostic (IVD) products for measuring NKG2D ligand expression have been validated as research use only (RUO) products, not as general purpose reagent (GPR) products, in line with USA regulatory standards [35]. Caution must be exercised in interpreting the findings of this study. Fifth, our study focused on examining the relationship between NKG2D ligand expression and recurrence-free survival, without exploring the detailed molecular mechanisms. In this study, an analysis of mRNA expression was not performed. It is important to note that the level of NKG2D ligand expression is not solely determined by its mRNA-mediated transcription and translation, but is also influenced by numerous other factors. Notably, to escape NKG2D-mediated immune surveillance, tumor cells release extracellular matrix (ECM) degrading enzymes such as matrix metalloproteinases (MMPs) or a disintegrin and metalloproteinases (ADAMs) [23]. These enzymes facilitate the shedding and removal of NKG2D ligands from the surface of tumor cells [23]. Additional research is essential to comprehensively elucidate the intricate mechanism, which includes the transcription and translation processes of NKG2D ligands and their subsequent shedding facilitated by ECM degrading enzymes. Sixth, we did not use image analysis devices for the quantification of immunohistochemistry. This decision was based on the fact that, compared to nuclear staining, image analyzers tend to be less accurate in detecting cytoplasmic staining than experienced pathologists. To address this, two oral pathologists independently interpreted the immunohistochemistry readings. Seventh, this study did not separately consider histopathological type. This is due to the current consensus that there is no correlation between histopathological type and tumor behavior or prognosis [36]. Lastly, because our study followed a retrospective design, it cannot establish definitive cause-and-effect relationships between prognostic factors and RFS in ameloblastoma.

In conclusion, our study showed that recurrent cases and loss of MICA/B expression are independent risk factors for early ameloblastoma recurrence following surgical resection. Our results suggest that diminished MICA/B expression may impair NKG2D-mediated immunosurveillance, potentially contributing to the early recurrence of ameloblastoma. Further in-depth and extensive research is required to understand the molecular mechanisms behind NKG2D ligand expression in ameloblastoma and to understand the cause-and-effect relationships between NKG2D ligand expression and ameloblastoma recurrence.

Acknowledgements
We express our sincere appreciation to the Biostatistics Medical Research Collaboration Center at Kyungpook National University Hospital and the Department of Medical Informatics at Kyungpook National University School of Medicine for their meticulous statistical review of our research.

Author contributions
Conception and design of study were conducted by M Kim, S Jeon, HJ Lee, AR ChoAcquisition of data was conducted by M Kim, S Jeon, HS Ri, JH KimAnalysis and/or interpretation of data was conducted by M Kim, S Jeon, HJ Lee, JS YeoDrafting the manuscript was conducted by M Kim, S Jeon, EJ Park, JS YeoRevising the manuscript critically for important intellectual content was conducted by S Jeon, HJ Lee, AR Cho.

Funding
This work was supported by Kyungpook National University Dental Hospital Institute for Dental Research (2023).

Data availability
The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The study protocol was approved by Institutional Review Board (IRB) of Kyungpook National University Hospital, Deagu, Korea (IRB No. 2023-05-010) and has been conducted in full accordance with the Declaration of Helsinki. The study was retrospective therefore the Institutional Review Board (IRB) of Kyungpook National University Hospital, Deagu, Korea (IRB No. 2023-05-010) waived the need for written informed consent.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 13 March 2024 / Accepted: 5 September 2024
Published online: 17 September 2024

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