Exploration and characterization of ameloblastoma and odontogenic keratocyst tumoroids

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Exploration and characterization of ameloblastoma and odontogenic keratocyst tumoroids

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박사 학위 취득을 앞두고 있는 지금 이 자리에 오기까지 저를 키워주시고 학문의 뜻을 잃지 않도록 지켜봐주신 부모님과 동생 우영이에게 존경과 사랑을 전합니다.

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ABSTRACT

Exploration and characterization of ameloblastoma and odontogenic keratocyst tumoroids

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Odontogenic tumors arise from the reciprocal interaction between the dental ectodermal mesenchyme and the epithelium, similar to the normal tooth formation process, but the mechanism is not currently known. Aggressive and frequently recurring epithelial odontogenic tumors and cysts, such as ameloblastoma (AM) and odontogenic keratocyst (OKC), are currently difficult to treat with radiation or drug therapy. In addition, since they frequently recur, extensive surgical treatment is often required to prevent this. As a result, a defect remains in the patient's jaw that impairs their quality of life.

The patient-derived tumor organoid model, which is mainly used in tumor research, can recapitulate the *in vivo* tissue microenvironment of the patient's

original tumor and can be used for the screening and verification of patient-specific anticancer drugs, thereby increasing treatment efficiency and reducing side effects. These tumor organoid models have been established and studied in various tumors, such as those of liver, lung, and breast cancer, but there have been no reports of odontogenic tumors. Therefore, we aimed to explore the microenvironment for constructing a tumor organoid model by using the tissues of patients with AM and OKC, for which there is no suitable disease model at present, and to explore the genetic characteristics of these conditions by using next generation sequencing (NGS).

In this study, AM-1 and tissues from primary AM (pAM) and OKC (pOKC) were collected and cultured in 2D and 3D in various extracellular matrices, including Matrigel and collagen gel, under various culture conditions. Tumoroid growth was observed and analyzed by histology and immunohistochemistry (IHC). In addition, through bulk RNA-sequencing, principal component analysis, gene ontology, and differentially expressed genes, differences in the transcriptomic characteristics of AM-1, pAM, and pOKC were investigated. As a result, the following conclusions were made:

1. 3D culture of AM-1, pAM, and pOKC was done successfully, but tumoroids did not maintain self-renewal capacity over passage 3.
2. The tumor microenvironment (ECM, calcium) could cause changes in cells that form tumoroids.
3. IHC results showed that cell-cell adhesion and the basement membrane were well-established in the pAM tumoroids.
4. General gene ontology results showed that the expression of genes related to proliferation was high in AM-1 cells; however, ECM expression was high in pAM.

5. Signaling pathway enrichment analysis showed that the expression of genes related to the MAPK and PI3K pathways was high in pAM and of those related to the VitD receptor-related pathway was high in pOKC.

This study is the first, to our knowledge, to establish a tumor organoid model for AM and OKC by using patient-derived tissue. Long-term culture conditions should be established in future studies. The results of this study confirm that the tumor microenvironment influences the differentiation or growth pattern of cells, and the possibility of establishing long-term culture conditions in future was confirmed through the results of NGS.

Keywords: tumor organoid, tumoroid, ameloblastoma, odontogenic keratocyst, 3D culture, RNA-sequencing, next-generation sequencing

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I. INTRODUCTION

1.1 Odontogenic tumors

Odontogenic tumors originate from the epithelial, ectomesenchymal, or mesenchymal tissues of the dentate apparatus, which are only found in jaw bones or soft tissues overlying the bones.[1,2] As with normal odontogenesis, odontogenic tumors represent reciprocal interactions between the odontogenic ectomesenchyme and epithelium.[3] The classification of odontogenic tumors is based on interactions between the odontogenic ectomesenchyme and epithelium. Therefore, odontogenic tumors are found in the jaw bones or the mucosal tissue covering the area of the tooth. Most odontogenic tumors are benign, but some act locally in a destructive

manner.[4] Although odontogenic tumors are relatively rare, they constitute 3.8% of head and neck pathology.

The first consensus on the classification of odontogenic tumors arose from a five-year study conducted by the World Health Organization (WHO) and published in 1971, which was accepted as the first international classification of these tumors.[5] The last updates of these odontogenic tumors were published in early 2017.[6]

1.2 Ameloblastoma

Odontogenic lesions can be divided into cysts (cavities lined with epithelium) and tumors (solid tissue masses).[7] Among odontogenic tumors, epithelial odontogenic tumors are composed of the odontogenic epithelium without the involvement of the odontogenic ectomesenchyme.

Ameloblastoma (AM) is the most common odontogenic tumor. Although its general biological behavior is similar to that of other benign tumors, AM is characterized by a high ability of local infiltration and a high risk of recurrence. The overall recurrence rate of AM is 31%, ranging from 65% for conservative surgery to 11% for definitive surgery.[8] Despite few reports of malignant transformations and/or metastases, the tumor causes expansion of the mandible, thinning and erosion of cortical bone, and subsequent infiltration into adjacent soft tissues. Invasive behavior requires more careful and often radical therapeutic interventions for AM treatment.[9,10]

AM can be originated from any odontogenic epithelium. The dental lamina (pre-odontogenesis), reduced enamel epithelium (post-odontogenesis), epithelial rests of Malassez and Serres (post-eruption), and the basal layer of the overlying epithelium (primitive source of thin layers of pre-embryonic and pre-dental teeth) could derive

AM.[11,12] Developmentally, AM can probably be derived from odontogenic epithelial rests, migrating odontogenic epithelium, migrating epithelium of the cervical loop, and the lining of dentigerous cysts.[13]

Epidemiological studies have highlighted the prevalence of jaw AM in the fourth decade of life, which primarily affects the molar area of the mandible. However, it is a rare neoplasm, with a global incidence of approximately 0.92 per million inhabitants per year.[14] Histopathological studies have highlighted the prevalence of solid forms of AM with major follicular and plexiform subtypes.

1.3 Odontogenic keratocyst

The odontogenic keratocyst (OKC) originates from odontogenic epithelium and dental lamina.[15,16] This lesion tends to have a more aggressive growth pattern and presents a higher recurrence rate than other cysts.[17,18] OKC is one of the most controversial pathological entities in the maxillofacial region since it was first described by Philipsen in 1956.[19] Due to its aggressive features, the revised categorization of head and neck tumors published by the WHO in 2005 reclassified OKC as a benign intraosseous tumor and coined the term keratocystic odontogenic tumor.[1] Most OKCs occur sporadically in a single form, but multiple lesions often occur in association with nevoid basal cell carcinoma syndrome (NBCCS).[20] Its histological features include a parakeratinized squamous epithelium about five-to-eight cells thick covered with a thin corrugated layer of parakeratin.[21,22] The basal layer shows a characteristic palisade pattern with uniform nuclei.[23] Previous OKCs have been reported to be prone to recurrence, describing a unique growth potential not found in other odontogenic lesions, providing a pronounced ability to resorb

bone. Although rare, malignant transformation into squamous cell carcinoma has been reported.[24]

1.4 Previous studies investigating the pathogenesis of AM & OKC

The molecular and genetic pathogenesis of AM were poorly understood prior to 2014. In 2014, an important study involving the genetics of this tumor was published, which provided a turning point in our understanding of the etiology of AM.[25-27] This study identified highly recurrent somatic mutations in genes related to the mitogen-activated protein kinase (MAPK) signaling, sonic hedgehog, phosphatidylinositol 3-kinase (PI3K), and Wnt signaling pathways in AM.[26,27] In particular, *BRAF* is the most commonly mutated gene involved in the regulation of the MAPK pathway.[27]

Similarly, in OKC, mutations in *PTCH1*, a tumor-suppressor gene, which maps to chromosome 9q22.3–q31, would lead to constitutive activation of the sonic hedgehog signaling pathway and result in aberrant cell proliferation.[28] However, germline mutations in *PTCH1* are less common in patients with sporadic OKC than in those with OKC associated with NBCCS. Therefore, the etiology of solitary OKC remains poorly characterized. Increased epithelial activity confirmed by previous studies comparing other odontogenic cysts may demonstrate the high recurrence rate of OKC.[29,30]

1.5 Organoids and tumoroids as tools for fundamental tumor research

To perform fundamental tumor research and develop effective therapies for tumor treatment, various strategies have been used, including analysis of clinical

samples, *in vivo* animal models, and *in vitro* models. Clinical samples are highly relevant to patients with cancer. However, their utilization is limited by the non-uniformity (heterogeneity) and small size of the tumor sample.[31]

Organoids, defined as three-dimensional self-organizing structures that can be grown from primary tissue or stem cells that are capable of self-renewal and able to replicate the complex structure of an organ, allow researchers to study organ structure and function in an *in vitro* culture system.[32,33] Organoids can also be grown long-term without genetic or phenotypical changes, a feat which was hitherto impossible. Thus, classical 2D cell lines require immortalization (e.g., AM-1 cell line). Therefore, organoids could be a useful experimental model for filling the gap between *in vitro* and *in vivo* model systems.[34]

In a traditional 2D culture system, cells are cultured as a monolayer on the flat surface of the plate. This gives each cell access to the same amount of growth factors and nutrients present in the medium, resulting in uniform growth and proliferation.[35] Therefore, 2D culture models cannot accurately mimic *in vivo* conditions and processes, such as tumor microenvironments, growth factors, or synthesis of cell adhesion molecules.[36] To overcome the limitations of 2D culture systems, a 3D cell culture model was developed that closely mimics the *in vivo* tissue microenvironment.[36] The 3D culture model maintains the interaction between cells and their extracellular matrix (ECM), creates gradient access for nutrients and pH, and builds tissue-specific scaffold cell combinations.[37] Therefore, the 3D tumor model more closely reflects *in vivo* human tumors and dictates the cell fate and differentiation, leading to the definition of tumor organoids as "tumoroids." The tumoroid model enables the modeling of the tumor microenvironment and maintains the major genetic and phenotypic features of individual tumors in an efficient and cost-effective manner.

1.6 The aim of the current study

Currently, many studies have been carried out that utilize sequencing to identify mutated genes in AM and OKC. These gene mutations could be the basis for targeted therapy in future. However, in addition to these intrinsic genetic factors, studies related to the tumor microenvironment, such as attachment to ECM or interactions with stromal cells, remain unelucidated. Recently, patient-derived organoid and tumoroid techniques have been widely used to not only construct *in vitro* disease models of various cancers but also implement drug discovery and personalized medicine. In lung, breast, liver, and gastric cancer, tumoroid models have been well-established; however, to date, no model has been reported for odontogenic tumors.

Therefore, the objective of the present study was to investigate the following by using patient-derived specimens:

1) Explore the effects of culture conditions and the tumor microenvironment (growth factors and the ECM) on primary AM and OKC tumoroid growth.
2) Next-Generation Sequencing (NGS) and RNA-sequencing (RNA-Seq) were performed to characterize gene expression in primary AM, OKC, and immortalized AM-1 cell lines.

II. MATERIALS & METHODS

2.1 Tumor sample collection and preparation

This study was conducted in accordance with the Declaration of Helsinki and the human subject research guidelines. This study was approved by the Ethical Review Board of Yonsei University Dental Hospital Institutional Review Board (IRB No. 2-2018-0050).

Six fresh tissue samples (three each of AM and OKC) were obtained during the surgical procedure. The tissue samples were divided into two sections. For cell isolation, one section was minced into small pieces (~1 mm^3) and dissociated in Dispase II (2.4 U, Thermo Fisher Scientific) and trypsin-EDTA (TrypLE, Gibco). The dissociated cells were embedded into gels for 3D culture or suspended in cell freezing medium (Recovery Cell, Gibco) and stored in a liquid nitrogen tank. The other section of the samples was processed for histological analyses. All samples were evaluated by a board-certified oral and maxillofacial pathologist, and diagnoses were classified based on the 2017 WHO Histologic Classification of Odontogenic Tumors.

2.2 2D and 3D *in vitro* cell culture

AM-1 cells were two-dimensionally cultured in a 10-cm cell culture dish (Nunc) with keratinocyte serum-free medium (KRCM) for maintenance. For the 3D culture, AM-1 cells were dissociated from the dish by treatment with trypsin-EDTA. The dissociated AM-1, pAM, or pOKC cells were counted, and 10,000 cells were suspended in 40 μL of Matrigel (Corning) or collagen gel (2 mg/mL, Corning) and seeded into 24-well cell culture plates (Nunc). The plates were incubated at 37 °C

to solidify the Matrigel or collagen gel for 10 min or 1 h, respectively. After solidification, 500 μL of the appropriate medium was overlaid on each gel. The embedded cells were incubated for two weeks in a CO_2 cell incubator at 37 °C with 95% humidity.

2.3 Bright-field and fluorescent imaging of live cells

Bight-field images of tumoroids were obtained using the EVOS cell imaging system (EVOS M5000, Thermo Fisher Scientific). For fluorescent imaging, the three-dimensionally cultured cells were stained using a live cell fluorescent dye, CellTracker Green CMFDA (Thermo Fisher Scientific), according to the manufacturer's instructions. Briefly, CellTracker was added to the medium directly to a final concentration of 1 μM and incubated for 30 min in a cell culture incubator. After incubation, the medium was replaced with fresh medium and fluorescence images were obtained using a confocal-based high-content analysis system CQ1 (Yokogawa).

2.4 Histology and immunohistochemistry

Tissue or tumoroid samples were fixed in cold 4% paraformaldehyde overnight. After processing to make paraffin blocks, the samples were sectioned to 4-μM thickness. For histological analysis, the deparaffinized sections were subjected to hematoxylin and eosin (HE) staining and subsequently visualized under an inverted microscopy system (Eclipse TE2000-U, Nikon). For immunohistochemistry (IHC), the deparaffinized sections were boiled with citrate buffer (pH 6.0) in a vegetable cooker (121 °C, 15 min) for antigen retrieval. After blocking with 5% goat serum

in phosphate-buffered saline at room temperature (RT) for 1 h, the sections were incubated with the following primary antibodies: E-cadherin (1:2000), p63 (1:2000), collagen IV (AbCam, 1:2000), and cytokeratin 14 (K14, AbCam, 1:2000) at 4 °C overnight. The sections were subsequently incubated with secondary antibodies conjugated with a fluorescence dye (1:2000, Alexa Fluor 488 and Alexa Fluor Alex 555, Thermo Fisher Scientific) at RT for 1 h. After counterstaining the nuclei with TO-PRO-3 (Thermo Fisher Scientific) and mounting with the mounting solution PermaFluor (Thermo Fisher Scientific), fluorescence images were obtained using CQ1 (Yokogawa).

2.5 Bulk RNA sequencing and analysis

Total RNA of the isolated cells from tissue samples was extracted using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop), and the 260/280 nm ratio was found to be between 1.7 and 2.0. The integrity of the total RNA samples was assessed using the Agilent 2100 (Agilent Technologies) and Tecan F2000 (Tecan Group Ltd.) devices, and samples with an RNA integrity number > 7.0 and high-quality RNA (28S/18S > 1) were utilized for subsequent analyses.

After reverse transcription, cDNA was synthesized using the 5′ adaptor forward and 3′ adaptor reverse primers. Libraries for Illumina sequencing were established from the cDNA as described by Trapnell et al.[38] Bulk RNA sequencing was performed by Theragen Bio Institute (Suwon, Korea) on an Illumina HiSeq 2000 high-throughput sequencer (Illumina) according to the manufacturer's specifications. The RNA sequencing data were analyzed as described previously.[44]

Briefly, reads were mapped to the *Homo sapiens* reference genome obtained from the University of California, Santa Cruz database using TopHat and Bowtie from Illumina iGenomes. Gene expression values were assessed for each gene from the Ensembl database as fragments per kilobase of exon per million mapped reads calculated using Cufflinks.[39] Principal component analysis and visualization were conducted using the R package for multivariate analysis, FactoMineR.[40]

Differentially expressed genes (DEGs) between AM and OKC were identified under the criteria of a p-value < 0.05 and fold-change of expression ≥ 2. The upregulated or downregulated DEGs were subjected to gene ontology (GO) analysis and visualized using an R package clusterProfiler to analyze and visualize functional profiles.[41] All analyses were performed in the R and R studio environment.

III. RESULTS

3.1 The effect of external factors on the 3D culture of AM-1 cells

To investigate the effect of ECM, we cultured AM-1 cells suspended or embedded in Matrigel or collagen gel. In the suspension culture, the AM-1 cells formed 3D cell masses that varied in size. The 3D cell masses were cultured in the form of a 3D spheroid without any distinguishable histological structure (Figure 1A).

The AM-1 cells grown in Matrigel formed a cyst-like structure with an empty inner space. KRCM, a previously established culture condition for the 2D culture of AM-1 cells[42], contains a low concentration of calcium (below 0.1 mM). To examine the effect of calcium on AM-1 3D culture, we supplemented the media with calcium chloride. When cultured in the presence of calcium, keratin was formed in the AM-1 cell tumoroid. This implies that cell differentiation is affected by the surrounding microenvironment (Figure 1B). On the other hand, when the ECM was a collagen gel, it was confirmed in the bright field image and cell tracking image that the arrangement of AM-1 cells changed to a cord shape-like plexiform type AM (Figure 2).

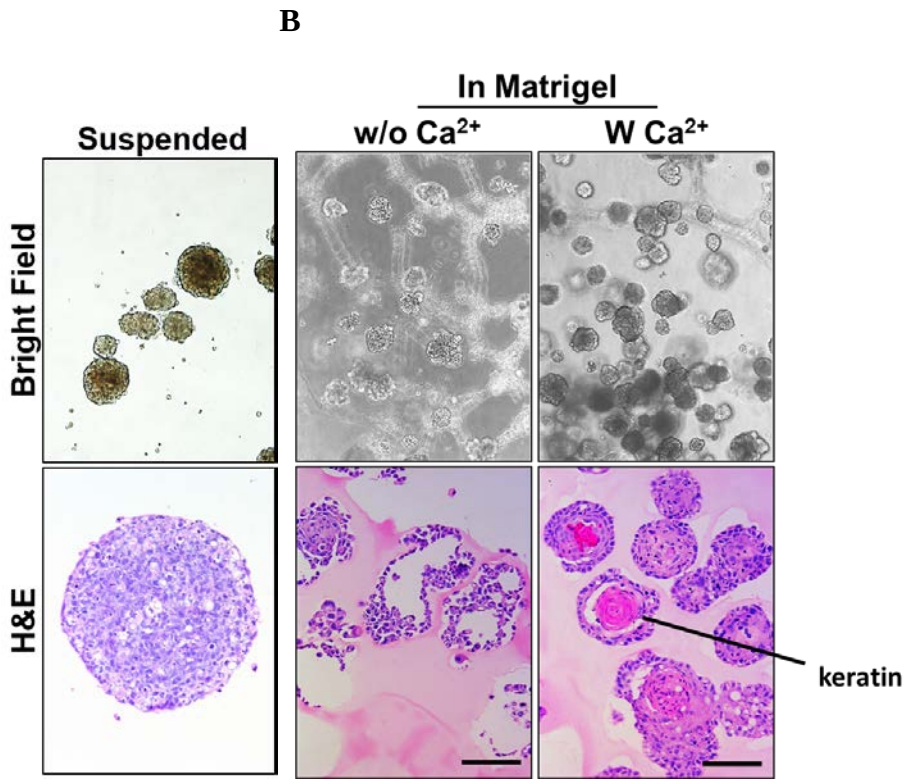


Figure 1. 3D culture of AM-1 in suspend culture and Matrigel
A: Representative bright-field image and HE staining of AM-1 suspended culture
B: Representative bright-field image and HE staining of AM-1 culture embedded in Matrigel with or without calcium. When cultured in the presence of calcium, keratin was formed.

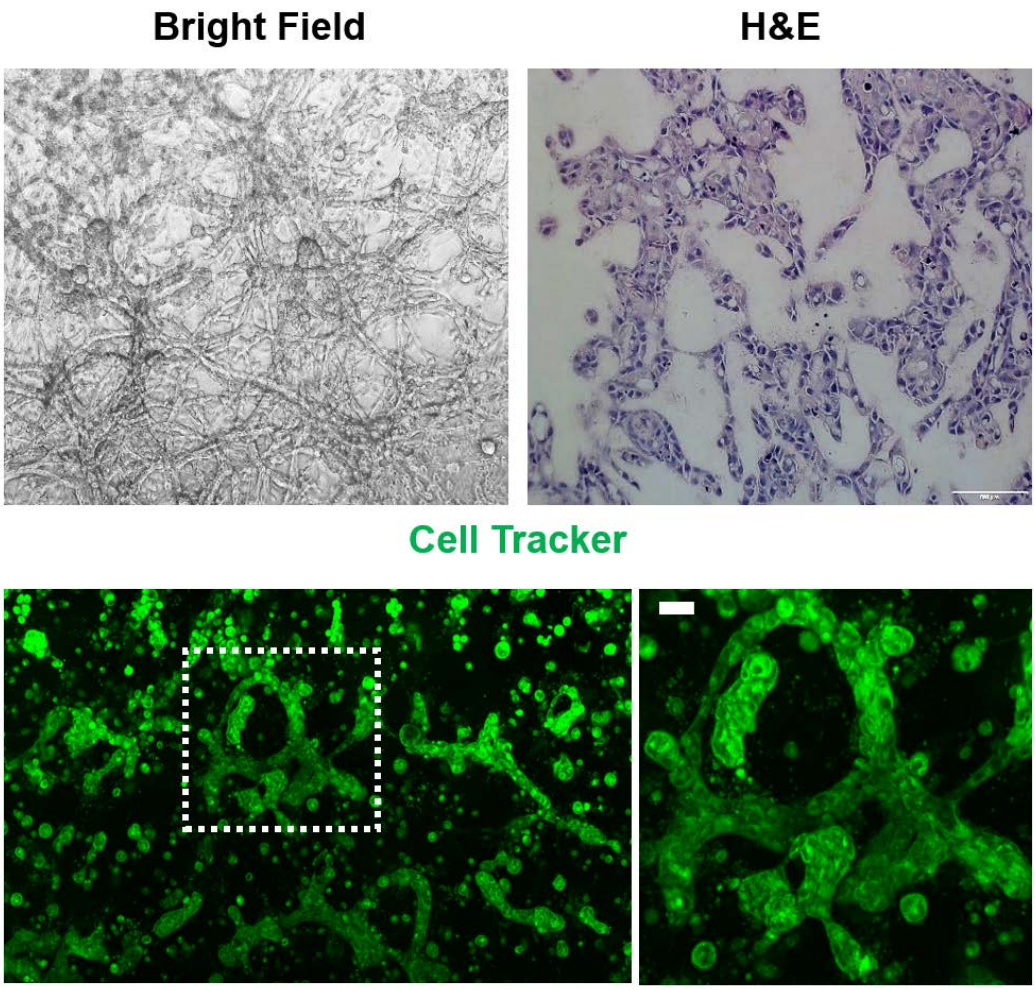
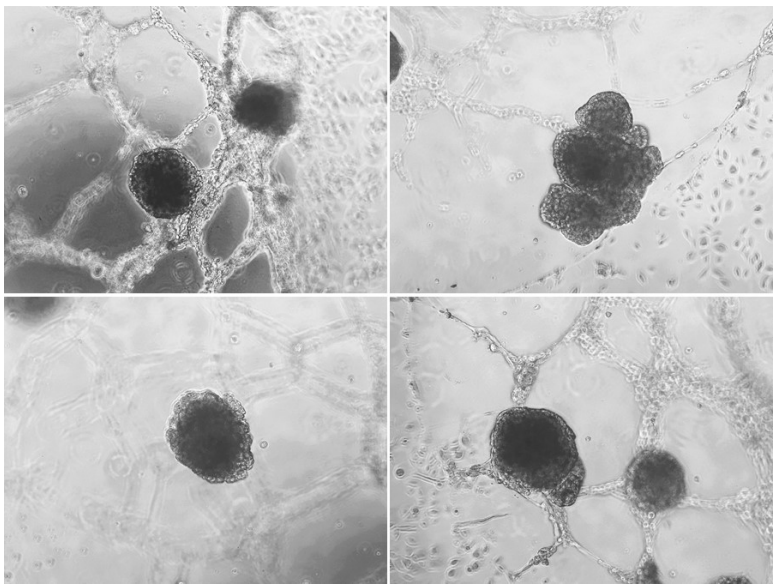


Figure 2. 3D culture of AM-1 in collagen gel
Bright field images, HE staining images, and fluorescent dye cell trackers showed that AM-1 cells were grown as they were arranged in a cord-like shape.

3.2 Tumoroid cultures derived from pAM

We cultured the 3D tumoroid in Matrigel with KRCM from the resected AM tissues of patients. Three tissue specimens of pAM were obtained. In the HE staining image, AM epithelial cells were spheroidal in shape, and there were two parts of the tumoroid (mass and cyst) (Figure 3A, B). For the characterization of tumoroids, IHC was performed after 21 days of culture at passage 1. E-cadherin immunoreactivity was observed at the cell-cell interface, and P-cadherin immunoreactivity was observed in the basal cell layer of the pAM tumoroid (Figure 4A, B). The expression of type IV collagen in the basement membrane (BM) of pAM tumoroids was also noted (Figure 4C).

A



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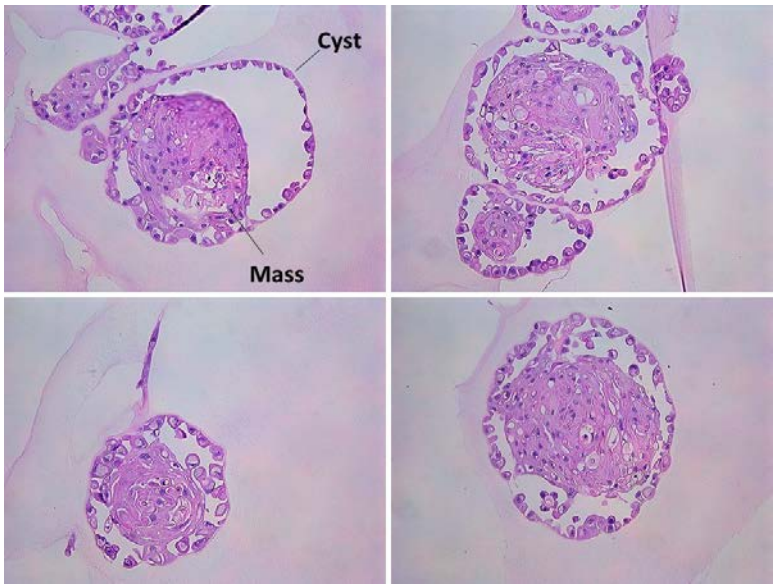
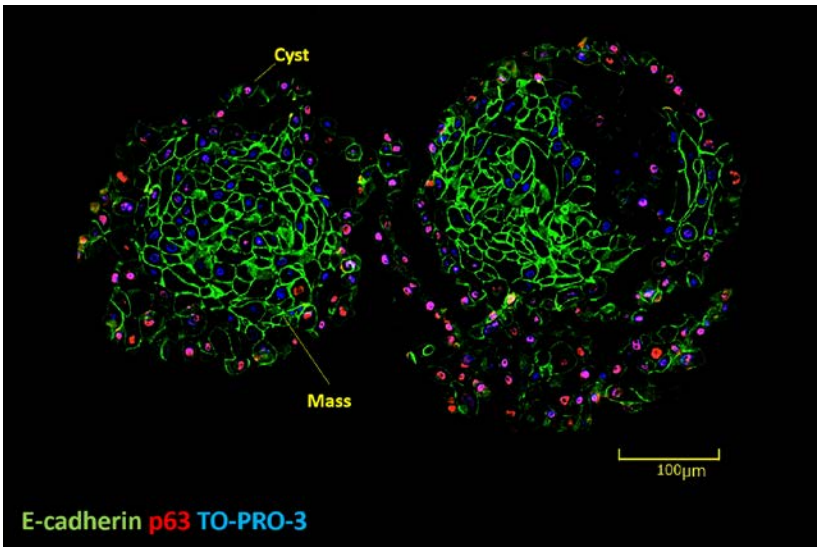


Figure 3. pAM tumoroid culture in Matrigel with KRCM, passage 1, day 21
A: The bright-field image of pAM culture.
B: Hematoxylin and eosin staining of pAM culture. There are two portions of the tumoroid model (mass and cyst).

A



B

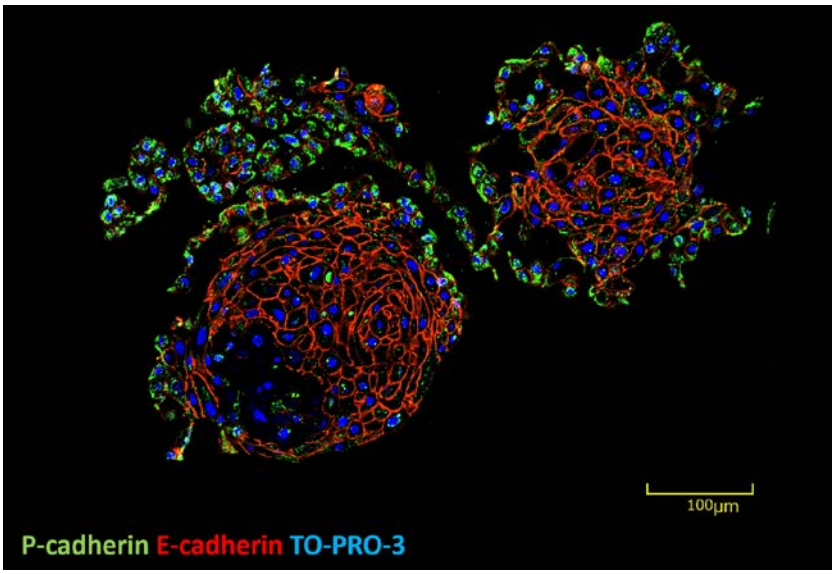


Figure 4. Immunostaining of pAM tumoroid

E-cadherin was expressed in the epithelial tissue of the pAM tumoroid in immunofluorescence staining.

A: Expression of E-cadherin showed epithelial cell adhesion in the pAM tumoroid.

B: Expression of P-cadherin in the basal cell of pAM tumoroid.

C

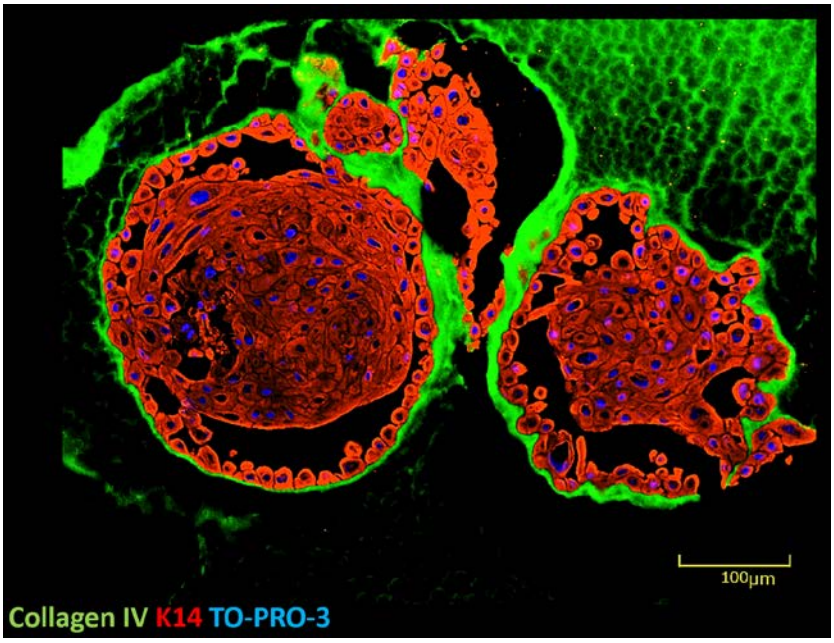
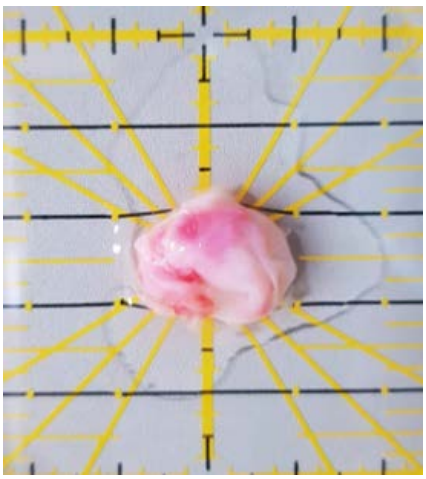


Figure 4. Immunostaining of the pAM tumoroid
C: Expression of type IV collagen in the basement membrane of the pAM tumoroid.

3.3 Tumoroid cultures derived from pOKC

We cultured the 3D tumoroid in Matrigel with KRCM from the resected OKC tissues of patients. Three tissue specimens of pOKC were obtained. HE staining showed that two-to-three layers of epithelial lining were formed with keratin formation after 14 days of culture at passage 1 (Figure 5).

A



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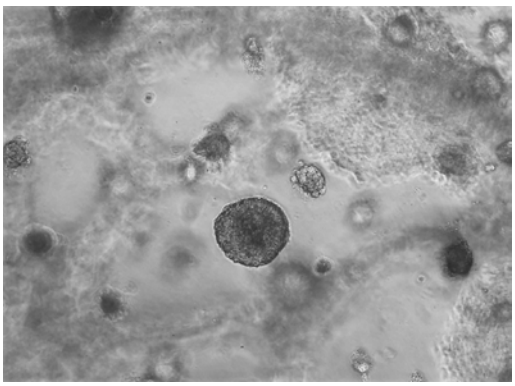


C

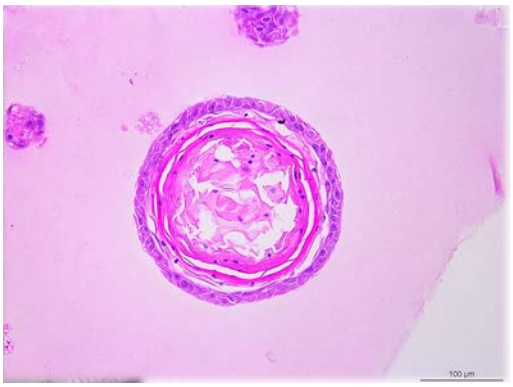


Figure 5. pOKC tumoroid culture in Matrigel with KRCM, passage 1, day 14

A: The tissue sample was collected from the patient.
B: Representative bright-field image of the pOKC tumoroid.
C: HE staining of the pOKC tumoroid. Keratin formation was noted.

3.4 Principal component analysis of RNA-sequencing data

For principal component analysis of RNA-seq data of the AM-1 cell line, three pAM and three pOKC were analyzed. To determine the contribution of the state to the total variability in gene expression, principal component analysis was performed. pAM clustered together and away from the AM-1 cell line. In addition, pOKC clustered together and away from the AM-1 cell line and pAM. There was one exception to this clustering in pOKC (Figure 5).

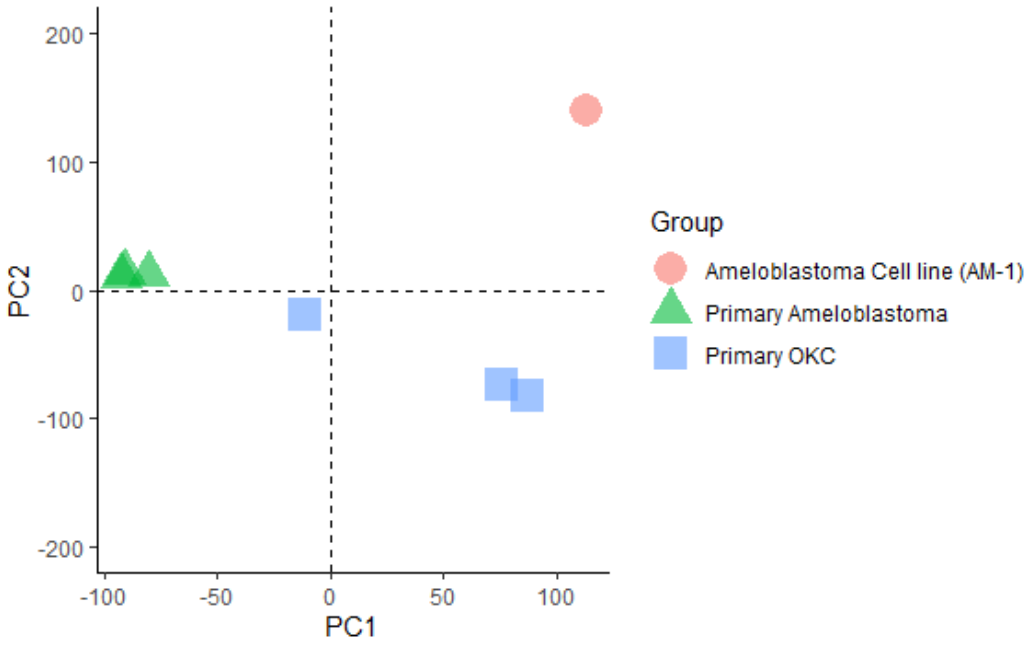


Figure 6. Principal component analysis of RNA-sequencing data
One AM-1 (red), three pAM (green), and three pOKC (blue) were analyzed. pAM clustered together and away from AM-1. pOKC also clustered together and away from AM-1 and pAM. There was one exception to this clustering.

3.5 Differentially expressed gene analysis, gene ontology, and signaling pathway enrichment analysis

The volcano plot reflects the difference in gene expression between AM-1 and pAM (Figure 7). A total of 1609 genes were upregulated in pAM and 834 genes were upregulated in AM-1. DEGs were established at fold-change ≥ 2 and $P < 0.05$. GO enrichment analysis showed that ECM-related genes were upregulated in pAM, while cell proliferation genes were upregulated in AM-1 (Figure 8). Signaling pathway enrichment analysis revealed that the PI3K and MAPK signaling pathways, which are the major pathways for related AM occurrence, were more enriched in pAM (Figure 9). The volcano plot can reflect the gene expression difference between AM-1 and pOKC (Figure 10). Additionally, 1141 genes were upregulated in pOKC and 631 were upregulated in AM-1. GO enrichment analysis showed that immune-related genes were upregulated in pOKC, while ECM-related genes were upregulated in AM-1 (Figure 11). The vitamin D receptor signaling pathway was enriched in pOKC (Figure 12).

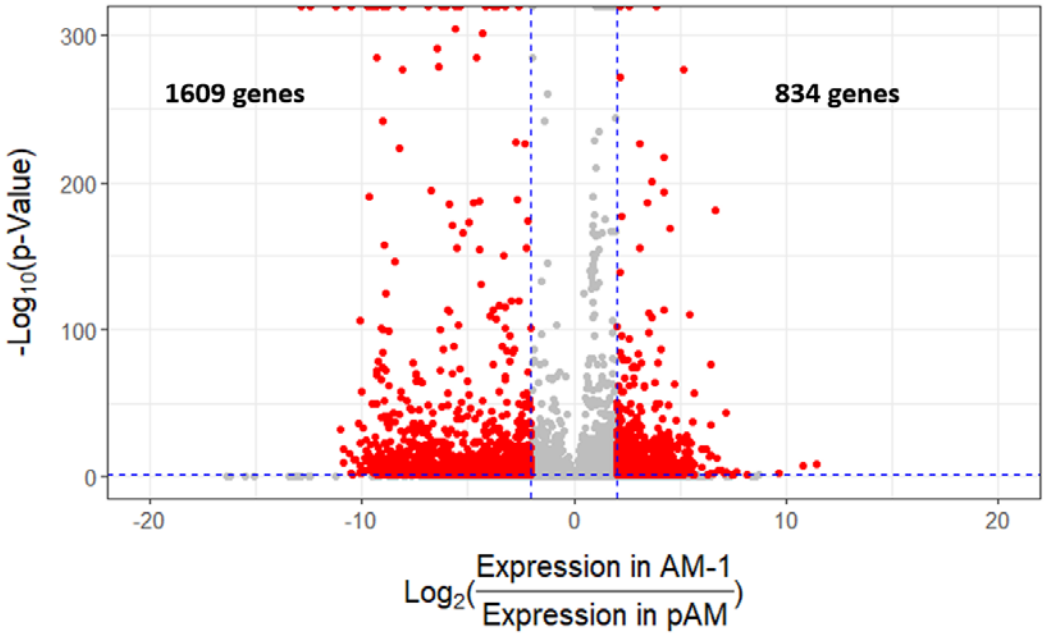
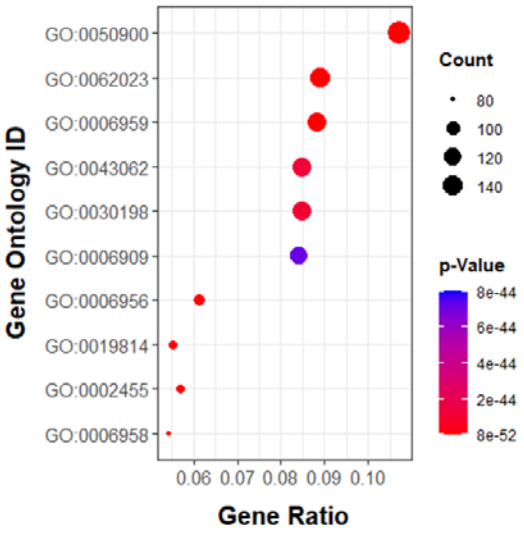


Figure 7. Differentially expressed gene analysis between AM-1 and pAM
Volcano plot. The dots on the left side represent the upregulated genes in pAM, and dots on the right side represent the upregulated genes in AM-1. The x-axis represents the differential expression profiles with the fold-induction ratios in a log2 scale, and the y-axis represents the P value of the T-test on a log10 scale. Differentially expressed genes were established at fold change ≥ 2 and $P < 0.05$.

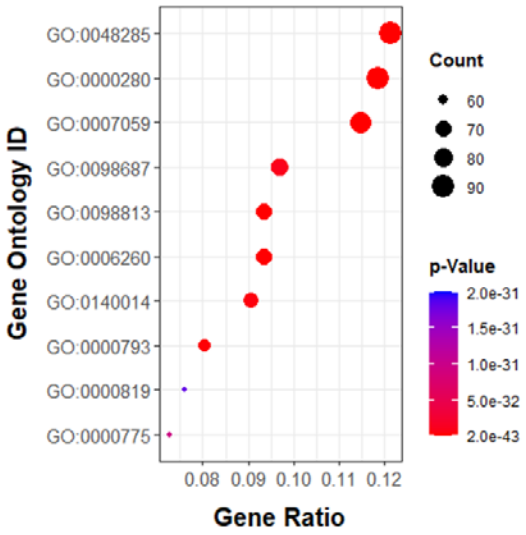
A



ID	Description
GO:0050900	leukocyte migration
GO:0062023	collagen-containing extracellular matrix
GO:0006959	humoral immune response
GO:0030198	extracellular matrix organization
GO:0043062	extracellular structure organization
GO:0006909	phagocytosis
GO:0006956	complement activation
GO:0019814	immunoglobulin complex
GO:0002455	humoral immune response mediated by circulating immunoglobulin
GO:0006958	complement activation, classical pathway

ECM related gene↑

B



ID	Description
GO:0048285	organelle fission
GO:0000280	nuclear division
GO:0007059	chromosome segregation
GO:0098687	chromosomal region
GO:0098813	nuclear chromosome segregation
GO:0006260	DNA replication
GO:0140014	mitotic nuclear division
GO:0000793	condensed chromosome
GO:0000819	sister chromatid segregation
GO:0000775	chromosome, centromeric region

Cell proliferation ↑

Figure 8. Gene ontology enrichment analysis between pAM and AM-1
A: ECM-related genes were upregulated in pAM.
B: Cell proliferation genes were upregulated in AM-1.

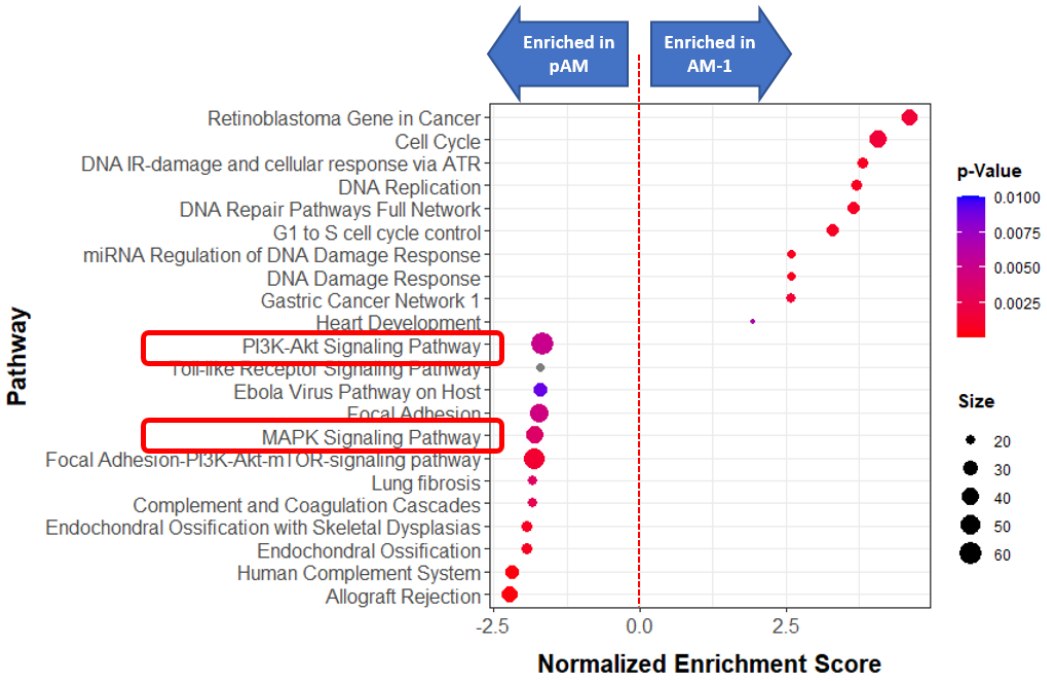


Figure 9. Signaling pathway enrichment analysis

A dot plot showing several enriched pathways in pAM. Among them, the PI3K-Ark signaling pathway and MAPK signaling pathway, the major pathways for relating AM occurrence, were enriched in pAM.

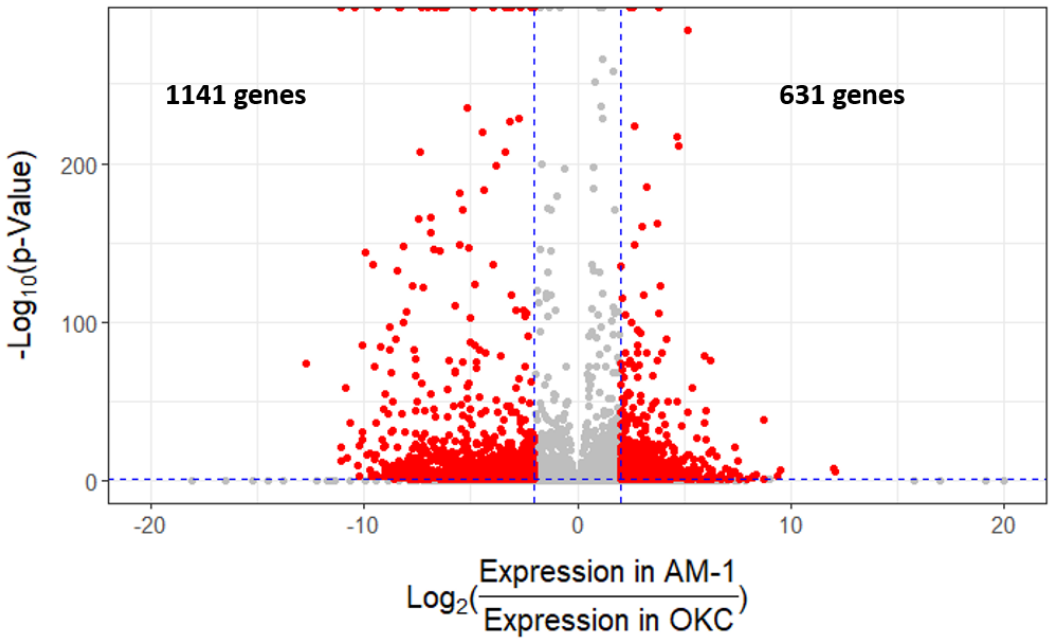
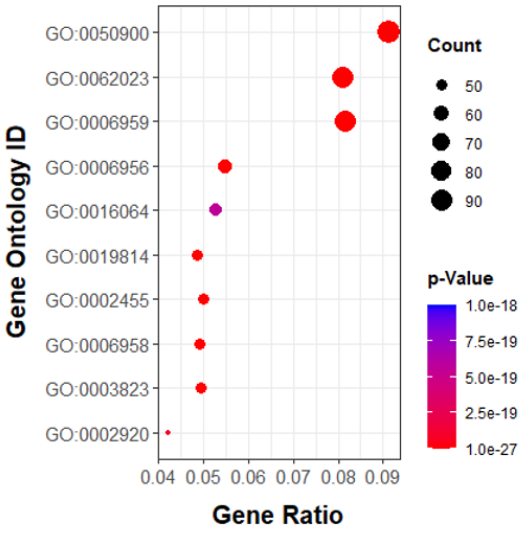


Figure 10. Differentially expressed gene analysis between AM-1 and pOKC
Volcano plot. The dots on the left side represent the upregulated genes in pOKC, and those on the right side represent the upregulated genes in AM-1. The x-axis represents the differential expression profiles with the fold-induction ratios in a log2 scale, and the y-axis represents the P value of the T-test on a log10 scale. Differentially expressed genes were established at fold-change ≥ 2 and $P < 0.05$.

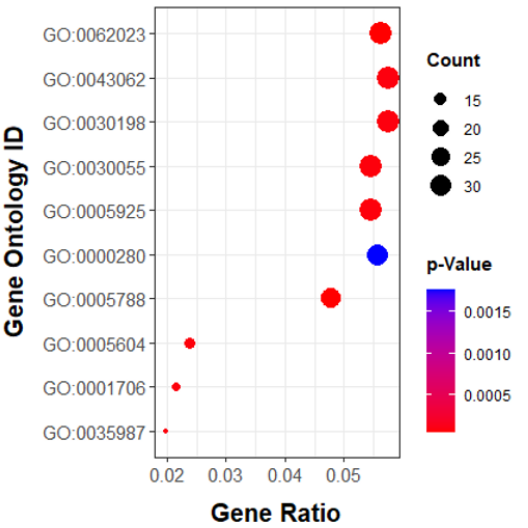
A



ID	Description
GO:0050900	leukocyte migration
GO:0062023	collagen-containing extracellular matrix
GO:0006959	humoral immune response
GO:0006956	complement activation
GO:0016064	immunoglobulin mediated immune response
GO:0002455	humoral immune response mediated by circulating immunoglobulin
GO:0019814	immunoglobulin complex
GO:0006958	complement activation, classical pathway
GO:0003823	antigen binding
GO:0002920	regulation of humoral immune response

Immune related gene↑

B



ID	Description
GO:0062023	collagen-containing extracellular matrix
GO:0030198	extracellular matrix organization
GO:0043062	extracellular structure organization
GO:0005925	focal adhesion
GO:0030055	cell-substrate junction
GO:0000280	nuclear division
GO:0005788	endoplasmic reticulum lumen
GO:0005604	basement membrane
GO:0001706	endoderm formation
GO:0035987	endodermal cell differentiation

ECM related gene↑

Figure 11. Gene ontology enrichment analysis between pOKC and AM-1
A: Immune-related genes were upregulated in pOKC.
B: ECM-related genes were upregulated in AM-1.

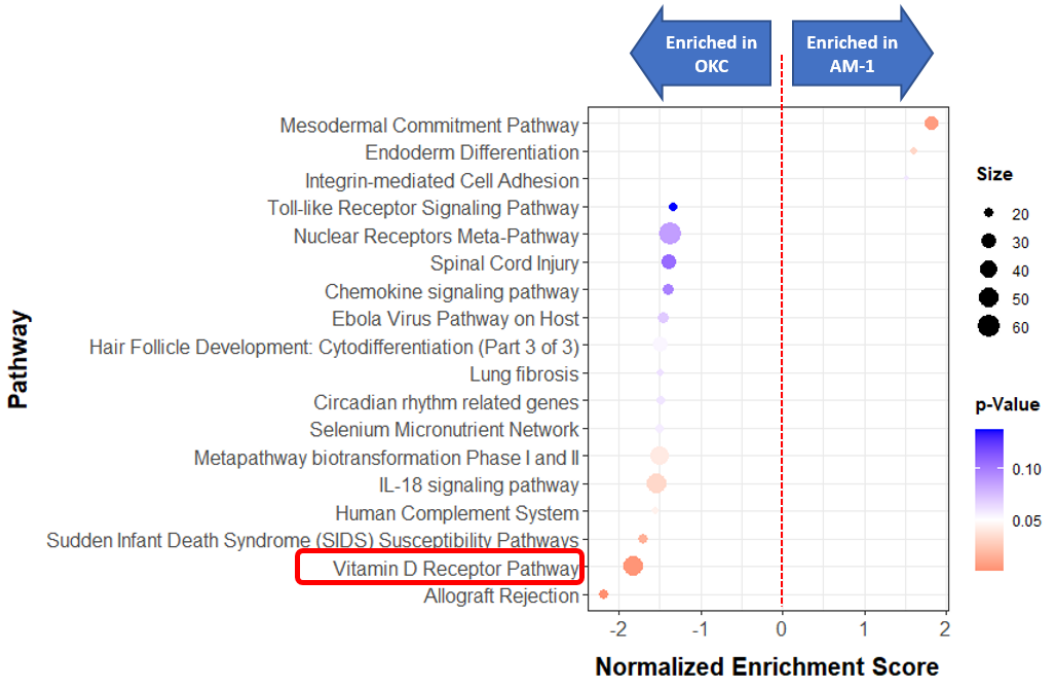


Figure 12. Signaling pathway enrichment analysis

A dot plot showing several enriched pathways in OKC. Among them, the vitamin D receptor pathway was enriched in pOKC.

V. DISCUSSION

4.1 Tumor microenvironment and tumoroid model in AM & OKC

The importance of the interaction between stromal and epithelial cells is well established in tumorigenesis. Tumor cells exist in a complex microenvironment, which includes ECM, diffusible growth factors, cytokines, and a variety of non-epithelial cell types.[43] The tumor microenvironment (TME) is composed of various stromal components such as cells and blood vessels that exert various regulatory functions that favor tumor growth.[44,45] Neoplastic epithelial cells together with stromal cells create a microenvironment essential for tumor invasion.[46] Furthermore, studies regarding tumor progression, local invasion of AM, and bone resorption induced by tumor cells have been reported.[47-49] Tumor cells in AM provide different types of molecules and growth factors, acting in both an autocrine and paracrine manner. These factors are responsible for the tumor growth and invasion of AM.[50]

Although AM has been studied, unsolved questions remain about its diverse clinical courses of its variants.[51] Angiogenesis could be involved in the specific behavior of conventional AM, as shown by recent studies reporting about microvascular decompression (MVD) than other odontogenic tumors analyzed.[52] All tumors contain abundant tumor stroma, including mesenchymal stromal cells surrounding the parenchyma. A recent study has found that interactions between AM cells and stromal fibroblasts are often transmitted by various cytokines, which affect a microenvironment favorable for tumor infiltration.[53]

In addition, although conventional studies on OKC have focused on the epithelial lining, recent studies have indicated that the stromal components of OKC may promote tumor growth, invasion, and angiogenesis.[54,55] Fibroblasts are the major

component of stromal cells and secrete various cytokines, chemokines, and ECM proteins to form a microenvironment that stimulates epithelial cell growth.[56,57] Several notable differences exist between OKC stroma and control tissues. For example, the MVD in the OKC lesion is greater than that in normal oral mucosa or dentigerous cysts.[58] Furthermore, greater osteoclastogenic capacity was detected in OKC fibroblasts co-cultured with Raw264.7 cells.[59] These findings support that the stroma of OKC should be recognized as a contributor to the neoplastic behavior.

Until now, tumoroid models have been studied in various tumors. Driehuis et al. established oral mucosal organoids and head and neck squamous cell carcinoma tumoroids.[60] Van de Wetering et al. were the first to describe a collection of well-characterized patient-derived organoids.[61] Gao et al. succeeded in culturing organoids from metastatic prostate cancer tissue as well as from liquid biopsies.[62] These models can be easily achieved within hydrogel-based matrices, containing a gelatinous mixture made of ECM compounds (including laminin, collagen, and proteoglycans) and growth factors (including TGF- and FGF).

AM and OKC currently do not have animal models, such as mice, which is a major limitation of several experimental studies. Therefore, only research through xenograft transplanting patients' cells can be conducted. The tumor can be cultured from biopsies and expanded *in vitro* using established culture conditions.

A critical limitation of tumor models is the lack of a TME, including stromal cells and immune cells, because tumoroids are derived only from epithelial cells.[63] Therefore, to investigate the interaction between tumor cells and the tumor microenvironment, co-culture systems with immune and stromal cells or xenograft models were required.[64] In this study, we cultured AM and OKC in KRCM, which is supplemented with epidermal growth factor and bovine pituitary extract. These factors supported the 3D growth of AM and OKC for two-to-three passages.

However, over the three passages the dissociated single cells of primary tissues stopped growing and failed to form tumoroids. In the case of OKC, basal layers of OKC tumoroids were poorly developed compared with OKC tissues. These results suggest that some factors supporting the efficient growth of tumoroids are missed in our current condition.

To recapitulate the 3D environment, ECM is used in organoid or tumoroid culture.[65] The most popular ECM for 3D culture is Matrigel, which is secreted by Engelbreth-Holm-Swarm mouse sarcoma cells containing various ECM proteins.[66] The epithelial cells cultured in Matrigel usually develop BMs, since the ECM proteins composed of BMs are abundant in the gel.[66] The BM is a highly organized ECM that typically serves as a barrier between the epithelium or endothelial cells and surrounding mesenchymal tissues. In addition, the BM mediates interactions between two different layers, so it plays important roles in diseases. The biological role of the BM is involved in cell proliferation, differentiation, adhesion, and migration.[67] In addition, alterations in BM protein expression contribute to the development and progression of tumors.[68] In many odontogenic tumor, the BM proteins are commonly expressed.[69] In AMs, collagen type IV plays a role in tumor cytodifferentiation and progression.[70] In this study, all tumoroids derived from AM-1, AM, and OKC developed a BM. Basal cell markers, such as p63 and P-cadherin, were expressed in most outer cells, which were in direct contact with the ECM. The accumulation of collagen IV, a major component of BM, was detected around the AM tumoroids.

Collagen is a major component protein of the bone matrix.[71] *In vitro* cultured AM-1 cells invade the collagen gel mimicking the invasive behavior of AM into the surrounding bone tissues.[42,72] To provide bone-like culture conditions, we cultured AM-1 cells in collagen gel. In this case, AM-1 cells formed a network, not spheroid,

which is reminiscent of plexiform AM. Therefore, the ECM affects cell differentiation and type. In addition, in the condition containing calcium, AM-1 cells formed keratin that was not formed in the original AM tissue. The effect of the microenvironment can affect cell differentiation and type.

In vitro tumoroid models representing individual patients can facilitate the development of personalized medicine for AM and OKC. AM-1 cell lines may undergo chromosomal rearrangements/duplications or mutations and epigenetic changes that make cell lines unable to recapitulate primary tumor behaviors.[73] Therefore, establishing a patient-derived tumoroid model may become more important.

4.2 Next-generation sequencing analysis in AM and OKC

The advent of large-scale parallel sequencing techniques has advanced a powerful tool for studying transcriptome changes via RNA-Seq.[74] By sequencing the transcriptome of tumors, their expression levels could be compared.[75,76]

Recently, NGS technologies announced a new era in genome studies.[77] NGS techniques will replace conventional array-based techniques with the falling costs of sequencing the human genome and data storage as well as the development of user-friendly software for analysis.[77] At present, NGS is revolutionizing various fields, such as genetic diseases, personalized medicine, and diagnostics, by providing a high-throughput option with the capability.[78] Moreover, NGS techniques are applied for gene expression (RNA-Seq) or methylation analyses, providing an ideal tool for cancer genetic studies.

Advanced NGS analyses identified high frequencies of *BRAF* and *SMO* mutations in AM.[25,26,79] Despite the existence of whole genomic sequence

information from patients with AM, the entire molecular signature and characteristics of AM cells remain obscure. Likewise, little is currently known regarding genetic aberrations in OKC, except that point mutations occur in the tumor suppressor gene, *PTCH*, in both the sporadic and syndrome-associated forms of the tumor.[28]

Our RNA-Seq analysis and NGS data showed that AM-1, pAM, and pOKC exhibit distinct gene expression patterns. To confirm the total variability in gene expression, PCA was performed. PCA revealed segregation of AM-1, pAM, and pOKC into different clusters, with the exception of pOKC clustering. GO enrichment analysis based on DEGs also showed that pAMs are mainly associated with the components of the ECM, suggesting that activation of the ECM plays a key role in the establishment of the tumoroid model. Even previously known AM-related signaling pathways, including the PI3K and MAPK pathways,[80] were more enriched in the 3D tumoroid culture of pAM than in the 2D culture of AM-1. These results indicate that the tumoroid model will recapitulate the microenvironment of AM. Therefore, the importance of establishing a patient-derived tumoroid model cannot be overemphasized.

4.3 Limitations and future research directions

The major limitation of this study was that tumoroids were cultivated under passage 3. Moreover, due to the limited number of specimens used in this study, further studies using other sophisticated methods and larger numbers of patient samples are required.

To our knowledge, a study that establishes a tumoroid model isolated from *in vivo* tumor biopsy tissue in patients with AM and OKC has never been reported. In

this preliminary study, we explored tumoroid culture conditions and investigated the importance of the ECM and stroma corresponding to the TME.

Our NGS data support the hypothesis that the PI3K and MAPK pathways are important for the development of AM. Therefore, based on the NGS results, it is necessary to introduce various factors that can implement the TME. In particular, additional analysis of the ECM enriched in pAM and the addition of various factors related to the PI3K and MAPK pathways can be conducted, and in pOKC, the application of vitamin D along with various immune-related factors can be added.

Precision medicine, also called personalized medicine, aims to identify effective treatment for individual patient through characterization of diseases at the molecular levels. If tumoroid and experimental models of AM and OKC are established, great advances will be made in the clinical treatment of tumors.[81,82]

V. CONCLUSION

In summary, this is the first study, to our knowledge, to attempt patient-derived AM and OKC tumoroid model making, and our experimental data demonstrate the following results:

1) The 3D culture of AM-1, pAM, and pOKC was conducted successfully, but tumoroids did not maintain the self-renewal capacity above passage 3.
2) The TME (ECM, calcium) caused changes in the cells that form tumoroids.
3) IHC results showed that cell-cell adhesion and the BM were well-established in pAM tumoroids.
4) General GO results showed that the expression of genes related to proliferation was high in AM-1 cells; however, ECM expression was high in pAM.
5) Signaling pathway enrichment analysis showed that the expression of genes related to the MAPK and PI3K pathways was high in pAM, and of those related to the VitD receptor-related pathway was high in pOKC.

These results provide clues necessary for establishing tumoroids and disease models of AM and OKC in future studies.

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ABSTRACT (in Korean)

법랑모세포종과 치성각화낭종의 환자 유래 종양 오가노이드 모델의 탐색과 특징

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치성 종양 (odontogenic tumor)은 정상적인 치아 형성과정과 유사하게 치성 외배엽성간엽과 상피사이의 상호작용으로부터 발생함이 알려져 있으나, 그 기전이 명확하게 알려지지 않았다. 이 중 침습적이고 재발이 잦은 상피성 치성 종양 및 낭종인 법랑모세포종(ameloblastoma, AM)과 치성각화낭종(odontogenic keratocyst, OKC)은 현재 수술적 치료 외에 방사선 요법이나 약물 요법으로 치료가 어려우며 재발이 잦은 특성을 가지고 있어, 재발을 막기 위한 광범위한 수술적 치료가 필요한 경우가 많다. 이는 결과적으로 환자의 악골에 결손을 남기며 삶의 질 저하로 이어지게 된다.

최근 종양 연구에 주로 사용하는 환자 유래 종양 오가노이드 모델(tumoroid model)은 환자 개개인의 종양의 생체 내 조직 미세환경을 구현할 수 있으며 맞춤 항암제 스크리닝 및 검증에 활용할 수 있어, 치료 효율을 높이고 부작용을 낮출 수 있게 되었다. 간암, 폐암, 유방암 등 다양한 종양에서 이러한 종양 오가노이드 모델이 확립되고 계속 연구되고 있으나

치성 종양에서는 보고된 바가 없다. 즉, 본 연구에서는 아직 적합한 질환 모델이 없는 primary AM (pAM)과 OKC (pOKC)에서 환자 조직을 이용한 종양 오가노이드 모델 구축을 위한 미세 환경의 탐색과 함께 차세대 염기서열 분석(next generation sequencing, NGS)을 이용한 유전적 특성의 탐색을 목표로 하였다.

본 연구에서는 인간 법랑모세포종 세포주인 AM-1 세포주와 환자 유래 법랑모세포종(pAM)과 치성각화낭종(pOKC)의 조직을 채취하여 Matrigel과 Collagen gel을 포함한 다양한 세포외 기질과 여러 조건에서 2차원 및 3차원 배양을 시행하여 성장을 관찰하였으며 면역형광염색을 이용하여 분석하였다. 또한 Bulk RNA-sequencing을 통한 주성분 분석 (principal component analysis) 및 유전자 온톨로지 (gene ontology), 차별 발현 유전자 분석 (differentially expressed gene)을 통해 AM-1과 pAM, pOKC의 유전자 특성의 차이를 분석하고 그 결과 다음과 같은 결론을 얻었다.

1. AM-1, pAM 및 pOKC의 3차원 배양은 성공적으로 수행되었으나 AM-1을 제외한 두 조직은 3계대 이하에서만 재생 능력을 유지했다.
2. 종양 미세 환경 (세포외 기질, 칼슘)은 종양 오가노이드를 형성하는 세포의 성질 변화를 일으킬 수 있다.
3. 면역형광염색 결과는 pAM 종양 오가노이드에서 세포-세포 접착 및 기저막이 잘 확립되었음을 보여준다.
4. 유전자 온톨로지 결과 AM-1 세포주에서는 세포 증식 관련 유전자의 발현이 높았으나 pAM에서는 세포외기질 관련 유전자 발현이 높았다.
5. 신호 전달 경로 농축 분석 (signaling pathway enrichment analysis) 결과 MAPK pathway 및 PI3K pathway와 관련된 유전자의 발현은

pAM에서 높았고, pOKC에서는 VitD 수용체 관련 경로의 비율이 높았다.

이번 연구는 환자 유래 조직을 이용한 AM과 OKC의 종양 오가노이드 모델 확립을 시도한 첫 번째 연구로 장기간 배양 조건은 추후 연구를 통해 더 추가 확립되어야 할 것이다. 본 연구의 결과들을 통해 종양 미세 환경이 세포의 분화나 성장 패턴에 영향을 주었음을 확인할 수 있었으며, 차세대 염기서열 분석 결과를 통해 추후 장기간 배양 조건 확립 가능성을 확인하였다.

핵심 되는 말: 종양 오가노이드, 튜머로이드, 법랑모세포종, 치성각화낭종, 3 차원 배양, RNA 시퀀싱, 차세대 염기서열 분석