

Gene analysis of ameloblastoma-derived cells treated with retinoic acid

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Ameloblastoma is one of the most common benign odontogenic tumors in the oral cavity, and may recur because it grows in a locally invasive manner. Although many studies have been performed on ameloblastoma, its bioactive characteristics remain unclear. Retinoic acid, a metabolite of fat-soluble vitamin A, which is used as a leukemia drug, has been demonstrated to have antitumor effects on some tumor types. We examined whether retinoic acid has potential as a new remedy for ameloblastoma. We found that retinoic acid inhibited the proliferation of cells derived from ameloblastoma and induced differentiation. However, its intracellular signaling and regulatory mechanisms remain unclear. In this study, we primarily cultured cells from ameloblastoma to elucidate signal transduction, and examined the differences in gene expression levels due to retinoic acid action by microarray analysis. Surgically removed specimens were obtained with consent of the patients from the Second Department of Oral and Maxillofacial Surgery, Osaka Dental University Hospital. Primary culture was started with medium for the epithelium, followed by cloning to be used as ameloblastoma cells. Subsequently, retinoic acid was added at a final concentration of 10^{-6} to 10^{-7} M and incubated for 6 hours to prepare an experimental group. A control group was also prepared without addition of retinoic acid. After culturing, the cells were collected to extract RNA from each group for microarray analysis.

In the experimental group, increase in the expression of the *EDF1* gene was most pronounced, whereas that of the *TM4SF10* gene was most markedly suppressed. Furthermore, the expression levels of the *MMP3* and *FGF2* genes were suppressed by 0.37- and 0.48-fold, respectively. The regulation of cell growth and differentiation comprises comprehensive regulatory mechanisms, such as protein synthesis, degradation, complex formation, and phosphorylation, in addition to gene expression. Our study suggested that changes in the expression of *FGF2* and *MMP3* genes are partly involved in the inhibition of growth and promotion of differentiation by retinoic acid in ameloblastoma-derived cells. (J Osaka Dent Univ 2020 ; 54 : 145-151)

Key words : Ameloblastoma ; Retinoic acid ; Microarray ; Cultured cells

INTRODUCTION

Ameloblastoma is one of the most common odontogenic benign tumors in the oral cavity, and it may recur because it grows in a locally invasive manner. Although many studies have been performed on ameloblastoma, its bioactive characteristics remain unclear. Retinoic acid, a metabolite of fat-soluble vi-

itamin A is used as a leukemia drug, and has anti-tumor effects on some tumor types.¹ Its potent physiological activity is achieved by DNA transcriptional regulation through the binding of retinoic acid to the retinoic acid receptor (RAR) (homodimer) or to retinoid X receptor (RXR) (heterodimer) in the cell nuclei.² We examined the anti-tumor effect of retinoic acid and its effect on tooth germs because

it may be useful for treating ameloblastoma, which may originate from the odontogenic epithelium. We found that retinoic acid inhibited the proliferation of ameloblastoma-derived cells and that it induced their differentiation.³ However, its intracellular signal transduction and regulatory mechanisms remain unclear. In the present study, we primarily cultured ameloblastoma and examined the differences in gene expression levels due to retinoic acid action using microarray analysis.

MATERIALS AND METHODS

Primary culture of ameloblastoma cells

Surgically removed specimens that were obtained with consent from patients diagnosed with ameloblastoma at the Second Department of Oral and Maxillofacial Surgery, Osaka Dental University Hospital, were used for primary culture (Fig. 1). After the tumors were excised, blood was removed and the tissue was immediately minced with a scalpel, followed by enzyme treatment with DMEM medium containing 0.1% collagenase/dispase (Fujifilm Wako Pure Chemical, Osaka, Japan) at 37°C in 5% CO₂ for 1 hour. Subsequently, the enzyme was inactivated and a cell suspension was prepared in DMEM medium containing four-times the amount of antibiotic agents (Thermo Fisher Scientific,

Waltham, MA, USA). The cell suspension was collected in a 50-mL centrifuge tube and allowed to stand vertically for 1 hour at 37°C in 5% CO₂. The supernatant was discarded as a fibroblast-containing layer. The cell residue was washed with the above medium, followed by centrifugation for three minutes at a maximum centrifugal acceleration of 60×g to precipitate and collect the cells. The obtained cells were primarily cultured using a medium for epithelial cells (Cnt-PR medium; CellIntec, Bern, Germany), containing an antibacterial (Thermo Fisher Scientific) at 37°C in 5% CO₂. The medium was changed every three days, followed by cloning on Day 14 after the start of the culture. Only the cell aggregates observed on the petri dish resembling pavement were collected using a cloning cup, and cultured again at 37°C in 5% CO₂ for a set period of time.

Fibroblasts were then removed by negative selection using a magnetic cell sorting (MACS) system. MACS buffer (0.025% EDTA-containing phosphate buffer and 1% bovine albumin) was prepared, mixed with anti-fibroblast antibody-bound magnetic beads (Miltenyi Biotec, Gladbach, Germany) at a ratio of 4 : 1, and reacted with the collected cultured tumor cells, followed by removal of fibroblasts through a column for MACS removal. Subsequently, the obtained cells were cultured again as ameloblastoma-derived cells. After stabilization of the culture for approximately seven days, a suspension of 5×10^5 cells/mL was prepared and seeded onto a culture dish and slide glass, and cultured again for 24 hours (Fig. 2).

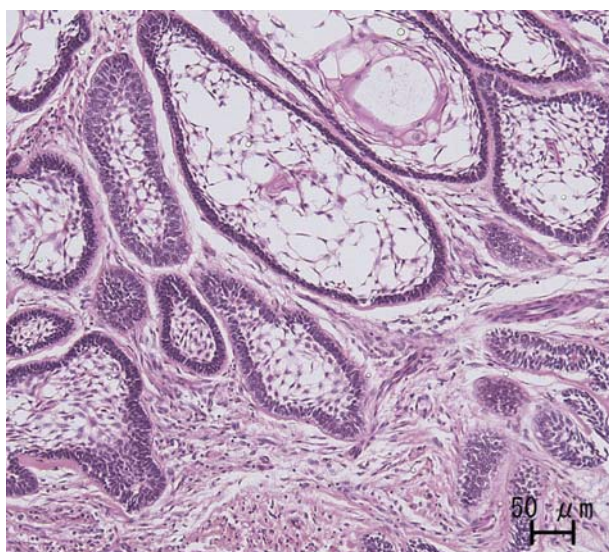


Fig. 1 Histopathological slide of a solid/multicystic type ameloblastoma (HE staining ×40).

Fluorescent immunostaining of the primary cultured cells

After completion of the culture, the cell surface was treated with 0.1% TritonX-100 (Union Carbide Corporation, Danbury, CT, USA), followed by blocking treatment to suppress nonspecific reactions. Anti-amelogenin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a primary antibody. After completion of the reaction, excess antibodies were washed off several times. The secondary antibody was reacted with the goat anti-rabbit

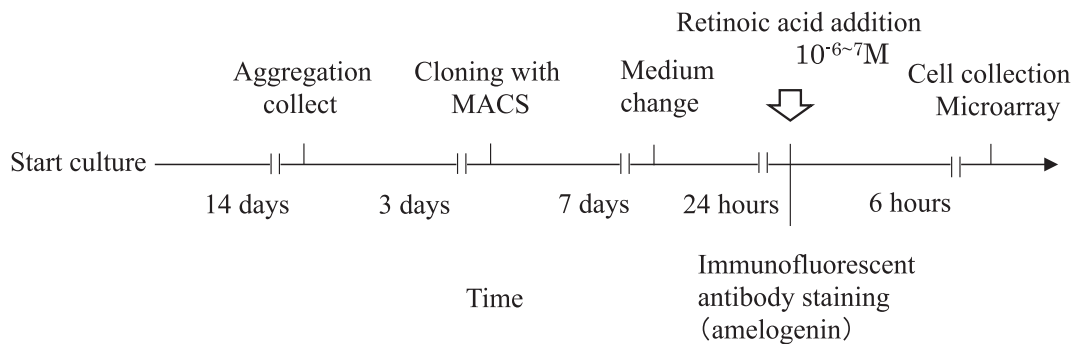


Fig. 2 Time course for this experiment.

antibody Alexa Fluor Plus 555 (Thermo Fisher Scientific), followed by nuclear staining with DAPI.

Microarray analysis of the cultured cells

Microarray analysis was performed using the cultured ameloblastoma-derived cells in the experimental and control groups with and without retinoic acid, respectively. In the experimental group, all-trans-Retinoic Acid (ATRA; Sigma-Aldrich Japan, Tokyo, Japan) was added to the cultured cells at a final concentration of 10^{-6} – 10^{-7} M (w/v 10^{-3} – 10^{-4}), followed by culture for 6 hours at $37^{\circ}C$ in 5% CO_2 . Immediately after culture, the cells were collected, and the cell mass obtained by centrifugation at $200 \times g$ for 10 min) was stored at $-80^{\circ}C$. RNA was collected from both experimental and control groups to outsource microarray analysis (Alliance Biosystems, Osaka, Japan). The obtained data were normalized by statistical methods before analysis. Genes without any difference in expression levels were discarded, and those with greater than two standard deviations were excluded. The remaining 6,858 genes were analyzed. Genes whose expression levels increased more than two-fold or decreased to less 0.5 in the experimental group compared with the control group were selected. This study was approved by the ethics committee of Osaka Dental University (Approval Nos. 110943 and 110916). The experiments were conducted in compliance with the Declaration of Helsinki. The authors declare no conflicts of interest regarding the experiment.

RESULTS

Polygonal cells were found on the petri dish after seven days of culture. The cells were adhered to each other like pavement, forming aggregates. Polygonal cells had a relatively high ratio of N/C, partially exhibiting a mitotic image (Fig. 3). After cloning, polygonal cells that may have originated from the epithelium were found on the petri dish, although they were scattered without aggregate formation. In addition, no spindle-shaped mesenchymal cells were observed around the polygonal cells (Fig. 4). To demonstrate that the cultured cells

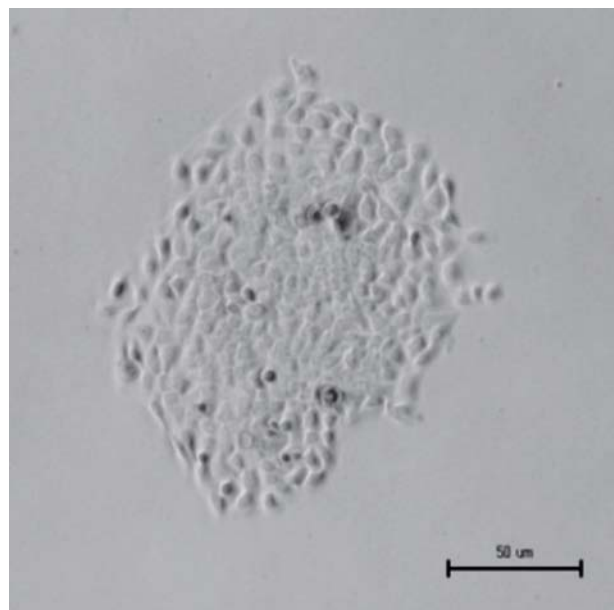


Fig. 3 Phase contrast micrograph showing aggregates looking like epithelial systems that grew to resemble pavement after 7 days of culture ($\times 100$).

originated from ameloblastoma, fluorescence immunostaining was performed using amelogenin antibody, an odontogenic protein, and DAPI to stain the cell nuclei. Some of the scattered nuclei exhibited protein expression of amelogenin as yellow fluorescence in the periphery or throughout the cytoplasm (Fig. 5). We examined 6,858 genes selected from a total of 21,939 genes by microarray analysis, and found 15 that exhibited a two-fold or

greater increase in expression level after the addition of retinoic acid (Table 1). Of these genes, endothelial differentiation-related factor 1 (EDF1) had the highest increase in expression of 3.31-fold (5,992 in the experimental group relative to 1,812 in the control group). The expression levels of cytochrome P450, family 26, subfamily A, and polypeptide 1 (CYP26A) increased 3.05-fold (1,957 in the experimental group relative to 641 in the control group).

On the other hand, the expression levels of 11 genes were more than halved relative to those in

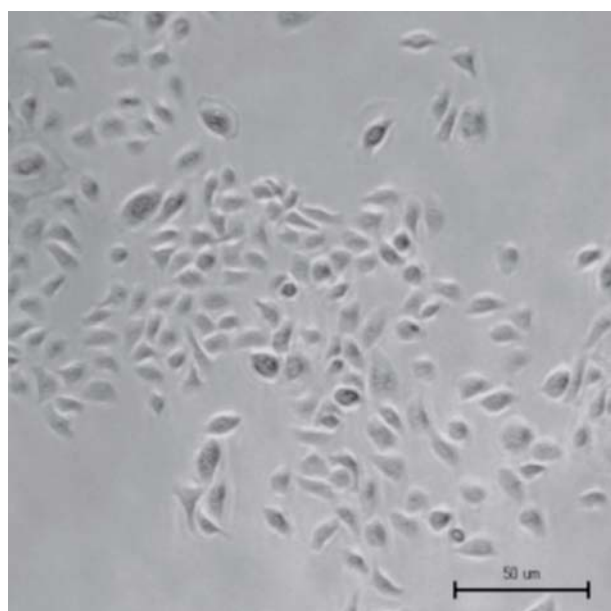


Fig. 4 Phase contrast micrograph after cloning. No epithelial colony was observed, although some of the cells grew like pavement ($\times 100$).

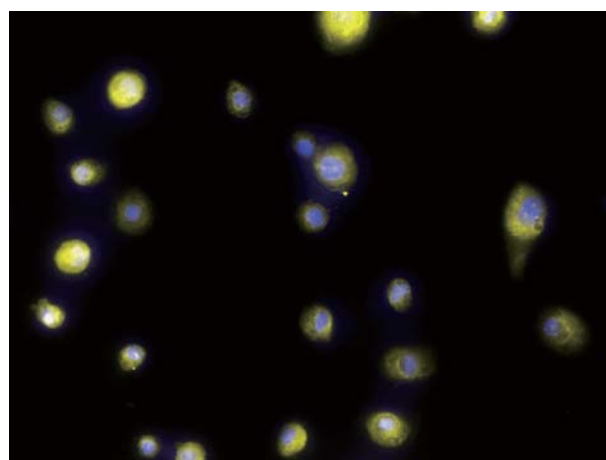


Fig. 5 Immunofluorescent antibody staining with amelogenin. Yellow-stained amelogenin protein was observed in the cytoplasm around the cell nucleus stained blue (Yellow: Amelogenin, Blue: Cell nucleus).

Table 1 Genes with two-fold or greater expression after retinoic acid addition

Systematic	Common	Normalized	Fold-change	Retinoic acid	Control
NM_M_003792	EDF1	3.3051171	3.31	5992	1812.9464
NM_M_004567	PFKFB4	3.232958	3.23	1664	514.6989
NM_M_057157	CYP26A1	3.0492344	3.05	1957	641.8004
XM_M_174227		2.9078364	2.91	1839	632.42896
NM_M_014047	HSPC023	2.7368507	2.74	3015	1101.6311
NM_M_003244	TGIF	2.6605177	2.66	3271	1229.46
NM_M_015414	RPL36	2.4347553	2.43	2517	1033.7794
NM_M_005318	H1F0	2.322808	2.32	30494	13128.076
NM_M_016167	NOL7	2.1877732	2.19	4832	2208.6384
NM_M_001750	CAST	2.1712127	2.17	2443	1125.1776
NM_M_002965	S100A9	2.1655083	2.17	45387	20959.053
XM_M_086495		2.1498954	2.15	2007	933.5338
NM_M_018327	C20ORF38	2.0297112	2.03	1820	896.6793
NM_M_003763	STX16	2.0236917	2.02	2360	1166.1855
XM_M_060704		2.0222113	2.02	5440	2690.1245

Table 2 Genes with decreased expression level of 0.5 times or less after retinoic acid addition

Systematic	Common	Normalized	Fold-change	Retinoic acid	Control
NM_M_002006	FGF2	0.4886059	0.48	8112	16602.338
XM_M_051860	KIAA1199	0.4759359	0.47	2750	5778.089
XM_M_171163	NA	0.47168052	0.47	827	1753.3054
NM_M_001146	ANGPT1	0.46589738	0.46	667	1431.6458
NM_M_013372	GREM1	0.465195	0.46	37065	79676.266
NM_M_057159	EDG2	0.43679115	0.43	541	1238.5782
NM_M_007036	ESM1	0.40331498	0.4	2120	5256.4375
NM_M_015133	MAPK8IP3	0.3996427	0.39	686	1716.5333
NM_M_001999	FBN2	0.3791504	0.37	1509	3979.9512
NM_M_002422	MMP3	0.3785346	0.37	5315	14040.989
NM_M_031442	TM4SF10	0.30254164	0.3	555	1834.4581

the control group after the addition of retinoic acid (Table 2). Of these genes, the expression of transmembrane 4 superfamily member 10 (TM4SF10) had the most significant decrease of 0.30-fold (555 in the experimental group relative to 1,834 in the control group). In addition, the expression of matrix metalloproteinase 3 (MMP3) and fibroblast growth factor 2 (FGF2) decreased 0.37-fold (5,315 in the experimental group relative to 14,040 in the control group) and 0.48-fold (8,112 in the experimental group relative to 16,602 in the control group), respectively.

DISCUSSION

Microarray was developed as a powerful tool to measure gene expression levels, and has been utilized as an effective method for gene profiling. Several reports have been published on its use for ameloblastoma.⁴⁻⁶ In the present study, we cultured tumor cells from ameloblastoma and analyzed the differences in gene expression levels due to retinoic acid action using a microarray to examine its bioactivity. We previously reported similar differentiation induction and growth inhibition using retinoic acid isomers such as 9-cis retinoic acid.³ First, the cells used in the study were demonstrated to be enamel-derived cells because the expression of amelogenin, an odontogenic protein, was confirmed by fluorescence immunostaining of the cultured cells derived from ameloblastoma.⁷⁻¹⁰ In the present experiment, CYP26A, whose expression level increased 3.04-fold relative to that in the control

group, belongs to the cytochrome P 450 family functioning in detoxification, and is highly involved in retinol metabolism along with CYPs.^{11, 12} The increased expression of the CYP26A gene in the present study suggests that CYP26A is rapidly synthesized to metabolize the added retinoic acid. On the other hand, most retinoic acid receptor RAR and RXR-related genes had markedly low expression among the excluded genes. Although the added retinoic acid binds the nuclear receptor, CYP 26A1 synthesis may be promoted to metabolize the excess. The 26 genes found in the study were annotated using the Database for Annotation Visualization and Integrated Discovery (DAVID), which is an online search tool. It is a comprehensive data bank that accumulates intracellular information networks and enables searching for relevant protein pathways (<https://david.ncifcrf.gov/>).¹³

The involvement of two pathways was suggested. In both of these pathways, ANGPT1, FGF2 and LPAR1 (EDG2) bind their receptors on the cell membrane. More specifically, one pathway leads to cell cycling and death by activating the PIP3 pathway, whereas the other activates the RAP1 pathway (Fig. 6). The phosphatidylinositol-3 kinase (PI3 K) pathway was activated by FGF2 in an experiment with chondrocytes, and functions in MMP1 regulation as a remodeling phenomenon.¹⁴ This suggests that the activation of the PI3 K pathway by FGF2 is essential for the effects of retinoic acid. In the experimental system of the primary culture of ameloblastoma using the same serum-free me-

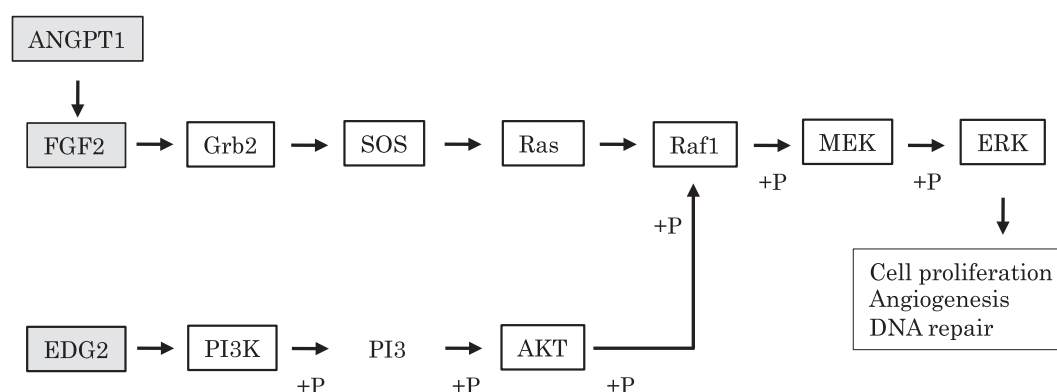


Fig. 6 Annotation by The Database for Annotation Visualization and Integrated Discovery (DAVID). Extract is part of PI3K pathway (+P: Phosphorylation)

Table 3 Some of the MMP-related genes found in the 6,858 genes detected in this study

Systematic	Common	Normalized	Fold-change	Retinoic acid	Control
NM_M_002421	MMP1	0.6995017	0.67	5605	8012.847
NM_M_004530	MMP2	0.7392801	0.74	11363	15370.358
NM_M_002422	MMP3	0.3785346	0.38	5315	14040.989
NM_M_004994	MMP9	0.7369047	0.73	1569	2129.1763
NM_M_002425	MMP10	0.6620744	0.666	7912	11950.319
NM_M_002426	MMP12	0.6746698	0.67	7676	11377.417

dium, FGF2 played a role in proliferation and the promotion of collagenase; therefore, MMP production may function in the regeneration of surrounding tissues and tissue invasiveness.¹⁵ As recently reported, FGF2 and its receptor, FGFR, exist in ameloblastoma, and may be correlated with the recurrence rate.¹⁶ In the present study, the inhibition of the *FGF2* gene by retinoic acid may have reduced autocrine signaling by which secreted substances act on the secreting cells, thereby inducing growth suppression. Retinoic acid may have inhibitory effects when proteins secreted by the tumor cells interact with the surrounding mesenchymal cells, in addition to its well-known anti-tumor effects on target tumor cells.

In the low expression group, TM4SF10 was most significantly decreased, followed by MMP3. MMPs play a major role in wound healing, bone remodeling, malignant metastasis, and invasive growth. A pathological study for comparison with calcified cystic odontogenic tumors demonstrated significantly increased MMP 9 expression in ameloblastoma.¹⁷ In

an experiment with AM-3, an immortalized cell line from follicular ameloblastoma, MMP 9 expression was increased by Wnt/ β -catenin signaling.^{18,19} The 6,858 genes detected in the present study were examined, demonstrating that the expression of MMP 1, 2, 3, 9, 10 and 12 in the experimental group were suppressed to a certain extent compared with in the control group (Table 3). As MMPs are activated through many processes, the reduced invasiveness of ameloblastoma cannot be explained only by the decreased expression. Considering the regulatory mechanisms of the tissue inhibitor of metalloproteinase, comprehensive examination may be necessary. However, the inhibited expression of the *MMP* genes should reduce the amount of protein and relevant enzyme activities, resulting in reduced tumor invasion. Numerous biological phenomena, such as cell proliferation, differentiation, and invasion, are regulated not only by gene expression. Different regulatory mechanisms, such as complex formation, modification and phosphorylation, in addition to protein synthesis and degrada-

tion, should be comprehensively examined. The changes in gene expression due to retinoic acid in ameloblastoma-derived cells partially explain the suppression of cell growth and promotion of differentiation.

CONCLUSION

It is thought that gene transcription of ANGPT1, FGF2, MMP3, CYP26 and LPAR1 (EDG2) is involved in the proliferation inhibition and differentiation of ameloblastoma-derived cells after retinoic acid addition.

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