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# Immunohistochemical Array for Clear Cell Type Mucoepidermoid Carcinoma

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**Background :** The protein expression profile of clear cell type mucoepidermoid carcinoma (MEC) is not well known. **Methods :** We examined a case of clear cell type MEC by immunohistochemical (IHC) array using 59 antibodies against oncoproteins, proliferation-related proteins, apoptosis-related proteins, growth factor-related proteins, angiogenesis-related proteins, and matrix proteins. **Results :** MEC tumor cells showed 40 to 60% more expression of BCL-2 and cyclin-dependent kinase 4 than normal gingival tissue, and 20-40% more expression of BCL-2-associated agonist of cell death, deleted in malignant brain tumors 1, E-cadherin, eIF5A, hypoxia-inducible factor, vimentin, and Wnt-1. Expression of other proteins, including p53, epidermal growth factor receptor, proliferating cell nuclear antigen, survivin, carcinoembryonic antigen, $\beta$-catenin, poly-ADP ribose-polymerase, etc. were relatively weak in MEC tumor cells. **Conclusions :** The IHC array for our MEC contained strong oncogenic signals involving Wnt-1/adenomatous polyposis coli, tumor necrosis factor $\alpha$/signal transducer and activator of transcription 3/BCL-2, and pAKT pathways, signals that could result in the prolonged survival of clear tumor cells.

**Key Words :** Oncogenes; Immunohistochemistry; Array

Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland tumor, accounting for about 3 to 15% of all salivary gland tumors and 12 to 40% of salivary malignancies.[1-3] Histologically, MEC has been classified as low, intermediate, or high grade, according to its cytological features, pattern of invasion, and cellular type.[4] As its name implies, it is composed of mucoid cells and epidermoid cells as well as intermediate cells.[5] The ratio of mucous to epidermoid cells predicts the biological behavior of the neoplasm and subsequent management. The low grade tumors are composed predominantly of mucous cells, whereas the high grade tumors have a greater epidermoid component.[3] Expression of some immunohistochemical (IHC) markers is also a prognostic factor in patients with MEC.[6,7] The clear cell type MEC may show a prominent population of intermediate and clear basal cells positive for the periodic acid-Schiff (PAS) reaction, cytokeratin (CK)7, CK14, and vimentin.[8] Clear cell changes in salivary tumors are usually found in pleomorphic adenoma, oncocytoma, and many malignant tumors including epithelial-myoepithelial carcinoma, polymorphous low grade adenocarcinoma, clear cell acinic carcinoma, clear cell squamous carcinoma, clear cell malignant melanoma, clear cell odontogenic carcinoma, clear cell rhabdomyosarcoma, sebaceous carcinoma and metastasis of renal carcinoma.[9] Moreover, the oncogenic pathogenesis of each tumor could be different from all the other tumors, because many different carcinogenic signaling pathways exist in complex cellular systems. However, cytodifferentiation between intermediate and clear cells of MECs are still controversial and do not easily explain the prognostic behavior of the clear tumor cells. Here, we compare IHC array results for a typical clear cell type MEC with results for normal gingival epithelium, demonstrating different IHC expression for 59 antisera in serial sections. Although this IHC array was done for only one case of clear cell type MEC, we were able to observe essentially the transient changes in protein expression

profile from the intermediate cells to the clear tumor cells in the whole serial sections of the tumor mass. The present study aimed to analyze the IHC results done simultaneously using the serial sections with the statistical methods of IHC tissue arrays. We also present a comparative analysis of IHC array results and molecular pathogenesis studies of intermediate and clear tumor cells of our MEC along with a literature review.

## MATERIALS AND METHODS

A clear cell type MEC arose in the left sublingual gland of a 63 year old female, producing a round mass about 3 × 4 × 5 cm, tightly attached on the lingual side of the mandible; it bulged out slightly into the oral cavity as a sessile mass. The surgically removed tumor mass showed a grayish yellow cut surface with multicystic spaces containing bloody coagulum (Fig. 1-1). The enlarged cervical lymph nodes were also removed. Histology indicated that two of five lymph nodes had tumor metastasis involvement. The excised specimen was fixed in 10% neutral formalin, embedded in paraffin, and sectioned into slices of 4 $\mu$m thickness. Serial microsections were made and routinely stained with hematoxylin & eosin, PAS reaction, and mucicarmine. This was followed by an IHC array. We compared findings to findings for normal gingival tissue obtained from the same patient was done using an indirect triple sandwich method[10] and 59 antisera corresponding to, proliferation-related proteins, apoptosis-related proteins, growth factors, growth factor-related oncoproteins, Wnt signaling oncoproteins, tumor oncoproteins, proinflammatory cytokines, angiogenesis-related proteins, extracellular matrix proteins, protective proteins, protein translation genes, and cytoskeletal proteins (Table 1). Immunostaining of $\alpha$-tubulin (1 : 100, DAKO, Glostrup, Denmark) was used as a control to normalize expression levels of different proteins. Background cross reactions were minimized by the use of negative control staining using no primary antibody during the same IHC procedures.[11] For analysis of IHC array histological images of representative tumor tissue were captured by digital camera (DP-70, Olympus, Tokyo, Japan). We then compared the distribution and intensity of the IHC reactions between the MEC and normal gingival tissue using ImageQuant ver. 5.2 program (Molecular Dynamic, Sunnyvale, CA, USA). The usage of the biopsy specimens filed in the Department of Oral Pathology, Gangneung-Wonju National University Dental Hospital was approved by the Life Ethic Committee of our Institution (IRB 2009-1-3).

## RESULTS

### Pathological examination

Microsections of the main tumor tissue showed a wide distribution of basaloid intermediate cells and frequent collections of

Table 1. Antibodies used in this study

| Groups | No. | Antibodies |
|---|---|---|
| Proliferation-related proteins | 9 | CDK4[a], c-Myc[a], MAX[b], MPM-2[a], p16[a], p21[a], PCNA[c], p53[a], Rb-1[a] |
| Apoptosis-related proteins | 12 | BAX[a], BAD[a], BCL-2[a], BID, FADD[a], FAS[a], FASL[a], FLIP, PARP[a], caspase-3[d], caspase-8[d], caspase-9[d] |
| Growth factor-related proteins | 8 | HGF-$\alpha$[a], EGFR[b], TGF-$\beta$1[d], AKT[c], pAKT[c], c-erbB2[b], JAK2[c], N-Ras[c] |
| Wnt signaling proteins | 4 | APC[a], $\beta$-catenin[b], E-cadherin[b], Wnt-1[b] |
| Tumor oncoproteins | 6 | CEA[c], DMBT1[c], maspin[a], PIM1[d], survivin[d], STAT3[a] |
| Proinflammatory cytokines | 2 | CD68[a], TNF-$\alpha$[a] |
| Angiogenesis-related proteins | 4 | Angiogenin[a], CD31[a], VEGF[d], HIF[d] |
| ECM proteins | 4 | Laminin $\alpha$5[a], MMP-1[c], MMP-2[c], MMP-9[a] |
| Protective proteins | 2 | HO-1[a], HSP-70[a] |
| Protein translation proteins | 2 | DOHH[c], eIF5A[c] |
| Cytoskeletal proteins | 6 | $\alpha$-actin[a], $\alpha$-SMA[b], CK14[a], S-100[b], tubulin[a], vimentin[a] |
| Total | 59 | |

[a]Santa Cruz Biotechnology, Santa Cruz, CA, USA; [b]DAKO, Glostrup, Denmark; [c]Neomarkers, Fremont, CA, USA; [d]ZYMED, San Francisco, CA, USA.
CDK, cyclin-dependent kinase; MPM, mitotic protein monoclonal; PCNA, proliferating cell nuclear antigen; BAD, BCL-2-associated agonist of cell death; FADD, FAS-associated death-domain protein; FASL, Fas ligand; FLIP, FLICE inhibitory protein; PARP, poly-ADP ribose-polymerase; HGF, hepatocyte growth factor; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; JAK, Janus tyrosine kinase; APC, adenomatous polyposis coli; CEA, carcinoembryonic antigen; DMBT, deleted in malignant brain tumors; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; MMP, metalloproteinase; HSP, heat shock protein; DOHH, deoxyhypusine hydroxylase; SMA, smooth muscle actin; CK, cytokeratin; ECM, extracellular matrix.

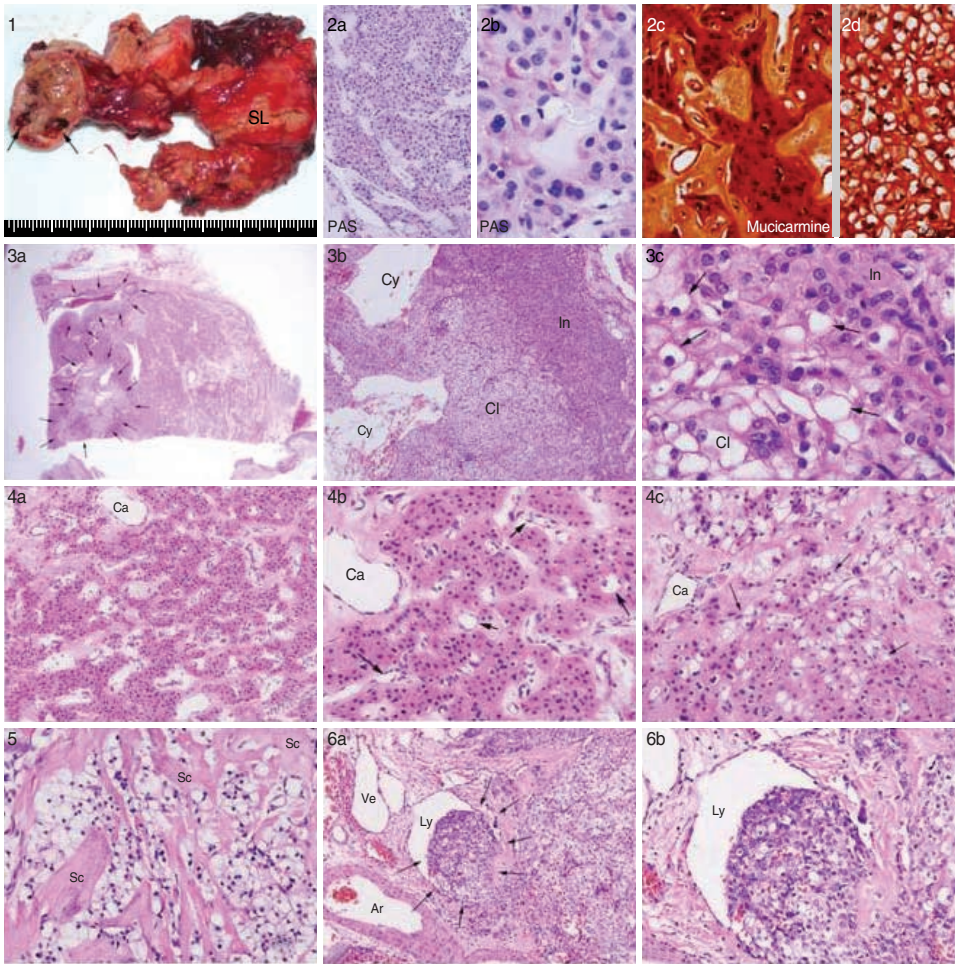


Fig. 1. Pathological examination of clear cell type mucoepidermoid carcinoma. 1: Gross observation. The round tumor mass (3 × 4 × 5 cm) is attached on the sublingual gland (SL). The cut surface is grayish yellow and reveals multicystic spaces (arrows). 2a-2b: Periodic acid Schiff (PAS) staining. 2a: Low magnification, the positive reaction (pink) for mucopolysaccharide is diffusely found in intermediate cells. 2b: The pinkish staining is condensed in the cytoplasm. 2c-2d: Mucicarmine stain. 2c: The epidermoid tumor cells are strongly positive (red) in their cytoplasm. 2d: Clear tumor cells are also positive (red) for mucicarmine stain. 3a: Low magnification of the lesion. Clear tumor cells are usually found near the cystic areas (arrows). Otherwise, the intermediate tumor cells were extensively distributed in the tumor tissue. 3b: The border area between the clear tumor cell (Cl) zone and the intermediate tumor cell (In) zone, show no clear distinction. Cy, cyst space. 3c: High magnification. Clear cell changes (arrows) are irregularly found in the area of the intermediate tumor cell (In) zone. 4: The area of intermediate tumor cells. 4a: The basaloid epithelial strands are frequently anastomosed in the vascular stromal tissue. Ca, capillary. 4b: High magnification. The polygonal intermediate tumor cells have abundant eosinophilic cytoplasms, and also show many tiny capillaries (arrows) near the tumor cells. 4c: A transitional area from intermediate to clear tumor cells (arrows), shows no clear borderline. Sc, sclerotic stromal tissue. 5: Many clear tumor cells are found in sclerotic stromal tissue. 6a: Tumor cells infiltratively grow towards lymphatic vessels (arrows), Ve, vein; Ar, artery; Ly, lymphatic vessel. 6b: High magnification of panel 6a. Infiltrating tumor cells are mostly polygonal intermediate cells.

clear cells around microcystic spaces and sclerotic stromal tissue (Fig. 1-3). Intermediate tumor cells were polygonal and contained cytoplasm with intense eosinophilic staining. They formed epithelial sheets or strands anastomosed frequently in well vascularized stromal connective tissue (Fig. 1-4), while the cytoplasm of the clear tumor cells showed vacuolated empty space with small round nuclei (Fig. 1-5). The individual clear tumor cells had huge cytoplasmic vacuoles, ones containing mucicarmine-positive materials, but were rarely stained by PAS reaction. The intermediate tumor cells frequently showed strong PAS reactions and cytoplasmic mucicarmine staining (Fig. 1-2, 1-3). The clear tumor cells scattered in the sclerotic connective tissue containing a few vascular channels gradually aggregated to form a mass containing a cystic space in its center. However, the border between the intermediate and clear cells was transitional, without a clear borderline. Some cytoplasmic clear changes were also

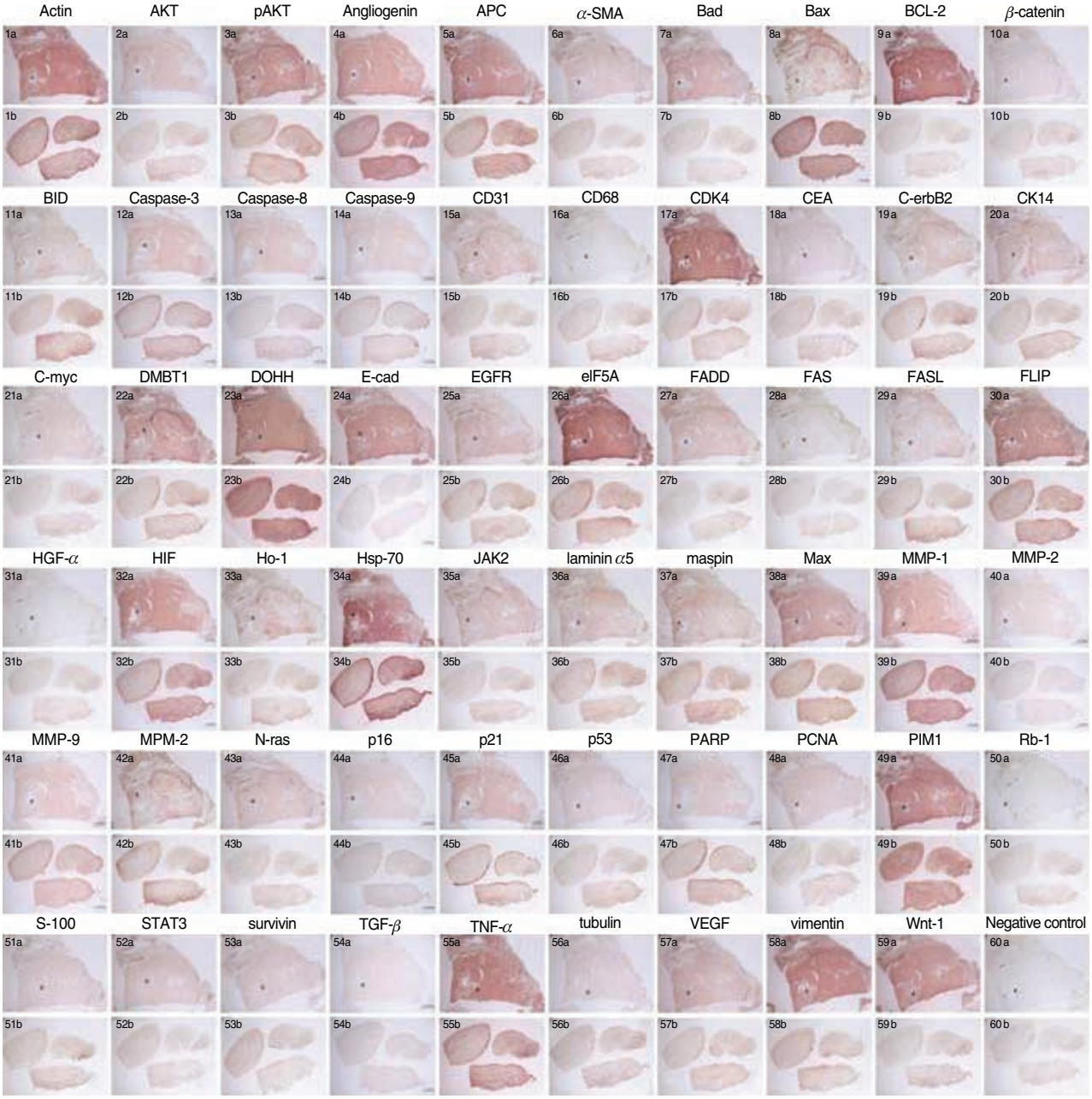


Fig. 2. Photomicrographs of immunohistochemical arrays of clear cell type mucoepidermoid carcinoma (a) and normal gingival tissue (b). 1-59: Strong positive reactions of tumor cells for BCL-2, cyclin-dependent kinase (CDK)4, deleted in malignant brain tumors 1 (DMBT1), E-cadherin, eIF5A, tumor necrosis factor (TNF)-$\alpha$, vimentin, and Wnt-1; marked positive reactions for pAKT, adenomatous polyposis coli (APC), BCL-2-associated agonist of cell death (BAD), DMBT1, FAS-associated death-domain protein (FADD), FLICE inhibitory protein (FLIP), p21, signal transducer and activator of transcription (STAT)3, TNF-$\alpha$, and vascular endothelial growth factor (VEGF). Expression of p53, epidermal growth factor receptor (EGFR), proliferating cell nuclear antigen (PCNA), survivin, carcinoembryonic antigen (CEA), $\beta$-catenin, poly-ADP ribose-polymerase (PARP), caspase-3, -8, -9, and hepatocyte growth factor (HGF)-$\alpha$ are weak. 60: Negative control. FASL, Fas ligand; FLIP, FLICE inhibitory protein; HIF, hypoxia-inducible factor; HSP, heat shock protein; JAK, Janus tyrosine kinase; MMP, metalloproteinase; TGF, transforming growth factor.

found in the polygonal intermediate cells. In the periphery of the tumor mass many epithelial sheets and strands of the intermediate tumor cells had infiltrated into the adjacent fibrous connective tissue, and some polygonal tumor cells in parenchymal tumor tissue had directly infiltrated into dilated lymphatic vessels (Fig. 1-6).

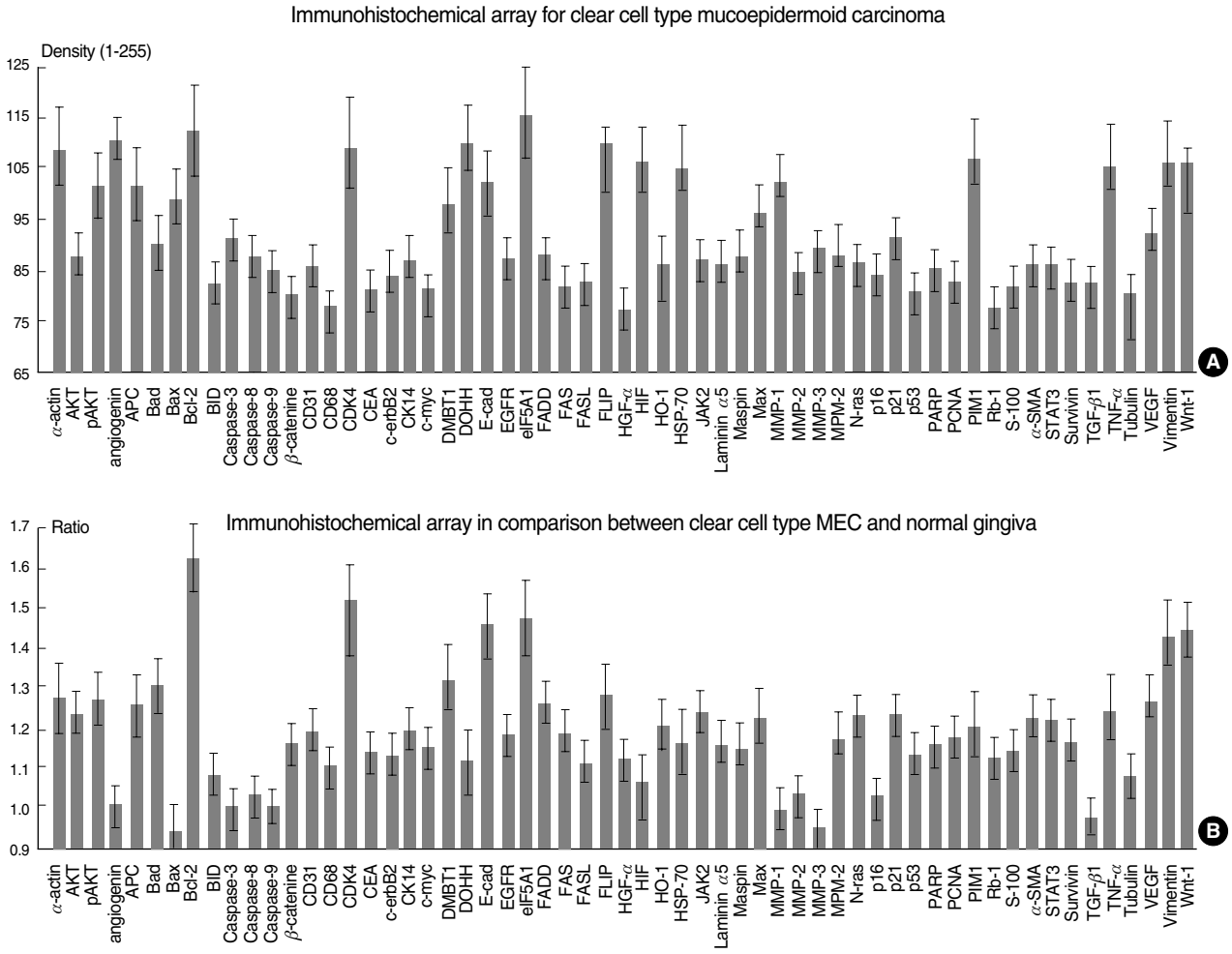


Fig. 3. Statistical analysis for the immunohistochemical (IHC) array. (A) An IHC array graph for clear cell type mucoepidermoid carcinoma (MEC) showing differential immunostaining for 59 dfferent antisera. (B) An IHC array graph comparing clear cell type MEC and normal gingival tissue, normalized by the expression level of $\alpha$-tubulin. It shows a clear contrast between the gene expression profile of the present MEC and normal gingival tissue.
APC, adenomatous polyposis coli; CDK, cyclin-dependent kinase; CEA, carcinoembryonic antigen; DMBT, deleted in malignant brain tumors; EGFR, epidermal growth factor receptor; FADD, FAS-associated death-domain protein; FASL, Fas ligand; FLIP, FLICE inhibitory protein; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; HSP, heat shock protein; JAK, Janus tyrosine kinase; MMP, metalloproteinase; MPM, mitotic protein monoclonal; PCNA, proliferating cell nuclear antigen; SMA, smooth muscle actin; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

## Immunohistochemical array

IHC array analysis indicated that MEC and normal gingival tissue had different levels of expression of the proteins examined in this study. BCL-2, cyclin-dependent kinase 4 (CDK4), deleted in malignant brain tumors 1 (DMBT1), E-cadherin, eIF5A, tumor necrosis factor (TNF)-$\alpha$, vimentin, and Wnt-1 were strongly positive in the MEC tumor cells compared to normal gingival tissue. AKT, pAKT, adenomatous polyposis coli (APC), BCL-2-associated agonist of cell death (BAD), DMBT1, FAS-associated death-domain protein (FADD), FLICE inhibitory protein (FLIP), HO-1, Janus tyrosine kinase (JAK)2, MAX, N-Ras, p21, signal transducer and activator of transcription (STAT)3, TNF-$\alpha$, and vascular endothelial growth factor (VEGF) were markedly positive in the tumor cells. p53, proliferating cell nuclear antigen (PCNA), survivin, carcinoembryonic antigen (CEA), and hepatocyte growth factor (HGF)-$\alpha$ were not significantly increased in the tumor cells. Regarding intracellular signaling pathways, proliferation-related oncoproteins, c-Myc, mitotic protein monoclonal (MPM)-2, p16, PCNA, p53, and Rb-1 were weak in tumor cells, while CDK4 and p21 were strong. Apoptosis-related proteins including BAX, BID, FAS,

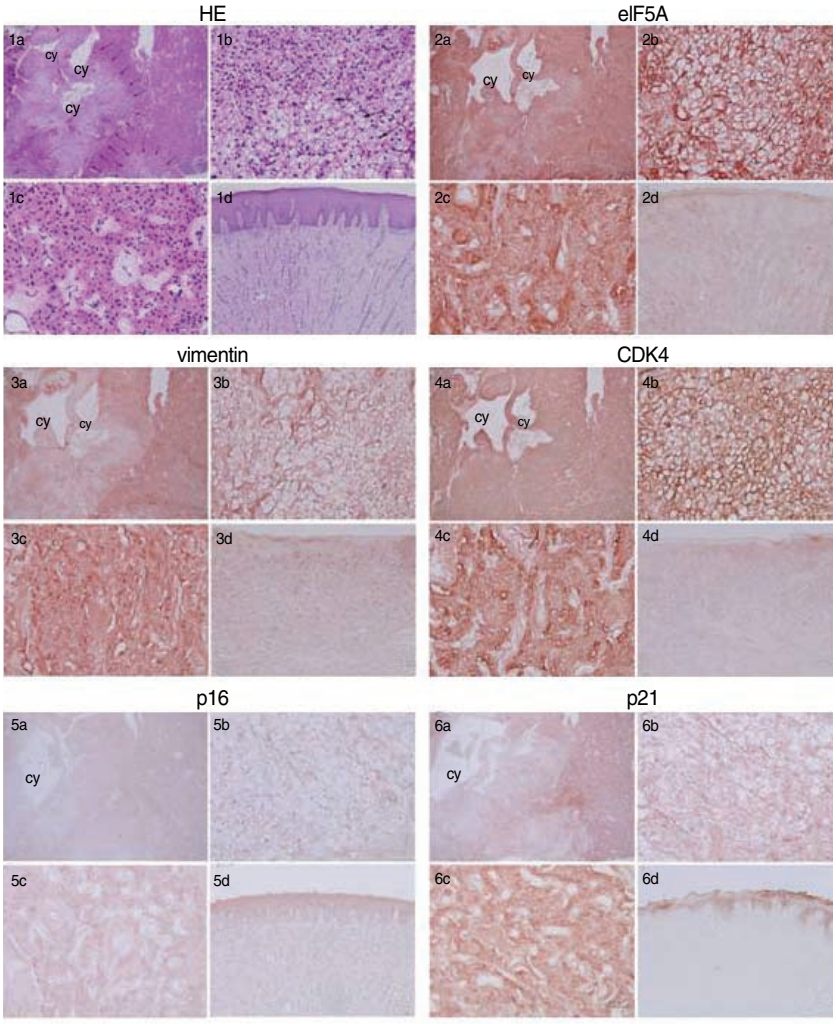


Fig. 4. Photomicrographs of routine histology and immunohistochemistry (IHC) comparing clear cell type mucoepidermoid carcinoma (MEC) and normal gingival tissue. 1: Hematoxylin and eosin stain, 1a: A low magnification view showing the clear tumor cells and intermediate tumor cells, 1b: clear tumor cell zone (arrows), 1c: intermediate tumor cell zone, 1d: epithelium and connective tissue of normal gingival tissue. 2: IHC of eIF5A, 2a, 2b, 2c: strong positive reaction in the intermediate tumor cells, and a marked reaction in the clear tumor cells, 2d: weak reactions in the normal gingival tissue. 3: IHC of vimentin, 3a, 3b, 3c: strong positive reactions in the intermediate tumor cells, but slight in the tumor cells, 3d: weak reactions in normal gingival tissue. 4: IHC of cyclin-dependent kinase (CDK)4, 4a, 4b, 4c: strong positive reactions in the clear and intermediate tumor cells, 4d: weak reactions in normal gingival tissue. 5: IHC of p16, 5a, 5b, 5c: slight positive reaction in the intermediate tumor cells, but a weak reaction in clear tumor cells, 5d: weak reaction in normal gingival tissue. 6: IHC of p21, 6a, 6b, 6c: strong positive reaction in both clear and intermediate tumor cells. 6d: marked reactions in the epithelium of normal gingival tissue. cy, cyst space.

Fas ligand (FASL), poly-ADP ribose-polymerase (PARP), caspase-3, caspase-8, and caspase-9 were weak, but BCL-2 was strongly positive. Growth factor-related proteins such as HGF-$\alpha$, epidermal growth factor receptor (EGFR), transforming growth factor (TGF)-$\beta$1, and c-erbB2 were weak; JAK2 and N-Ras were slightly positive, while pAKT was markedly positive in tumor cells. Among tumor oncoproteins, CEA, maspin, and survivin were weak, PIM1 and STAT3 were slightly positive, and DMBT1 was markedly positive. Wnt signaling proteins including APC, E-cadherin, and Wnt-1 were strongly positive in tumor cells, and $\beta$-catenin was slightly positive. The proinflammatory cytokine, TNF-$\alpha$ was strongly positive, but the macrophage marker, CD68 was rarely positive. Angiogenesis-related proteins, including VEGF, were markedly positive. Extracellular matrix proteins (laminin $\alpha$5, metalloproteinase [MMP]-1, MMP-2, and MMP-9) were weak. The protective protein, HO-1 was slightly positive in the tumor cells, but heat shock protein (HSP)-70 was weakly positive. Protein translation molecules, deoxyhypusine hydroxylase and eIF5A were strongly positive, and the cytoskeletal protein, vimentin was consistently positive in tumor cells, but $\alpha$-smooth muscle actin (SMA), CK14, and S-100 were almost negative (Fig. 2).

The graph for the expression of proteins in the IHC array, normalized by $\alpha$-tubulin, shows the difference in expression between MEC tumor cells and normal gingival tissue (Fig. 3). BCL-2 and CDK4 were expressed by 40-60% more in MEC; BAD, DMBT1, E-cadherin, eIF5A, hypoxia-inducible factor (HIF), vimentin and Wnt-1 were expressed by 20-40% more; AKT, pAKT, APC, $\beta$-catenin, CD31, CD68, CEA, c-erbB2, c-myc, EGFR, FADD, FAS, FASL, FLIP, HGF-$\alpha$, HO-1, HSP-70, laminin $\alpha$5, JAK2, maspin, MAX, MPM-2, N-Ras, p21, p53, PARP, PCNA, PIM1, Rb-1, S-100, $\alpha$-SMA, STAT3, survivin, TNF-$\alpha$, and VEGF were expressed by 0-20% more; angiogenin, BAX, caspase-3, -8, -9, MMP-1, -2, -3, and TGF-$\beta$1 were expressed less than in normal

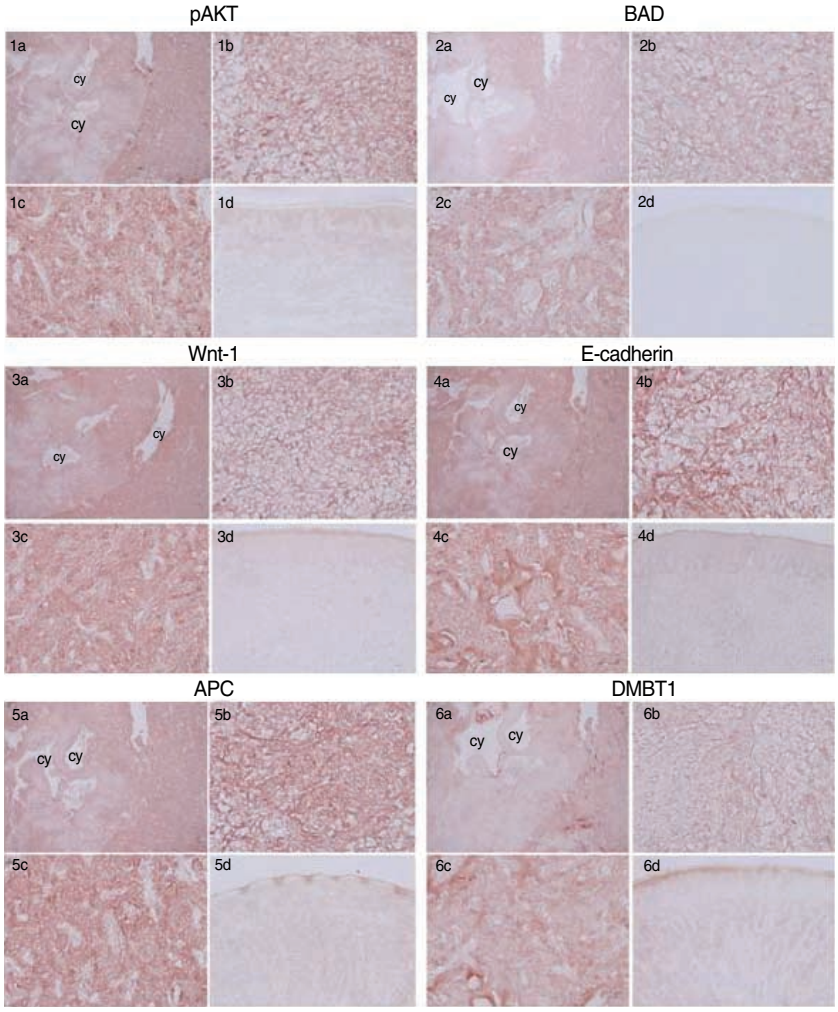


Fig. 5. Photomicrographs of immunohistochemistry (IHC) comparing clear mucoepidermoid carcinoma cell types and normal gingival tissue. 1: IHC of pAKT, 1a, 1b, 1c: positive in the clear and intermediate cells, 1d: weak in normal gingival tissue. 2: IHC of BCL-2-associated agonist of cell death (BAD), 2a, 2b, 2c: positive in the clear and intermediate cells, 2d: rare in normal gingival tissue. 3: IHC of Wnt-1, 3a, 3b, 3c: positive in the clear and intermediate tumor cells, 3d: weak in the normal gingival tissue. 4: IHC of E-cadherin, 4a, 4b, 4c: positive in the clear and intermediate tumor cells, 4d: weak in normal gingival tissue. 5: IHC of adenomatous polyposis coli (APC), 5a, 5b, 5c: strong positive reactions in clear and intermediate tumor cells, 5d: weak in normal gingival tissue. 6: IHC of deleted in malignant brain tumors 1 (DMBT1), 6a, 6b, 6c: strong positive reactions in intermediate tumor cells, but slight reactions in clear tumor cells, 6d: weak in normal gingival tissue. cy, cyst space.

gingival tissue.

Generally, clear tumor cells were distributed around the microcysts in the MEC tumor tissue (Fig. 4-1a). Intermediate tumor cells formed anastomosing epithelial strands admixed with well vascularized mesenchymal tissue (Fig. 4-1c). Clear tumor cells gradually aggregated and formed a solid mass with scanty mesenchymal tissue (Fig. 4-1b). Normal gingival tissue (the control) showed slight basal hyperplasia of its epithelium and mild inflammatory cell infiltration in its connective tissue (Fig. 4-1d). At the high magnification of immunohistochemistry, the intermediate and clear tumor cells were strongly positive for eIF5A, vimentin, and CDK4 (Fig. 4-2, 4-3, 4-4). p21 was moderately positive in intermediate tumor cells, but slightly positive in clear tumor cells, while p16 was rarely positive in tumor cells (Fig. 4-5, 4-6). pAKT and BAD were consistently positive in intermediate and clear tumor cells (Fig. 5-1, 5-2). Wnt-1, E-cadherin, and APC were strongly positive in intermediate and clear tumor cells (Fig. 5-3, 5-4, 5-5). DMBT1 was moderately positive in intermediate tumor cells and slightly positive in clear tumor cells (Fig. 5-6). Intermediate and clear tumor cells were strongly positive for HIF, but slightly positive for VEGF (Fig. 6-1, 6-2). Intermediate and clear tumor cells were strongly positive for TNF-$\alpha$, PIM1, and BCL-2, but slightly positive for STAT3 (Fig. 6-3, 6-4, 6-5, 6-6).

## DISCUSSION

MEC histology showed that clear tumor cells were usually aggregated around microcysts, which were irregular in shape and different from salivary ducts or ordinary epithelial cysts. Both clear tumor cells and intermediate tumor cells showed positive cytoplasmic PAS reactions and cytoplasmic mucicarmine. These epidermoid features of MEC tumor cells led us to select the normal gingival epithelium as a negative control for the IHC array. It is known that intermediate tumor cells of MEC origin express

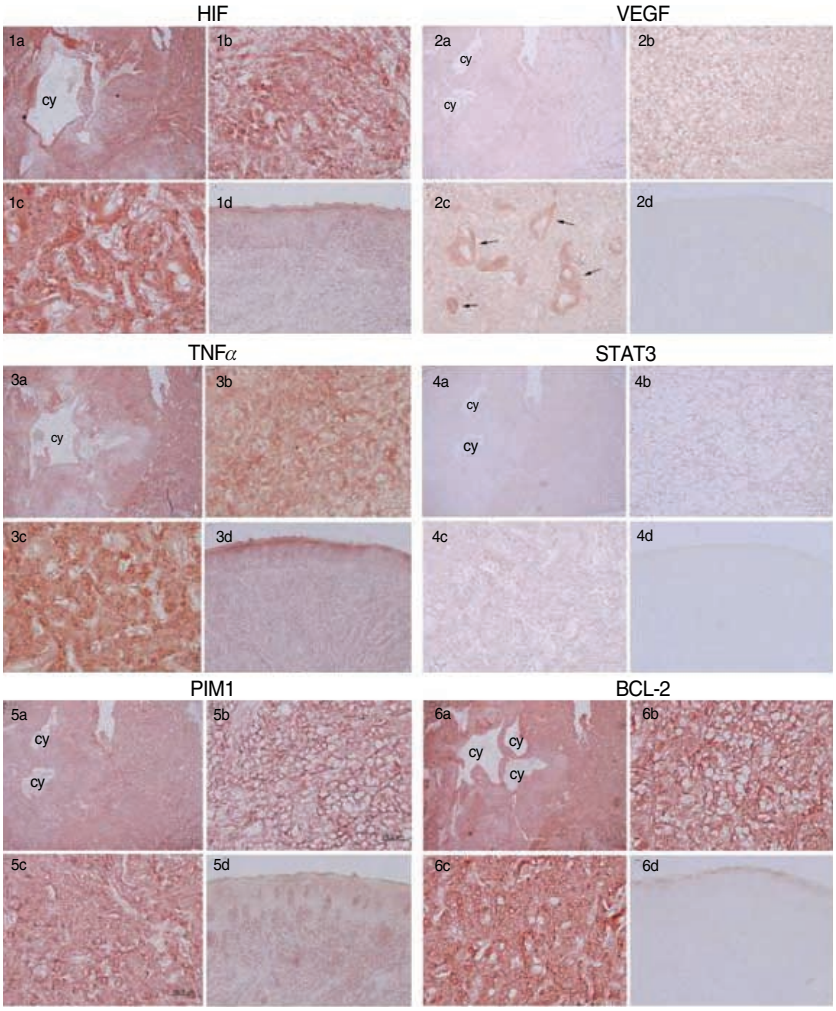


Fig. 6. Photomicrographs of immunohistochemistry (IHC) comparing clear MEC cell types and normal gingival tissue. 1: IHC of hypoxia-inducible factor (HIF), 1a, 1b, 1c: strong positive reaction in intermediate and clear tumor cells, 1d: weak in normal gingival tissue. 2: IHC of vascular endothelial growth factor (VEGF), 2a, 2b, 2c: positive in intermediate and clear tumor cells, showing localization to vascular mesenchymal tissue (arrows), 2d: rare in normal gingival tissue. 3: IHC of tumor necrosis factor (TNF)-$\alpha$, 3a, 3b, 3c: positive in intermediate tumor cells but increased in clear tumor cells, 3d: weak in normal gingival tissue. 4: IHC of signal transducer and activator of transcription (STAT)3, 4a, 4b, 4c: slightly positive in intermediate and clear cells, 4d: rare in normal gingival tissue. 5: IHC of PIM1, 5a, 5b, 5c: strongly positive in intermediate and clear cells, 5d: weak in normal gingival tissue. 6: IHC of BCL-2, 6a, 6b, 6c: strongly positive in intermediate and clear tumor cells, 6d: rare in normal gingival tissue. cy, cyst space.

mucin (MUC)1 and MUC4 all along their cell surface and MUC2 and MUC5AC in their cytoplasm, but the content of vacuolated cytoplasm in clear tumor cells was negative for the PAS reaction and for mucin markers.[12] We found that clear changes occurred transiently from the zone of the intermediate tumor cells to the zone of the clear tumor cells, and the clear tumor cells were conspicuously positive for mucicarmine staining despite a sparse PAS reaction.

Patients with an intraoral MEC have a reduced survival expectation if they are male, with regional metastasis, with a high grade malignancy, strong expression of PCNA, p53, and EGFR, or weak expression of c-erbB2.[7,13-15] The MEC we studied occurred in a female and showed a low grade of malignancy, tumor metastasis into cervical lymph nodes, and weak expression of PCNA, p53, Rb-1, MPM-2, EGFR, and c-erbB2. Therefore, we thought that the oncogenic signals of our MEC would be different from other MECs reported previously. In our IHC array, CDK4 was strongly positive in MEC tumor cells, while other proliferation-related proteins, i.e., PCNA, Rb-1, MPM2, c-Myc, and MAX, were weakly positive. The strong reaction for CDK4 coincided with a positive reaction for p21 (WAF1), a cyclin dependent kinase inhibitor. It was reported that overexpression of CDK4 has oncogenic activity in CDK4 transgenic mice,[16] and that alteration of the cyclin-dependant kinase inhibitor p21 (WAF1) can cause uncontrolled proliferation leading to cancer.[17] In contrast, our tumor cells intensely expressed both CDK4 and p21, and did not undergo further cell cycle progression. Therefore, we thought the strong reaction for CDK4 was not relevant to the causative oncogenic signal of this tumor due to the increased inhibition by p21 (WAF) through a p53-independent pathway.

The immunoreactivity of $\beta$-catenin showed a significant correlation with histologic differentiation and MEC tumor staging, because $\beta$-catenin might play a crucial role in the Wnt-signaling pathway.[18,19] It is also known that the neoplastic histologic phenotype of E-cadherin is lost in more undifferentiated and invasive epithelial salivary gland tumors.[20,21] In the present study,

immunostaining of $\beta$-catenin showed a slight positive reaction in the cytoplasm of intermediate and clear tumor cells, and E-cadherin, which is an extracellular counter part of $\beta$-catenin, was strongly positive in intermediate and clear tumor cells. These findings suggest that the epithelial tumor cells were well stabilized in epithelial sheets and strands. On the other hand, Wnt-1 and APC were strongly positive in intermediate tumor cells, with the reaction being slightly decreased in clear tumor cells. Therefore, we presumed that the Wnt-1/APC pathway plays a role in the tumorigenesis of this MEC.

With higher mean CEA concentrations in the saliva of patients with MEC compared to healthy controls the CEA immunoreactivity was usually detected in the cytoplasm of epithelial cells and luminal contents of neoplastic glands.[6,22] Although it is known that higher expression of CEA is frequently found in high grade MECs,[23] the present MEC showed a weak reaction of CEA both in the clear and intermediate tumor cells. And immunoreactivity for p53, Rb-1, and survivin were consistently weak in tumor cells. Therefore, we thought the present MEC was a relatively low grade malignancy according to its oncogene expression profile.

The protein DMBT1, also known as glycoprotein-340 (gp340) or salivary agglutinin (SAG), plays a role in local innate immune defense.[24] DMBT1 belongs to the scavenger receptor cysteine-rich group B protein family and was described first as a putative tumor suppressor gene in medulloblastoma and glioblastoma multiforme.[25] Then, glycoprotein-340 (DMBT1/gp340) and salivary agglutinin (DMBT1/SAG) were detected as alternative splicing forms of DMBT1.[26] In the present study, DMBT1 was markedly positive in intermediate tumor cells, and its positive reaction was decreased in clear tumor cells. However, the positive reaction was usually localized to ductal and microcystic areas. Therefore, it was presumed that the marked positive reaction of DMBT1was relevant to the rare inflammatory reaction and to a role of the tumor suppressor in the MEC tumor.

Despite the rare inflammatory reaction in the parenchymal tissue of the MEC tumor, the tumor cells were strongly positive for TNF$\alpha$, and continuously positive for the proteins of the STAT3/PIM1/BCL-2 pathway, which does not belong to p53 mediated signaling.[27] In particular, BCL-2 was strongly positive both in intermediate and clear tumor cells, and might play an important role in the antiapoptotic survival of tumor cells. On the other hand, the tumor cells of the MEC were markedly positive for pAKT, which is known to be involved in cell survival reactions to various metabolic stresses.[28] The strong coexpression of TNF-$\alpha$, BCL-2, and pAKT may directly mediate the antiapoptotic survival of the tumor cells in the MEC, and may affect tumorigenesis and transformation from intermediate tumor cells into clear tumor cells. Therefore, we presumed that the present MEC was relatively resistant to the apoptotic degradation of tumor cells and was rather able to be transformed into clear tumor cells that persistently survived. However, during histological observations the clear tumor cells occasionally revealed atrophic nuclei and rupture of cytoplasmic vacuoles, followed by cellular degradation. Although these features of the clear tumor cells resemble retrogressive degeneration, we found that clear tumor cells still showed strong immunoreactivity for eIF5A, CDK4, BCL-2, TNF-$\alpha$, and pAKT, and decreased immunoreactivity for VEGF, angiogenin, and CD31 compared to intermediate tumor cells. In the IHC array for MEC the differential expression of oncoproteins, proliferation-related proteins, apoptosis-related proteins, angiogenesis-related proteins, etc., were usually found in both intermediate and clear tumor cells, but their expression levels were gradually decreased in the clear tumor cells. Therefore, we assumed that the clear tumor cells were of the same linage as the intermediate tumor cells, and that the clear tumor cells were still active and long-lived, but that they would be gradually aggregated and, away from vascular support, inevitably undergo degenerative processes that produce huge vacuolizations in their cytoplasms.

The increased angiogenesis in tumor tissue usually resulted in a poor prognosis, with regional metastasis of tumor cells. The present MEC showed an abundance of capillaries distributed near the epithelial strands of intermediate tumor cells with positive reactions for angiogenesis-related proteins, i.e., HIF, VEGF, and MMP-2. HIF was strongly positive both in intermediate and clear tumor cells, and VEGF was markedly positive in intermediate tumor cells but slightly positive in clear tumor cells. Therefore, we thought that the intense expression of HIF may be related to the increased production of VEGF, followed by the production of MMP-2 for *de novo* angiogenesis.[29] Moreover, maspin, a serine protease inhibitor suggested to have inhibitory effects on tumor-induced angiogenesis, tumor cell motility, invasion and metastasis,[30] was sparsely positive in tumor cells. Therefore, we assumed that the tumor cells of the present MEC can undergo regional metastasis through the abundant vascular channels in the parenchymal tumor tissue, even though their proliferative activity as assessed by immunostaining for PCNA, Rb-1, MPM-2, and c-Myc was relatively low. Actually the individual tumor cells of the present MEC were benign looking in cytology, with a low mitotic ratio and no hyperchromatic nuclei, but some tumor cells grew infiltratively into lymphatics and peripheral stromal connective tissue. The IHC array was helpful for comparing expres-

sion of antiapoptotic and survival proteins, angiogenesis-related proteins, proliferation-related proteins, and oncogenic proteins. These findings might explain the aggressive behaviors of the present MEC such as infiltrative growth and regional metastasis.

IHC array analysis of the present clear cell type MEC revealed strong oncogenic signals via Wnt-1/APC, TNF-$\alpha$/STAT3/BCL-2, and pAKT signaling pathways, and increased tumor angiogenesis, which is different from findings for other MECs reported previously. Expression of these oncogenes is highly relevant to the prolonged survival of clear tumor cells and to the continuous proliferation of intermediate tumor cells in the present clear MEC cell type.

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