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Failure of immune response and genetic changes as a cause of recurrent squamous cell carcinoma of oral cavity

구강 편평상피암의 재발 원인으로서 종양 면역 반응의 실패와 유전자 변이 연구

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Failure of immune response and genetic changes as a cause of recurrent squamous cell carcinoma of oral cavity

by

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ABSTRACT

Introduction: Recurrence and spread of cancer is the main cause of cancer-related death. But recent staging system mainly focuses on anatomic considerations, so it has limitations in reflecting host immune responses against transformed cells or different potential of individual tumors to recur or to spread. Considering carcinogenesis begins with genetic changes, finding genetic alterations of host genome can be more basic and physical way of research. Genes promoting the progression of tumor were reported in some previous studies but the importance of interactions between tumor cells and host immune system was usually neglected. Considering that oral squamous cell carcinoma arise from the epithelium of aerodigestive tract mucosa where intensive mucosal immune system functions, the role of host immune response against transformed cells gains more importance.

Methods: Patients who were diagnosed with squamous cell carcinoma of oral cavity and underwent successful surgical resection

were included. Gene expression profiles were compared between recurrent and non-recurrent group of the patients in both tumor and local tumor-free lymph node samples by cDNA microarray (Selection criteria: p<0.01 or 0.05, fold difference ≥2.0 or ≤0.5). Immunohistochemical analysis of tissue samples was carried out for phenotypic analysis.

Results: Widespread immune dysfunctions in local lymph node samples of recurrent group were most distinctive features. Genes associated with antigen presentation (HLA-DPA1, CD1E), antigen recognition of T cell (CD3D, CD2, CD5), co-stimulators (CD28, B7.1) and chemotaxis of immune effectors (CCR4) were down-regulated. In tumor samples, FAS was over-expressed, which induces apoptosis of immune effectors. Genes promoting tumor progression were also identified. COX-2(=PTGS2), a well-known gene that promotes angiogenesis and cell proliferation and MMP25, a metalloproteinase known to have relation with metastasis, were over-expressed. Immunohistochemical analysis revealed that recurrent group had relatively high immuno-reactivity for tumor-promoting genes and non-recurrent group showed more obviously

high immuno-reactivity for genes related with tumor immunity in local lymph node samples.

Conclusions: Our study revealed that unsuccessful clearance of migrating tumor cells in local lymph nodes could be the main cause of tumor progression and also confirmed that almost whole processes of antigen presentation, antigen recognition, subsequent immune reaction were affected. Aggressive genetic natures of tumor itself were also identified in the process of tumor recurrence and progression although it was not that obvious than their role in the initial malignant transformation suggested by previous studies.

Keywords: Oral cavity, squamous cell carcinoma, Genetic change, Gene expression, Immune reaction

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CONTENTS

LIST OF TABLES AND FIGURES

Table

Figure

INTRODUCTION

Oral and oropharyngeal squamous cell carcinoma is a significant worldwide health problem. It is eighth most frequently diagnosed malignancy in the United States (1) and one of the most prevalent cancers in some Asian countries (2). However the overall prognosis of patients has not been improved despite recent therapeutic advances (1, 3). In recent staging system, disease is classified according to anatomic extent, tumor size, lymph node involvement and distant seeding. This staging system mainly focuses on anatomic aspects without any sufficient consideration of basic physiology during carcinogenesis and metastasis. But in the previous leading studies, it is suggested that a primary tumor is consisted of various subpopulations that have different metastatic potential (4, 5). At this point, recent staging system has limitations in reflecting different potential of individual tumors to grow and to metastasize. As a result, we can see many cases that take different clinical courses despite they were in the same stage and underwent successful standard treatment. To overcome this kind of problems we are needed to change our way of

approach to pay more attention to basic changes during tumor progression. Considering carcinogenesis begins with genetic changes, finding genetic alterations of host genome can be more basic and physical way of research. Nowadays, with the improvement of genetic research techniques, we can directly identify the genetic changes associated with tumor progression and information is growing accumulated (6, 7).

Most oral and oropharyngeal cancers arise from the epithelium of aerodigestive tract mucosa where intensive mucosal immune system functions. For this reason, interactions between newly emerged transformed cells and host immunity plays very important role during carcinogenesis in this region. Recently successful treatment options like monoclonal antibody against EGF receptor (cetuximab) were approved for immunotherapy of head and neck cancer (8). Reported strategies for tumor cells to escape host immune response includes altered antigen presentation or down regulation of major histocompatobility complex(MHC) (9), promoting apoptosis of T cells by Fas ligand expression (10), secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) which inhibits the activation of effector cells (11)

and defective function of T cells (12).

In this study, we compared genetic expression profiles between recurrent group and non-recurrent group of oral squamous cell carcinoma in both tumor specimen and adjacent tumor-free lymph node specimen and confirmed the phenotypic concordance by immunohistochemical evaluation on tissue samples. By this way, we could identify not only genetic changes associated with progression of tumor but also interactions between tumor and host immunity.

MATERIALS AND METHODS

1. Patients' information and tissue collection

A total of eight patients who were diagnosed with squamous cell carcinoma of oral cavity were included with approval of the institutional review board at Seoul National University Bundang Hospital (No. B-1205/153-304). All patients were received successful surgical resection. Histopathologic confirmation obtained from formalin-fixed, paraffin-embedded (FFPE) sections of surgical specimen by pathologists who were not involved in the study. All tumor margins were carefully reviewed and concluded as free of tumor. Disease was recurred in four patients. Tissues were obtained from tumor and adjacent tumor-free lymph node simultaneously in every patient. All lymph nodes were also confirmed as tumor cell-free by a pathologist who was not involved in the study.

2. RNA isolation

Total RNA was extracted from the formalin-fixed, paraffin-embedded (FFPE) samples using an RNeasy Mini Kit with DNase treatment (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA integrity and purity were checked by agarose gel electrophoresis and Agilent Bioanalyzer 2100™. Samples of acceptable quality fulfilled the following criteria: OD260/280 > 1.8, OD260/230 > 1.8 and RIN > 7.

3. Probe preparation and hybridization

Amplification and labeling of each total RNA sample (300ng) were carried out using Transplex Whole Transcriptome Amplification kit ™ (Sigma, MO, USA) and Genomic DNA ULS Labeling kit ™ (Agilent technologies, CA, USA). Dye incorporation was controlled by a Nanodrop spectrophotometer. Cy3- or Cy5-labeled cDNAs were resuspended in 50㎕ of hybridization solution (Agilent technologies, CA, USA) and were placed on Agilent SurePrint G3 Human GE 8x60K array ™ (Agilent technologies, CA, USA). Hybridization was performed according to the manufacturer's instructions for 17 hours at 65 ℃. The hybridized slides were washed in 2 X SSC, 0.1 %

SDS for 2 minutes, 1 X SSC for 3 min, and then 0.2 X SSC for 2 minutes at room temperature. The slides were centrifuged at 3000 rpm for 20 seconds and were scanned by Agilent 2505C scanner ™ (Agilent technologies, CA, USA).

4. Data analysis

Gene expression levels were calculated with Feature Extraction v10.7.3.1™ (Agilent technologies, CA, USA). Relative signal intensity of each gene was generated using the robust multi-array average algorithm. The data were processed based on Quantile normalization method using the GeneSpring GX 12.1 ™ software (Agilent technologies, CA, USA). The normalized, and log transformed intensity values were then analyzed using GeneSpring GX 12.1 ™ software (Agilent technologies, CA, USA). To explore the difference in gene expression, we conducted a comparative analysis with the unpaired unequal variance t-test (Welch's t-test) method in GeneSpring GX 12.1 ™ software (Agilent technologies, CA, USA). To correct for multi-testing errors, the false discovery rate (FDR) was employed following a permutation based bootstrap step-down

procedure using GeneSpring GX 12.1 ™ software (Agilent technologies, CA, USA). Genes that were significantly up or down regulated (>2 fold, p-value < 0.05) were selected for hierarchical clustering. Hierarchical clustering tree was created based on Euclidean distance under each experimental condition. DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov) and GeneSpring GX 12.1 ™ software (Agilent technologies, CA, USA) were both used for further functional analysis. The Gene Ontology database (URL: http://www.geneontology.org) was used to assign biological relevance to our data and to identify genes by ordering them in relevant biochemical pathways.

5. Immunohistochemical analysis of tissue samples

From patients' formalin-fixed, paraffin-embedded (FFPE) blocks, 4 ㎛ thick section were transferred to poly-L-lysine-coated glass slides and incubated in a dry oven at 60℃ for 1 hour. These sections were then dewaxed in xylene (three changes), rehydrated in a graded series of ethanol solutions with decreasing concentrations and rinsed in tris-buffered saline (TBS; pH 7.4). The endogenous peroxidase activity was

inactivated with 3% hydrogen peroxide in methanol for 15 minutes at 37 ℃. The slides were then placed in citrate buffer (10% citrate buffer stock in distilled water, pH 6.0) and microwaved for 25 minutes. Non-reactive staining was blocked using 1% horse serum in TBS (pH 7.4) for 3 minutes.

The over-night probing of primary antibodies which were anti-BCL1A1 antibody (Cell signaling technology Inc., Danvers, USA), anti-FAS antibody (Cell signaling technology Inc., Danvers, USA), anti-H1F1A antibody (Abcam Inc., Cambridge, USA), anti-MST4 antibody (Abcam Inc., Cambridge, USA) among tumor-promoting genes and anti-CD40L antibody (Abcam Inc., Cambridge, USA), anti-CD80 antibody (Abcam Inc., Cambridge, USA), anti-STAG2 antibody (Abcam Inc., Cambridge, USA) among genes associated with immune reaction were conducted at 4℃. After probing primary antibodies, the slides were soaked into 0.1% PBST twice for 5 minutes each. The biotinylated secondary probing was done in room temperature (RT) for 30 minutes. The slides were incubated with ABC kit (Vector laboratories, Burlingame, USA) in RT for 30 minutes, After the first incubation, the slides were soaked into 0.1% PBST twice for 5 minutes and were incubated

again with the ABC kit in humid chamber. After two times of the incubation, the slides were soaked into 0.1% PBST twice for 5 minutes again. Then, Diaminobenzidine (Dako, Glostrup, Denmark) was applied on the slide and the staining was closely monitored with following five times of washing for 3 minutes each. After immunohistochemical staining, the slides underwent dehydration and mounting. The dehydration was conducted as the exact reverse process of the rehydration procedure described before. After mounting, the slides were dried up in RT for several hours.

The stained slides were evaluated by two independent clinical pathologists who were blinded to patients' information and clinical data. Semi-quantitative scale was calculated by HSCORE suggested by Allred *et al.* (13). The HSCORE was based on the percentage of positive cells (Pi) and their staining intensity (i). The score was calculated according to the formula: HSCORE=Σ(i x Pi), where i=1,2, or 3, and Pi varies from 0 to 100% finally resulting in scores ranging 0 to 300.

RESULTS

1. Patients' Characteristics

For this study, we analyzed eight oral squamous cell carcinoma patients (four patients in recurrent group and four patients in non-recurrent group). Five tongue cancer and three buccal cancer were included and all patients were classified as stage IVa according to current TNM staging system (14). Average age of the patients was 59.3 years old and male to female ratio were 3 to 5. Clinical characteristics of patients are shown in Table 1.

2. Functional analysis of genes

According to selection criteria ($p<0.05$, fold difference ≥ 2.0 or ≤ 0.5), a total of 1867 genes were identified in local lymph node samples (834 genes were up-regulated and 1033 genes were down-regulated) and a total of 674 genes were identified

in tumor samples (393 genes were up-regulated and 281 genes were down-regulated). We could find several patterns by classifying the selected genes according to the Gene-Ontology (GO) (15). Functional processes such as cell cycle, cell migration, cytokine signaling pathway, signal transduction were up-regulated in tumor sample, which might contribute to tumor cell proliferation, invasion and metastasis. Whereas functional processes such as immune response, transcription, signal transduction were down-regulated in local lymph node samples, which might imply dysfunction of host immune response. Strangely apoptosis were up-regulated in tumor samples, but it might be the effect of FAS ligand expression in tumor samples implying that it might be the result of up-regulated apoptosis of T-cells (16). The result of functional analysis is summarized in Table 2.

3. Down-regulated genes in host lymph node

According to the selection criteria (p<0.01, fold difference < 0.5), 135 genes were identified in lymph node analysis (Table 3, Table 4, Table 5). Because microarray analysis checks whole

human genomes, selected genes usually include almost whole biological processes. So selection of interested genes was necessary. Genes involving general biological processes such as hormonal response, biosynthetic process, metabolic process, sensory perception, organ/cell development and transport, gene expression, signal transduction, cell growth and extracellular matrix/cytoskeleton organization might be thought to have relation with maintenance of proper lymph node function. But down-regulation of immune response drew attention because it contained the largest number of genes and was the principal function of local lymph node especially against tumor cells. So we focused on involved genes and functions of immune function.

Widespread dysfunction of host immune response was identified. When we carefully looked into it, we could find out that most down-regulated processes were related with innate immune response, antigen presentation and T cell function. These processes were known to play essential role in tumor immunity (17). Regarding antigen presentation, HLA-DPA1 which consists the major histocompatibility complex (MHC), CD1E which plays key role in the presentation of hydrophobic non-peptide and glycolipid and many other genes were

included in this group (18) (Table 3). Down-regulation of genes involving T cell receptor complex (TCR) and its subsequent signaling pathway was found. CD3D which produces δ subunit of CD3 molecule was also down-regulated (19). CD3 plays key role in initiating signal transduction of TCR which initiates antigen-specific T cell responses (19-21). Co-stimulators were significantly down regulated. Principal co-stimulators, CD28 of T cell and its paired ligand, CD80 (=B7.1) of antigen presenting cell were significantly down-regulated. Without proper co-stimulation, T cells might fell into anergy even if they successfully recognized tumor specific antigen (22). Other molecules that have various roles with T cell receptor function (CD2 and CD5) were also down-regulated (23, 24). Especially CD2 was reported as having a role in immune regulation through T cell differentiation and its regulatory role was also reported in oral cancer (25). CD40L, a member of the TNF family, was down-regulated. It is now thought that CD40-CD40L interactions play a general role in immune regulation (26). Chemokines such as CCR4 were also significantly down-regulated, which could impede the migration and tissue homing of T cells (27).

When $p<0.05$ was applied, many other genes that works for the communication between antigen presenting cell and T cell and its subsequent pathway were down-regulated. Genes and its biological functions were summarized in Table 6 and Figure 1.

4. Up-regulated genes in tumor

We could find two main genetic changes that might contribute to tumor progression. First, a well-known tumor immune-escape mechanism, FAS was over-expressed in tumor tissue with a significance of $p<0.01$, that might result in powerful suppression of T lymphocytes by promoting apoptosis (10, 16). Second, genes working for tumor progression were identified even though we compared two cancer group patients. (Table 7, Table8) Among them, COX2 (=PTGS2), MMP25 were over-expressed with a significance of $p<0.01$. COX2, originally principal enzyme of prostaglandin metabolic pathway, contributes to tumor progression by promoting angiogenesis and cell proliferation. Its role in the progression of tumor was frequently reported in many cancers (28). For about the

extracellular matrix-degrading-enzyme, MMP25, the important role of metalloproteinase in lymph node metastasis was already confirmed in previous studies (29).

When we expand our search up to $p<0.05$, a number of oncogenes, oncogenic signal transduction cascades were identified. ERBB3(=HER3) of epidermal growth factor family was over-expressed, which was reported to have relations with short survival and relapse in head and neck carcinoma (30). It is a potent inducer of MAPK(Ras/Raf/MEK/ERK) signaling cascade, which plays an important role in cell proliferation and oncogenesis in many cancers (31). We also identified significant over-expression of MAP2K1 (=MEK1) which plays a central role in MAPK (Ras/Raf/MEK/ERK) signaling cascade and GRB2 which produces adaptor molecule connecting epidermal growth factor receptor and its subsequent G-protein, Ras of MAPK (Ras/Raf/MEK/ERK) signaling cascades (32)(Figure2). Another growth factor receptor, IGF2R which produces insulin-like growth factor-2 was significantly over-expressed. The gene was reported to have relation with poor prognosis of head and neck cancer (33). We could also identify significantly over-expressed genes

encoding transcriptional factors that were reported to have oncogenic potential. MYBL1 is reported as a oncogene in various leukemia, lymphoma and other solid cancers like colon and breast cancer (34) and LYL1 is also reported to have oncogenic potential in acute myeloblastic leukemia (35). Genes that were reported to have relation with invasion and metastasis were identified such as CXCL3 (36) and ITGA4 (37). Significant over-expression of TGM2 gene was identified. This gene originally plays a role in extracellular matrix stabilization, cellular differentiation and apoptosis and is reported to have relation with poor prognosis of many malignancies including laryngeal cancer (38).

5. Up-regulated genes in host lymph node

For about genes involving cell proliferation, fibroblast growth factor related gene (FGF1) was up-regulated (p<0.01). Applying p<0.05, two epidermal growth factor related genes, ERBB (=HER2/Neu) and EGFR were included. Some genes contributing to the immune process were found such as genes related to interleukin, interferon or complement system. But

few genes that could directly elicit innate or adaptive immune response were identified. Principal genes were summarized in Table 9.

6. Down-regulated genes in tumor

Genes involving cell migration and adhesion were down-regulated (CCL26, CADM1, $p<0.01$). CCL26 (=Eotaxin3) is a specific agonist to C-C chemokine receptor-3(CCL3) and its main role is reported as attracting and activating immune cells including Th2 T lymphocytes (39). CADM1 is principally known to be a tumor suppressor gene by working for cell adhesion and it is preferentially inactivated in invasive cancer (40). When we expand our search up to $p<0.05$, we could find some down-regulated genes that is related to the regulation of T cell and natural killer cell function (41), activation of complement system. Some growth factor binding proteins that were reported to have anti-tumor effect were down-regulated (42). Principal genes were summarized in Table 10.

7. Cluster analysis

Cluster analysis was performed in tumor samples and local lymph node samples separately. For local lymph node samples, the expression patterns showed rather uniform patterns with clinical parameters (Figure 3A). The patients in non-recurrent group were clustered together, which means members of non-recurrent group had different gene expression profiles distinguished from recurrent group. Genes involved with composition of the major histocompatibility complex (MHC), intracellular signal transduction of T cell receptor and composition of T cell receptor complex were closely clustered together (Figure 3A). Regarding tumor samples, genes involved with MAPK (Ras/Raf/MEK/ERK) signaling cascade were closely clustered (Figure 3B). But genes of tumor samples showed less distinct feature of clustering according to clinical characteristics than local lymph node samples (Figure 3B).

8. Immunohistochemistry in tissue samples

For tumor samples, tissues of aggressive group showed relatively high immune-reactivity than non-recurrent group.

(especially p=0.05 for HIF1A, Mann-Whitney test) In contrary, non-recurrent group showed obviously higher immune-reactivity than recurrent group for lymph node sample analysis. (especially p= 0.01 for CD40 in germinal center of lymph nodes, p=0.05 for medullary cord of lymph nodes) (Figure 4). All of the tumor-promoting genes (BCL2A1, FAS, HIF1A, MST4) were stained in epithelial layer of tumor specimen. For subcellular location, BCL2A1, FAS, MST4 from genes highly expressed on lymph node samples showed immune-reactivity in cytoplasm whereas HIF1A mostly in nucleus which means the tumor cells were suffering from hypoxia inducing the nuclear translocation of HIF1A (43). More evidently higher immune-reactivity of non-recurrent group over recurrent group was identified in germinal center than medullary cord and generally more active immune-reactivity was found in germinal center for tumor suppressing genes (CD40L, CD80, STAG2) in lymph node sample. Most genes showing relatively low expression profiles on lymph node samples were more densely stained in germinal center than medullary cord. For subcellular location, CD40L showed immune-reactivity in cytoplasm and CD80 on cell membrane and STAG2 mainly in nucleus.

Representative patterns of immunohistochemical staining were summarized in Figure 5.

DISCUSSION

Basic purpose of this study was the comparison of gene expression profiles between recurrent group and non-recurrent group of oral squamous cell carcinoma, which means all group already developed pathologically proven squamous cell carcinoma of oral cavity. In this aspect, abundant down-regulated genes related to immune responses in local lymph node samples might contribute to the failure of tumor cell clearance before they metastasize to another portion of the body rather than the formation of primary tumor. The same principle can be applied to up-regulated genes in tumor samples which are known to have function in promoting proliferation and migration of transformed cells might contribute to the recurrence or metastasis of the tumor rather than initial malignant transformation. Although detected differentially expressed genes in tumor samples were not so abundant than previous studies that compared genetic profiles between normal oral mucosa and squamous cell carcinoma of oral cavity (44), identified genes could be important because they were selected genes for the aggressive nature or the recurrence of the tumor.

Few down-regulated genes were identified that might be related with immune reaction in tumor samples of recurrent group (Table 10). That could be also explained by similar mechanism. Both groups were composed of patients who were already diagnosed with oral squamous cell carcinoma, so transformed cells already evaded local immune surveillance in their original sites. As a result, comparison of genes related with immune reaction of tumor samples had little chances to show noticeable differences because they commonly failed to prevent emerging transformed cells from forming clinically apparent carcinomas. Few up-regulated genes were found that are known to have roles in cell proliferation or migration in lymph node samples of recurrent group (Table 9). This result could have chances to be thought as genetic profiles of metastasized tumor cells. But we already strictly confirmed that the lymph nodes were not contaminated by any tumor cells. If we assume that there were missed tumor cells in local lymph nodes, the genetic profiles of contaminated lymph node should be similar to that of tumor samples because previous report already confirmed that genetic expression profiles of primary head and neck tumor were maintained in their corresponding

lymph node metastasis (45). However, we could not confirm any similar pattern of genetic profiles. By these reason, we could conclude that the result was mainly proper genetic expression profiles of cells composing lymph nodes.

Above-mentioned pattern of genetic expression profiles was also confirmed in the results of cluster analysis. We can easily identify that clusters of patients in local lymph node samples clearly discriminated between recurrent group and non-recurrent group (Figure 3A). And the relative levels of gene expression showed apparently different features according to the clusters (Figure 3A). In contrast, the cluster of tumor samples showed discrete patterns regardless of recurrence (Figure 3B).

Previous leading studies reported that the involvement of local lymph node almost always precede distant metastasis in head and neck carcinoma (46). So we focused on the role of local lymph nodes in blocking migration and spread of tumor cells. As a result, we could confirm widespread down regulation of immune function in local lymph node samples of recurrent group. Most tumor cells are not derived from professional antigen-presenting cells and therefore the tumor cell do not express

co-stimulators such as B7-1 or B7-2. So the tumor cell itself usually cannot stimulate T cell responses. So the process is mainly mediated by professional antigen presenting cells (47). The antigen presenting cells pick up the tumor cells or its antigens and process them. Peptides derived from the tumor antigens are displayed on class I or class II MHC molecules. The peptides presented by class I MHC molecules are recognized by CD8+ T cells and differentiated CD8+ cytotoxic T cells can recognize tumor cells and kill them. The peptides presented by class II MHC molecules are recognized by CD4+ T cells and differentiated CD4+ helper T cells can provide the signals needed for differentiation of CD8+ T cells into anti-tumor cytotoxic T cells (17). In this study we confirmed down regulation of genes working for composition of MHC complex, co-stimulators including B7-1, T cell receptor complex and its signaling pathway, subsequent adaptive immune response (Table 3, Table 6). Another important component in tumor immunity, innate immune system also had problems. Genes for innate immune response, NK T cell function were down-regulated (Table 3). These results support the concept that the local lymph node forms first-line defence mechanism against

transformed cells that already evaded local immune surveillance of its originated oral mucosa.

We could also find mechanisms to evade host immunity in tumor samples. There was significant over-expression of Fas ligand in the tumor samples of aggressive group, which might cause powerful immune suppression by inducing apoptosis of T cells. Previous studies reported the expression of FAS ligand on tumor cell surface as a mechanism of active immune modulation caused by tumor itself (48).

The malignant nature of tumor itself also has important roles in its progression. Clearly it plays key role in forming clinically visible tumor in its origin and it could also be one of the mechanisms of immune evasion when the rapid growth of tumors overwhelm the capacity of immune clearance (17). In this study we could identify lots of genes that were known to work for proliferation of tumor such as COX2, ERBB3, IGF2R, and genes composing the MAPK signaling cascade. Genes related to migration of cells could contribute to invasion and metastasis of tumor cell were identified such as MMP25, CXCL3. They were reported as having relation to the poor prognosis of cancer (29, 49).

One of the interesting aspects of this study was that we could easily find functionally paired down-regulation of genes, which gave chances for synergic effect in the functional impairment. For example, we can confirm significant down-regulation of various genes involving antigen presentation of antigen presenting cells and antigen recognition of T cells, which could result in powerful suppression of immune reaction to specific antigen. Similar results could be confirmed in co-stimulation of T cells. CD80 (=B7.1) of antigen presenting cell and its complementary molecule, CD28 of T cell were simultaneously down-regulated, which drove T cells into anergy. Considering that antigen recognition through MHC - TCR interaction is almost the only way to recognize the tumor antigen, the dysfunction of co-stimulators could cause significant immune suppression (17). We could also find genes commonly up-regulated in the same signaling cascade. In the tumor samples we could identify commonly up-regulated genes for MAPK signaling cascade. That might accelerate aggressive proliferation of tumor cells.

We extracted RNA from formalin-fixed, paraffin-embedded (FFPE) samples. It was newly developed methods for cDNA

microarray analysis. There have been a few studies conducting cDNA microarrays with FFPE samples. They reported more than 80% concordance level between the genome profiling data from FFPE samples and that from fresh frozen samples (50-52). We can also confirm similar patterns of phenotypic expression by immunohistochemical analysis of tissue specimen. This study can contribute to give confidence to the application of cDNA microarrays with FFPE samples of cancer. If cDNA microarray techniques with FFPE samples become widely used, it could provide enormous opportunity for research by overcoming the limitations of tissue storage.

There have been some reports that searched the genetic changes associated with various cancers, but most of them only checked genetic changes of tumor cells associated with initial malignant transformation or progression to lymph nodes involvement. In contrary, we can hardly find any reports about genetic changes of host immune function, especially there is almost none about genetic changes of host immune function associated with progression of head and neck squamous cell carcinoma. But since Ehrlich first proposed the concept that transformed cells arise continuously in our bodies and the

immune system eliminates these cells before they are manifested clinically (53), more organized reports have been continuously updated. Suggested mechanisms include alterations in signal transduction molecules on immune effector cells, immunological ignorance and tolerance (54). In our study, we could confirm all categories of above-mentioned mechanisms.

Our study provided information about various roles of immune system in tumor progression. We could confirm that failure of immune clearance can be an important factor with aggressive nature of tumor in its progression. We could also confirm active mechanisms of immune modulation initiated by tumor that promoted its progression.

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Table 1. General characteristics of patients and diseases

Group	Age/Sex	Site	T	N	M	Overall	Recurrence	Outcome
Aggressive	76/F	Buccal	4a	0	0	4A	Distant	AWD
recurrent	58/M	Tongue	4a	0	0	4A	Regional	AWD
group	29/F	Tongue	4a	1	0	4A	Regional	AWD
	49/F	Tongue	4a	2	0	4A	Local	DOD
Non	61/M	Tongue	4a	0	0	4A	-	NED
recurrent	66/F	Buccal	4a	2	0	4A	-	NED
group	71/M	Tongue	2	2	0	4A	-	NED
	64/F	Buccal	4a	2	0	4A	-	NED

AWD alive with disease, DOD: died of disease, NED: no evidence of disease

Table 2. Functional classification of genes selected by the selection criteria (fold change >2 or <0.5, p<0.05) according to the Gene-Ontology (GO)

	Lymph Node		Tumor	
	over	down	over	down
apoptosis	11	16	13	4
cell cycle	12	30	19	7
cell adhesion	34	18	8	8
behavior	6	8	5	3
cell proliferation	13	11	2	6
cell growth	3	9	3	2
cell migration	15	10	12	2
cell differentiation	68	66	22	23
inflammatory response	9	6	8	5
immune response	17	25	9	7
cytokine signaling pathway	7	7	3	1
homeostasis	24	21	7	6
lipid metabolism	21	20	11	10
neurogenesis	29	25	12	7
transcription	16	40	13	10
translation	3	4	3	0
transport	78	69	37	29
signal transduction	76	119	44	23
total	834	1033	610	281

Table 3. Genes significantly down-regulated (p<0.01, fold change<0.5) in lymph node sample analysis (1)

Category	Biological process[1)]	Genes (p<0.01)[2)]
Immune response	Innate immune response	CYLD, MALT1, RPS6KA5
	Antigen processing and presentation	CD1E
	Antigen processing and presentation of peptide antigen via MHC class I	PSMD10
	Antigen processing and presentation, exogenous lipid antigen via MHC class Ib	AP3B1
	Antigen processing and presentation of exogenous peptide antigen via MHC class II	HLA-DPA1, KIF3A
	Antigen processing and presentation, exogenous lipid antigen via MHC class II	KIF2A
	T cell costimulation	CD28, CD5, CD80
	T cell receptor signaling pathway	CD3D, PTPRC
	T cell activation	CD2
	Activated T cell proliferation	SATB1
	Positive regulation of immune response to tumor cell	CD40LG
	NK T cell differentiation	TXK
	T cell differentiation	PTPRC
	B cell differentiation	PTPRC
	Chemotaxis	CCR4
	Monocyte chemotaxis	PTPRO
	Interferon gamma production	TXK
	Inflammatory response	IL17D, SEMA4D
	Immune response	BMPR1A, CD96
	Regulation of immune response	CD200

1) classification according to Gene-Ontology(GO)

2) significance level

Table 4. Genes significantly down-regulated (p<0.01, fold change<0.5) in lymph node sample analysis (2)

Category	Biological process[1)]	Genes (p<0.01)[2)]
Gene expression	Transcription, DNA dependent	ZKSCAN1, ZNF529, ZNF585B, SETD1B, ATXN1, SCAI, CHTOP, ZNF559, ZNF254, ZNF425, ZSCAN30
	Regulation of transcription, DNA dependent	ZNF780A, ZNF880, MTERF, ZFP82, ZNF277, KLF12, SCML4
	Positive regulation of DNA replication	CTC1
	DNA repair	XPA
	Response to DNA damage stimulus	ATAD5
	mRNA processing	MAGOHB
	Ribosome biogenesis	GTPBP10
Signal transduction	G protein-coupled receptor signaling pathway	GPR171, GPR18
	Signal transduction	SPOCK2
	Intracellular signal transduction	GPR155
	Activation of MAPK activity	TAB1
	Small GTPase mediated signal transduction	RAB9B
Cell growth	Cell proliferation	MAP7, DUSP22
	Positive regulation of cell proliferation	CNOT6L
	Multicellular organism growth	APBA2
	cell differentiation	CATSPER2, RBM11, KDM3A
	Mitotic cell cycle	STAG2, CENPK
	Chromosome segregation	DSN1
	Cell cycle	POC5
	Apoptotic process	CASP8, UNC5C
	Induction of apoptosis	ACVR1B

1) classification according to Gene-Ontology(GO)

2) significance level

Table 5. Genes significantly down-regulated (p<0.01, fold change<0.5) in lymph node sample analysis (3)

Category	GO term[1)]	Genes (p<0.01)[2)]
Hormonal response	Cellular response to gonadotropin-releasing hormone	GNRHR
	Regulation of glucagon secretion	PASK
Biosynthetic process	Melanin biosynthetic process	PMEL
	ATP biosynthetic process	ATP2B1
Metabolic process	Ubiquitin-dependent protein catabolic process	NUB1, USP45, USP25
	GMP metabolic process	PDE6B
	Cellular protein metabolic process	MGAT5, PMPCB
Sensory perception	Detection of chemical stimulus involved in sensory perception of bitter taste	TAS2R43, TAS2R4
	Visual perception	EYS
	Sensory perception of pain	FAM134B
Organ/Cell development	Epithelial cell differentiation	ANKRD62
	Cilium morphogenesis	CC2D2A
	Epithelial cell differentiation	ANKRD62
	Epidermis development	BTD
Transport	Long chain fatty acid transport	SLC27A6
	Potassium ion transport	KCNA3
	Protein transport	SYS1
	Protein import into nucleus, translocation	ARNTL
	Cell fate commitment	SOX12
ECM/ Cytoskeleton organization	Tight junction assembly	MPP7
	Extracellular matrix assembly	GPM6B
	Actin cytoskeleton organization	MON2
Others	protein folding	DNAJC30
	Cell fate commitment	SOX12
	Muscle contraction	SNTB1

1) classification according to Gene-Ontology(GO)

2) significance level

Table 6. Genes significantly down-regulated (p<0.05, fold change<0.5) in lymph node sample analysis

Biological process/Molecular function[1)]	Genes (p<0.05)[2)]	Genes (p<0.01)[2)]
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	HLA-DQA1, HLA-DQB1, HLA-DPA1, TAP2	HLA-DPA1
Antigen processing and presentation	CD1E	CD1E
T cell costimulation	CD80(B7-1), CD28	CD80(B7-1), CD28
T cell receptor signaling pathway	CD3D, CD247, LCK	CD3D
T cell activation,	CD2	CD2
Positive regulation of immune response to tumor cell	CD40LG	CD40LG
T cell differentiation	IL7R	
Positive/negative T cell selection	THEMIS	
Positive thymic T cell selection	ITPKB	
Chemotaxis	CCR4	CCR4
Lymphocyte migration into lymph node	CCR7	

1) classification according to Gene-Ontology(GO)

2) significance level

Table 7. Genes significantly up- regulated (p<0.01, fold change>2) in tumor sample analysis

Biological process[1)]	Gene(s)
Cation transport	ATP13A3
Apoptotic process	TNFRSF10C
Positive regulation of FGF/PDGF/TGFβ production	PTGS2(=COX2)
Sensory perception of taste	TAS2R42
Regulation of transcription	RBM4B
Carbohydrate metabolic process	ATHL1
Actin skeleton organization	CAPZB
Negative regulation of cell proliferation	MXI1(suppressor)
Spermatogenesis	SPA17, SPAG4
Molecular hydrogen transport	ADHFE1
Neuron=neuron synaptic transmission	TMOD2
Glycoprotein metabolic process	ERP44
Chemotaxis	FPR2
Metalloendopeptidase activity	MMP25
Regulation of GTPase activity	ALS2CR12
Activation induced cell death of T cells	FAS
Mitochondrion degradation by induced vacuole formation	SPATA18
Adenylate cyclase-activating G-protein coupled receptor signaling pathway	GNA13

1) classification according to Gene-Ontology(GO)

Table 8. Genes significantly up-regulated (p<0.05, fold change>2) in tumor sample analysis

Biological process/Molecular function[1)]	Genes (p<0.05)[2)]	Genes (p<0.01)[2)]
Regulation of cell proliferation	ERBB3	
Positive regulation of cell proliferation	IL6R	
Epidermal/fibroblast growth factor receptor signaling pathway	GRB2	
Insulin-like growth factor receptor signaling pathway	IGF2R	
Positive regulation of FGF/PDGF/TGFβ production: cell migration involved in sprouting angiogenesis	PTGS2(=COX2)	PTGS2(=COX2)
Cellular response to hypoxia	HIF1A	
Angiogenesis	HIF1A, IL8	
Activation of MAPK activity	MAP2K1	
Regulation of MAPK cascade	GRB2	
Intracellular protein kinase cascade	MAP4K4	
Protein phosporylation	TAOK1, RPS6KB1, MST4	
Metalloendopeptidase activity	MMP25	MMP25
Chemokine activity	CXCL3	
Cell adhesion, Positive regulation of cell adhesion	ITGA5, TGM2	
Cytoskeleton organization	FGD4	
Activation induced cell death of T cells	FAS	FAS
Apoptotic process	FAS, TNFRSF10C TNFSF10, PRUNE2, DIABLO, BCL2A1	FAS , TNFRSF10C
Immune response	IL8	

1) classification according to Gene-Ontology(GO)

2) significance level

FGF: fibroblast growth factor, PDGF: platelet-derived growth factor, TGFβ: transforming growth factorβ

Table 9. Genes significantly up-regulated (fold change>2) in lymph node analysis

Biological process/Molecular function[1)]	Genes ($p<0.05$)[2)]	Genes ($p<0.01$)[2)]
Positive regulation of cell proliferation	ERBB2(=HER2/Neu), EGFR, FGF1,	FGF1
Transcription, DNA-dependent	TLX2	
Extracellular matrix organization	COL1A1, COL4A1, COL15A1, COL6A3	COL15A1
Positive regulation of cell migration, Extracellular matrix disassembly	MMP14	
Proteolysis	FAP, MME	
Cell-matrix adhesion	ITGB5	
Structural constituent of cytoskeleton		
IgG binding, IgA binding	FCGR1B, FCAR	FCAR
T cell activation	CD276(=B7-H3)	
Type I interferon mediated signaling pathway	IFI27	
Interleukin-22 receptor activity	IL22RA2	IL22RA2
Interleukin-1 receptor antagonist activity	IL36RN	
Interleukin-2 biosynthetic process	IL1RAP	
Leukocyte migration	CD177	
Inflammatory response, bradykinin receptor activity	BDKRB2	BDKRB2
Complement activation, classical pathway	C2	
Apoptotic process	LTBR	LTBR
Growth factor activity	GDF7	
Protein binding	IL17RE	

1) classification according to Gene-Ontology(GO)

2) significance level

ECM: extracellular matrix; Ig: immunoglobulin; TNF: tumor necrosis factor; TGF: transforming growth factor

Table 10. Genes significantly down-regulated (fold change<0.5) in tumor sample analysis

Biological process/Molecular function[1)]	Genes ($p<0.05$)[2)]	Genes ($p<0.01$)[2)]
Positive regulation of natural killer cell mediated cytotoxicity/differentiation/cytokine production	IL21	
Innate immune response	C4BPA	
Leukocyte migration	CD177	
Complement activation, classical pathway	C2, C4BPA	
Cellular defence mechanism	KIR2DS3	
Regulation of transcription, DNA-dependent	MYCL1	
Growth factor binding	FGFBP2	
Regulation of cell growth	IGFBPL1	
Positive regulation of cell migration	CCL26	CCL26
Cell adhesion, homophilic cell adhesion	EDIL3, CADM1	CADM1

1) classification according to Gene-Ontology(GO)

2) significance level

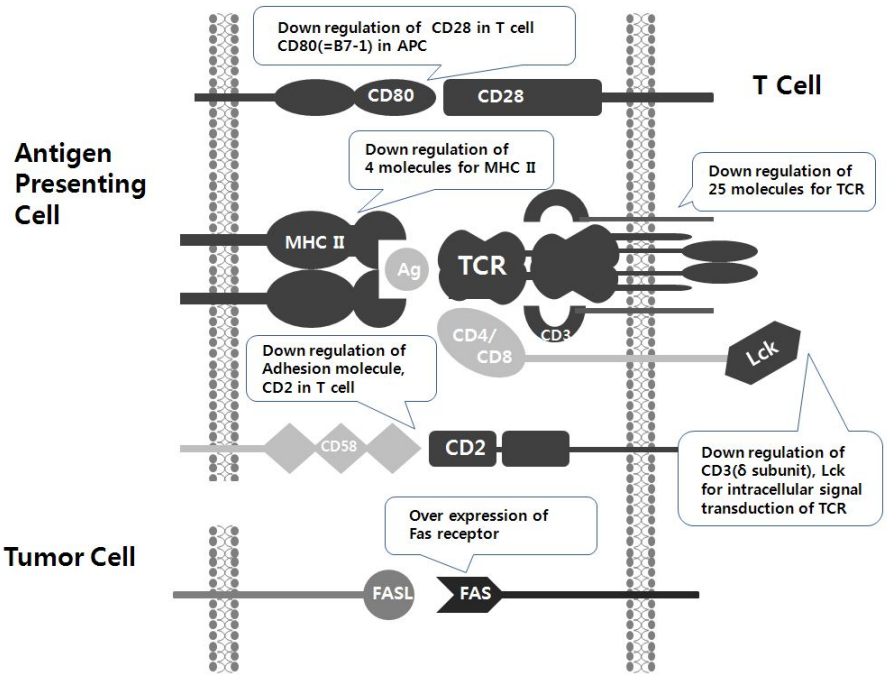


Figure 1. Genetic alteration of host immune reaction (p<0.05, fold change >2 or <0.5)

Almost whole steps including antigen presentation, recognition and subsequent process for adaptive immune response were down-regulated. Co-stimulators (CD80, CD28) were also down-regulated which might drive T cells into anergy. Adhesion molecule (CD2) was down-regulated resulting in unstable contact between immune cells. Over expression of Fas receptor might cause apoptosis of T cells

Ag: Antigen, MHC: Major histocompatibility complex, TCR: T cell receptor

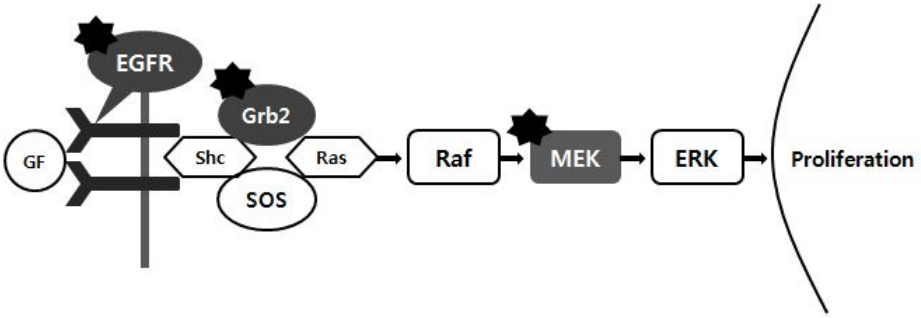


Figure 2. MAPK(Ras/Raf/MEK/ERK) signaling cascade

ERBB3(=HER3) of epidermal growth factor receptor, GRB2, MAP2K1(=MEK1) were up-regulated. (Involved genes were marked with an asterisk.)

GF: growth factor, EGFR: epidermal growth factor receptor

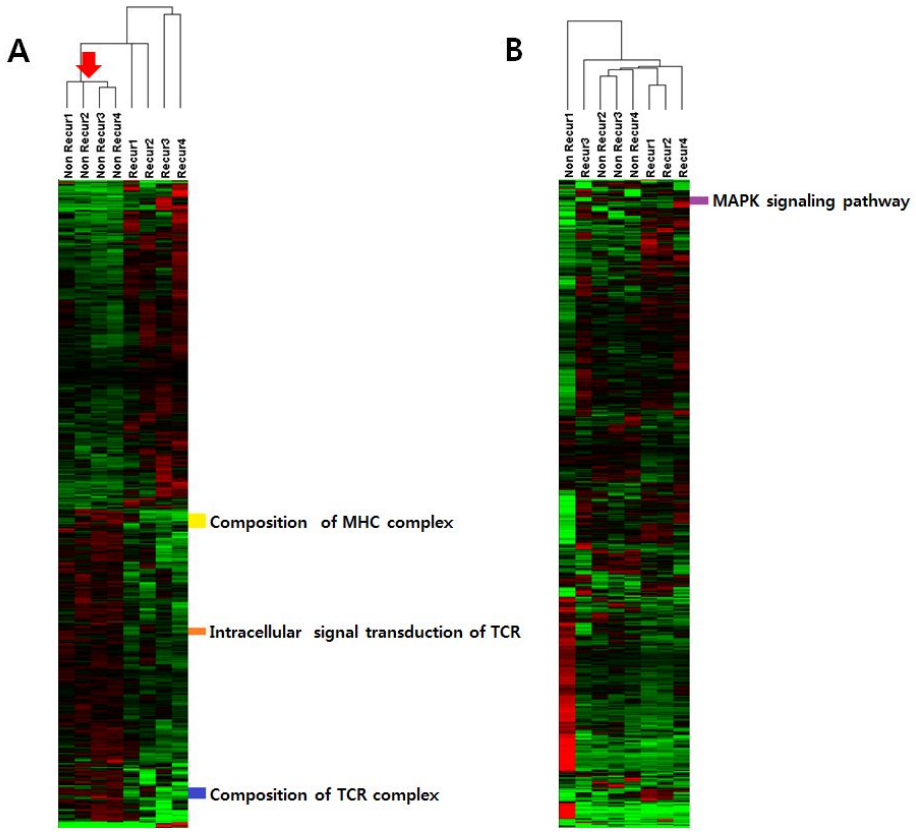
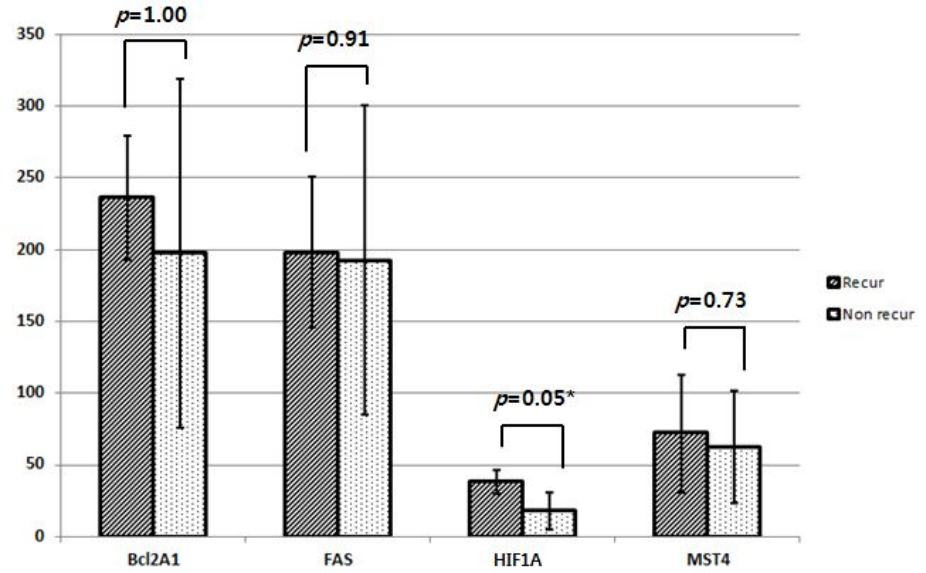


Figure 3. Hierarchical clustering of the gene expression data of local lymph nodes (3A) and tumors (3B)

Each column represents the relative expression levels for all genes in a particular patients and each row represents that of a particular gene across all patients. The patients of non-recurrent group were clustered together in the analysis of local lymph nodes, (red arrow) which means they have similar gene expression patterns showing quite different features from recurrent group. Genes involved with composition of major histocompatibility complex (MHC), intracellular signal

transduction of T cell receptor complex (TCR) and composition of T cell receptor complex (TCR) were closely clustered together. (3A, marked in the right side of the column). For tumor sample, there was no distinct feature of clustering as local lymph node sample except close cluster of genes for MAPK signaling cascade. (Figure3 B, marked in the right side of the column)

A



B

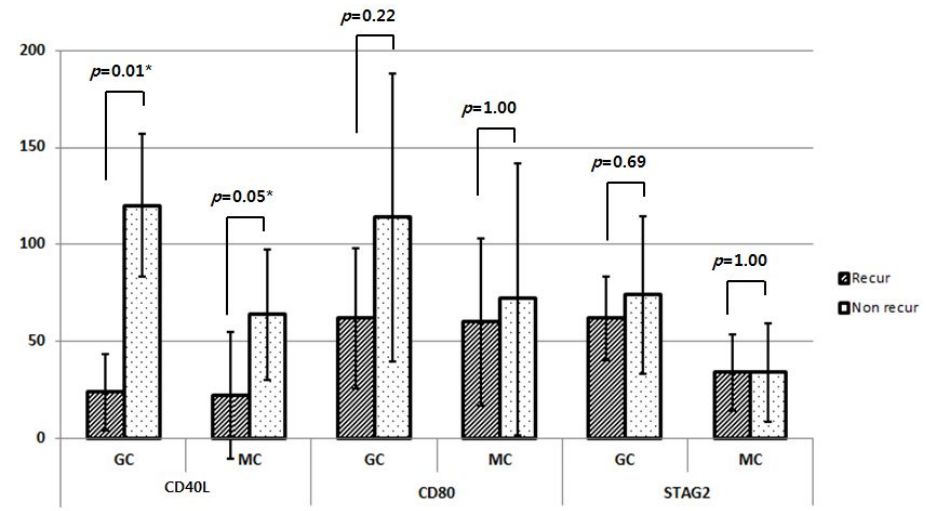


Figure 4. Results of immunohistochemistry

For tumor samples, tissues of aggressive group showed relatively high immune-reactivity than non-recurrent group. (especially p=0.05 for HIF1A, Mann-Whitney test) (A) In

contrast, non-recurrent group showed more obviously high immune-reactivity than recurrent group for lymph node sample analysis. (especially p= 0.01 for CD40 in germinal center of lymph nodes, p=0.05 for medullary cord of lymph nodes) (B) GC: germinal center; MC: medullary cord

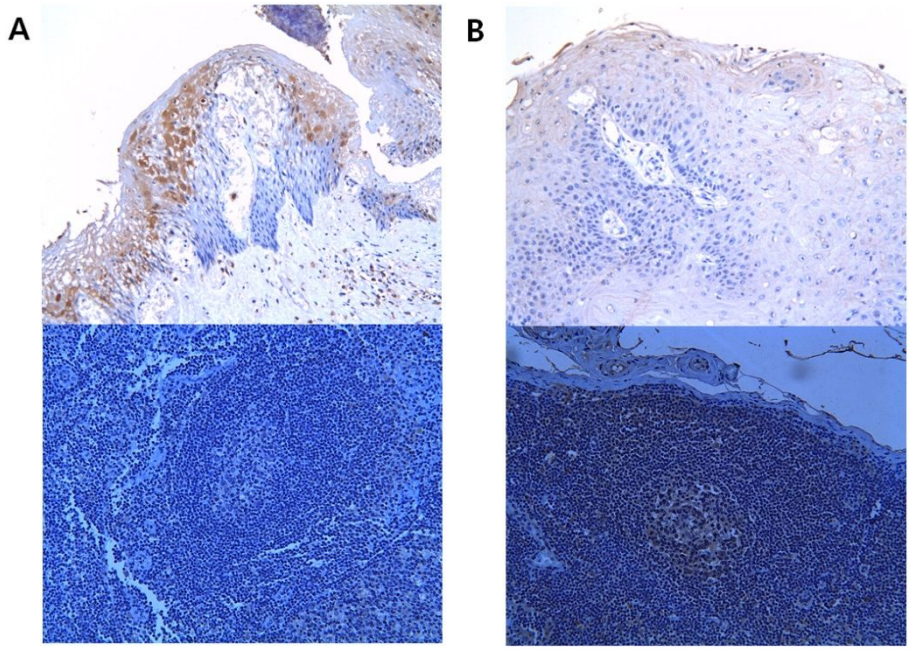


Figure 5. Immunohistochemistry findings

Figure 5A shows a sample of a patient in recurrent group that shows strong immuno-reactivity (HSCORE: 200) for BCL2A1 and low immuno-reactivity for CD40L (HSCORE: 30). In contrast, figure 5B shows a sample of a patient in non-recurrent group with relatively low immuno-reactivity for BCL2A1 (HSCORE: 60) and relatively high immnuno-reactivity for CD40L (HSCORE: 120).

국문 초록

서론: 종양의 재발과 전이는 종양과 연관된 사망의 주요 원인이다. 하지만 현재의 종양에 대한 병기 설정 및 이에 따른 치료법은 주로 종양의 해부학적인 요소에 의해 결정된다. 하지만 이러한 방법은 개별 종양의 서로 다른 재발 및 전이 능력을 평가하거나 종양에 대한 개체의 면역 작용을 평가하는 데에 한계를 지닌다. 종양의 발생이 유전자의 변이로부터 시작됨을 고려한다면 개체의 유전자 변이를 분석하는 일은 보다 더 근본적이고 생리적인 연구 방법이 될 것이다. 특히 본 연구의 대상인 구강암의 경우 강력한 점막면역체계가 작동하고 있는 구강 점막이 원발 부위임을 고려한다면 새롭게 발생한 종양 세포에 대한 면역 작용은 종양 발생에 있어 그 중요성이 매우 높다고 할 수 있다.

방법: 구강에 발생한 4 기 편평상피암 환자 중 성공적으로 병변을 절제 받은 환자를 대상으로 하였다. 이 후 종양이 재발한 환자들을 환자군으로 하고, 재발하지 않고 치료된 환자들을 대조군으로 설정하였다. cDNA microarray 법을 사용하여 상기 환자들의 종양과 국소림프절의 유전자 발현을 각각 분석하고 비교하였다. 실제 분석은 $p<0.01$ 혹은 $p<0.05$ 이하의 유의성을 가지고 각군 간에 2 배 이상의 발현 차이를 보이는 유전자를 선별하여 분석하였고, 이 과정을

통해 종양의 재발과 연관된 유전자를 확인하고 종양에 대한 면역 방어의 문제점을 확인하였다. 또한 실제 조직 표현형에서 같은 결과가 확인되는 지 알아보기 위해 조직에 대한 면역조직화학염색을 통한 분석을 시행하였다.

결과: 종양이 재발한 환자군의 국소림프절에서 광범위한 면역 작용의 실패가 확인 되었다. 주요 유전자로는 항원 제시에 핵심적인 역할을 하는 HLA-DPA1, CD1E 등의 유전자, T 림프구의 항원 인식에 핵심적인 역할을 하는 CD3D, CD2, CD5 등의 유전자, T 림프구의 활성에 핵심적인 공동자극분자인 CD28, B7.1 (=CD80), 림프구의 주화성에 중요한 CCR4 등의 유전자의 유의한 발현 억제가 확인되었다. 종양의 진행을 촉진하는 다양한 유전자도 확인되었는데 종양의 증식과 신생 혈관 생성에서 중요한 역할을 하는 COX-2 (=PTGS-2), 종양의 전이와 연관된 MMP25 등의 유전자의 과발현이 확인되었다. 종양 조직의 면역조직화학염색 결과에서 재발군이 비재발군에 비해 종양 촉진 유전자의 면역형광활성도가 높게 나타났으며, 반대로 림프절 조직에서는 종양 면역에 관계하는 유전자에 대해서 비재발군의 면역형광활성도가 재발군에 비해 확연히 높게 나타났다.

결론: 본 연구는 국소 림프절에서 종양 세포 제거의 실패가 종양 진

행의 주요 원인임을 확인하였고, 국소 림프절에서의 면역 작용의 실패는 항원 제시, 항원 인지, 이어지는 면역 작용의 모든 과정에 걸쳐 발생하였음을 확인하였다. 또한 본 연구에서는 종양의 진행을 촉진하는 여러 유전자도 확인하였으며, 이제까지 주로 연구된 종양의 신생 과정에 관여하는 유전자가 아닌 이미 발생한 종양의 진행과 재발에 관여하는 유전자라는 점에서 그 의미가 있다.

주요어 : 구강암, 유전자, 유전자 발현, 종양 면역

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