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Original Article

Activation of PI3K-AKT Pathway in Oral Epithelial Dysplasia and Early Cancer of Tongue

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Abstract

Increasingly, cancers are being diagnosed at an early stage. Leukoplakia, a precancerous lesion, progresses to cancer in 5–10% of cases. We performed genetic analysis using cDNA microarray and immunohistochemistry in 6 patients, 3 with precancerous lesions and 3 with early tongue cancer, to evaluate the usefulness of these methods in the diagnosis of precancerous lesions and early cancer. Samples of normal epithelium, epithelial dysplasia, and cancer tissues were collected by laser microdissection, RNA was extracted, and the signals converted to numerical values. Immunohistochemical analysis was performed using antibody against phospho AKT (p-AKT), a component of the phosphoinositide 3-kinase (PI3K) signal pathway. Five genes showed a 2 times or greater level of increase in expression in epithelial tissue in comparison with in normal tissue, while 4 genes showed a 2 times or greater increase in early cancer tissues. In cancer tissues and epithelial dysplasia tissues, PI3K class III was expressed at 2.5 times and 11 times the level of that found in normal tissue, respectively. Histochemistry using p-AKT antibody revealed no positive cells in normal tissue. Positive cells were noted in the basal and parabasal cell layers, and partially in the spinous layer of epithelial dysplasia tissues, and in the spinous layer of early cancer tissues. These findings suggest that activation of the PI3K-AKT signal pathway is associated with oral carcinogenesis.

Key words: PI3K-AKT—cDNA microarray—Epithelial dysplasia—Early cancer—Tongue

Introduction

Leukoplakia is an oral precancerous lesion

that progresses to cancer in 5–10% of cases²²⁾, and therefore requires long-term, periodic follow-up. In 2005, WHO examined

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the indices of carcinogenesis and established diagnostic criteria for dysplasia consisting of 13 factors that can be used to determine clinical type and degree of epithelial dysplasia; these are the current criteria for histopathological diagnosis²³⁾. The presence or absence of dysplasia and its degree are also significant for diagnosing malignant potential²⁸⁾. Early cancer has been increasingly diagnosed due to advances in diagnostic techniques, societal recognition of the importance of oral lesions, and the shortening of the period between discovery of a lesion and visiting a physician. Borderline lesions require careful investigation to establish a definitive diagnosis.

With progression of the genome project, genetic techniques have recently been used to diagnose and treat oral cancers. In cellular carcinogenesis, various genes interact with each other, altering in a complex way⁹⁾. If carcinogenesis occurs with the accumulation of certain cancer-related genes, then the genetic analysis of lesions at each stage of progression is necessary. cDNA microarray is useful for elucidation of the expression system⁷⁾.

A cDNA microarray is capable of collectively analyzing gene expression patterns using several hundred to tens of thousands of DNA probes spotted on a slide glass. The usefulness of the cDNA microarray for diagnosing leukemia has been reported²⁾. The cDNA microarray has also been used to diagnose and investigate anticancer drug sensitivity of invasive carcinoma in the head and neck region¹⁹⁾. Although cancer-related genes associated with invasive carcinoma of the tongue have been reported⁴⁾, there have been only a few reports of cancer-related genes that could be used for the diagnosis of oral precancerous lesions and early tongue cancer⁵⁾.

In this study, we used gene analysis to investigate whether phosphoinositide 3-kinase (PI3K), known to be closely involved in carcinogenesis¹⁵⁾, was associated with oral carcinogenesis. We performed immunohistochemical staining analysis using phospho AKT (p-AKT) antibody. We performed gene analysis and immunohistochemical staining to establish which factors could be used as criteria to diagnose precancerous lesions and early cancer.

Materials and Methods

1. Human tissue samples

Primary epithelial dysplasia (Dys, Case 1–3) and early cancer (ECa, Case 4–6) cases were examined in this study. Written informed consent was obtained and ethical clearance (approved no. 102) was provided by the Ethics Committee of Tokyo Dental College. Dys or ECa and their corresponding normal epithelium (NT) were obtained from 6 patients by excisional biopsy (Table 1).

Epithelium that did not show any apparent morphologic abnormality was classified as NT. Epithelium with no apparently abnormal epithelial structure that showed pleomorphism and division of nuclei in the basal layers and hyperplasia of the spinous layers was classified as Dys. Epithelium with relatively atypical cells that did not produce a clearly alveolar structure with many cells showing division of the nucleus and dyskeratosis in tumor areas was classified as ECa.

2. Preparation of frozen human tissue samples

Two-mm-thick sections were embedded in OCT compound, fixed by isopentane preserved in liquid nitrogen, and stored at -80°C . Six- μm -thick sections were cut from the frozen samples and prepared for pathological examination using the Breadloaf step sectioning (BLSS) method. The pathologist was informed of the patients' clinical findings.

3. Laser microdissection method

We used the Application System Laser Microdissection System (AS LMD, Leica Microsystems, Wetzlar, Germany). The specimen was mounted on film slide glass (Meiwa Shoji, Tokyo, Japan) used for laser microdissection and stained with 1% toluidine blue for gene analysis. We procured a few hundred cells of Dys and ECa according to definition. In brief, epithelium with no apparently abnormal epithelial structure that showed

Table 1 Clinical characteristics of leukoplakia and squamous cell carcinoma of tongue

	Age	Sex	Diagnosis
Case 1	72	F	Leukoplakia with mild dysplasia
Case 2	70	M	Leukoplakia with mild dysplasia
Case 3	75	M	Leukoplakia with moderate dysplasia
Case 4	31	M	Squamous Cell Carcinoma questionable early invasive
Case 5	80	M	Squamous Cell Carcinoma questionable early invasive
Case 6	64	M	Squamous Cell Carcinoma questionable early invasive

pleomorphism and division of nuclei in the basal layers and hyperplasia of the spinous layers was classified as Dys. Epithelium with relatively atypical cells that did not produce a clearly alveolar structure with many cells showing division of the nucleus and dyskeratosis in tumor areas was classified as ECa.

4. RNA extraction and amplification

Total RNAs were extracted from each sample of laser-microdissected cells into 350 μ l buffer RLT (Qiagen, Hilden, Germany), and β -mercaptoethanol was added to a concentration of 1%. Total RNAs (0.4–0.6 μ g) were purified using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. All of the total RNA was subjected to T7-based RNA amplification; 2 rounds of amplification yielded 30–50 μ g amplified RNA from each sample. In brief, whole total RNA was reverse-transcribed by using oligo-dT T7 primer containing the T7 RNA polymerase binding site (5'-AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGCT15-3'). Next, second strand cDNA synthesis was performed with RNase H, *Escherichia coli* DNA polymerase I and T4 DNA polymerase (TaKaRa Shuzo, Shiga, Japan). The cDNA was then purified and transcribed with the MEGA script T7 Transcription Kit (Ambion, Austin, TX)²⁰. The purity and concentration of the RNA were determined using the Ultraspec 3300 Pro (Amersham Biosciences, San Francisco, CA, USA).

5. cDNA microarray

Reverse-transcription and fluorescence label-

ing were performed to synthesize fluorochrome-labeled cDNA probes using the CyScribe First-Strand cDNA Labeling Kit with Cy5-dUTP (red) and Cy3-dUTP (green) (Amersham Biosciences). We used a commercially available cDNA microarray, the IntelliGene Human Cancer CHIP Version 4.0 (TaKaRa Biomedical, Tokyo, Japan) on which 868 cancer-related gene fragments had been arrayed.

Labeled probes were mixed with a hybridization solution. After hybridization for 16 hr at 55°C, the slides were washed twice in 2 \times SSC (standard saline citrate; 1 \times SSC = 150 mM NaCl, 15 or 17 mM sodium citrate, pH 7.0), and 0.2% sodium dodecyl sulfate (SDS) for 5 min at 55°C, and then washed in 0.05 \times SSC for 5 min at room temperature. The fluorescence intensity of each spot was measured using the Affymetrix 428 array scanner (Affymetrix, Santa Clara, CA, USA). The data were analyzed by ImaGene software, Version 5.0 (BioDiscovery, Marina del Rey, CA, USA). Gene expression was quantified as the tumor-to-normal fluorescence ratio (T:N ratio). The gene was considered to be overexpressed when the T:N ratio was greater than 2.0 and underexpressed when the ratio was less than 0.5. The criteria for detecting genes were the same as those used in a study by Nakamura *et al.*¹⁸.

6. Immunohistochemical analysis

The labeled streptavidin-biotin (LSAB) method was used. Immediately before immunohistochemical staining, the samples were returned to normal room temperature and fixed with acetone at 4°C for 10 min. They

were then pre-incubated with 0.3% hydrogen peroxide in methanol for 5 min to inactivate endogenous peroxidase. After blocking endogenous peroxidase and nonspecific reactivity, the sections were incubated with the Polyclonal p-AKT (Ser473) Antibody (Cell Signaling Technologies, Beverly, MA, USA) at room temperature for 1 hr. Next, the sections were incubated with biotinylated secondary antibody for 10 min, and with peroxidase-conjugated streptavidin for 10 min (LSAB 2 kit, DAKO, Tokyo, Japan) according to the manufacturer's instructions. Immunolabeling was visualized by incubation in 3,3'-diaminobenzidine (DAB) solution. The sections were finally counterstained with Mayer's hematoxylin. Negative controls using PBS instead of the primary antibody were processed in the same manner. More than 500 epithelial cells were counted in each lesion, and were characterized as early cancer, dysplasia, or normal epithelium. The counts were performed on sections observed in one microscopic field at 200 \times magnification. The percentage of positive cells was calculated, and the mean values were obtained from 20 lesions in each case; these values were used as the labeling index (L.I.)¹⁶⁾ and compared statistically using the Student's *t*-test. A *p*-value of less than 0.01 was considered significant.

Results

1. Gene analysis by cDNA microarray

Compared to NT levels, twice or higher overexpression was noted in 5 genes, and 0.5 times or lower underexpression was noted in 10 genes in Dys. Compared to NT levels, twice or higher overexpression was noted in 4 genes, and 0.5 times or lower underexpression was noted in 9 genes in ECa (Tables 2 and 3).

Among the overexpressed genes, PI3K class III and BCL2-related protein A1 were commonly expressed in Dys and ECa; the signal intensity of BCL-2-related protein A1 was similar in Dys and ECa. The expression level of PI3K class III in ECa was 2.5 times that in NT,

but in Dys, it was 11 times that in NT, suggesting that PI3K is a carcinogenesis-related gene. In Dys, the signal intensities of keratin 19, cadherin 3 type 1, and keratin 13 were twice or higher than NT levels, and in ECa, the signal intensities of keratin 14 and matrix metalloproteinase 3 were twice or higher than NT levels.

Among the underexpressed genes, genes expressed at a 0.5 times or lower level in Dys compared to in NT were also inhibited to 0.5 times or lower compared to NT in ECa; there was no significant difference in the signal intensity among the genes between Dys and ECa.

2. Immunohistochemistry using p-AKT antibody

p-AKT-positive cells were absent in NT, but present in Dys and ECa. In Dys, p-AKT-positive cells were present in the cytoplasm of the basal and parabasal cell layer, and partially present in the spinous layer at a higher expression. In ECa, p-AKT positive cells were present among the basal cell layers and the spinous layer, but the expression rate was lower than in Dys; many of the p-AKT-positive cells showed cytoplasmic staining, and a few cells showed nuclear staining (Fig. 1). The mean \pm SD p-AKT L.I. was 2.3% \pm 1.6% in NT, 78.6% \pm 14.2% in Dys, and 48.9% \pm 10.6% in ECa (Fig. 2). The p-AKT L.I. was significantly higher in Dys and ECa compared to in NT, and in Dys compared to in ECa (*p*<0.01).

Discussion

In the analysis of gene expression profiles in carcinoma cells, the use of laser microdissection is required for the procurement of carcinoma cells and normal epithelial cells¹²⁾. Using laser microdissection and cDNA microarray may contribute to cancer diagnosis and therapy and improvement in the quality of life of cancer patients.

1. Gene analysis using cDNA microarray

PI3K is a lipid kinase that phosphory-

Table 2 Overexpressed genes in epithelial dysplasia (Dys) and early cancer (ECa)
Dys (Case 1–3)

GenBank Accession No.	Gene name	Ave.*	SD**
NM_002647	Phosphoinositide-3-kinase, class 3	11.770	2.909
NM_002276	Keratin 19	3.684	1.410
NM_001793	Cadherin 3, type 1	3.008	1.271
NM_002274	Keratin 13	2.784	1.377
NM_004049	BCL2-related protein A1	2.536	1.280

ECa (Case 4–6)

GenBank Accession No.	Gene name	Ave.*	SD**
NM_000526	Keratin 14	5.909	3.175
NM_002422	Matrix metalloproteinase 3	4.162	1.345
NM_004049	BCL2-related protein A11	2.698	1.770
NM_002647	Phosphoinositide-3-kinase, class 3	2.571	1.660

*Ave.: Average of Dys/ECa: NT fluorescence intensity, ** SD: standard deviation

Table 3 Underexpressed genes in Dys and ECa

Dys (Case 1–3)

GenBank Accession No.	Gene name	Ave.*	SD**
NM_000110	Bone morphogenetic protein 4	0.172	0.120
NM_001202	Dihydropyrimidine dehydrogenase	0.203	0.007
NM_001315	Mitogen-activated protein kinase 14	0.241	0.146
NM_000372	Tyrosinase	0.242	0.099
NM_000584	Interleukin 8	0.249	0.100
NM_001779	CD58 antigen	0.273	0.164
NM_001709	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	0.323	0.150
NM_004448	v-erb-b2 avian erythroblastic leukemia viral oncogene	0.327	0.265
NM_000597	Interleukin-like growth factor binding protein	0.338	0.173
NM_000575	Interleukin 1, alpha	0.351	0.052

ECa (Case 4–6)

GenBank Accession No.	Gene name	Ave.*	SD**
NM_001202	Bone morphogenetic protein 4	0.263	0.050
NM_001779	CD58 antigen	0.269	0.087
NM_000372	Tyrosinase	0.289	0.034
NM_000110	Dihydropyrimidine dehydrogenase	0.298	0.080
NM_004448	v-erb-b2 avian erythroblastic leukemia viral oncogene	0.306	0.034
NM_000597	Interleukin-like growth factor binding protein	0.347	0.049
NM_000575	Interleukin 1, alpha	0.371	0.061
NM_000584	Interleukin 8	0.386	0.045
NM_001315	Mitogen-activated protein kinase 14	0.411	0.020

*Ave.: Average of Dys/ECa: NT fluorescence intensity, ** SD: standard deviation

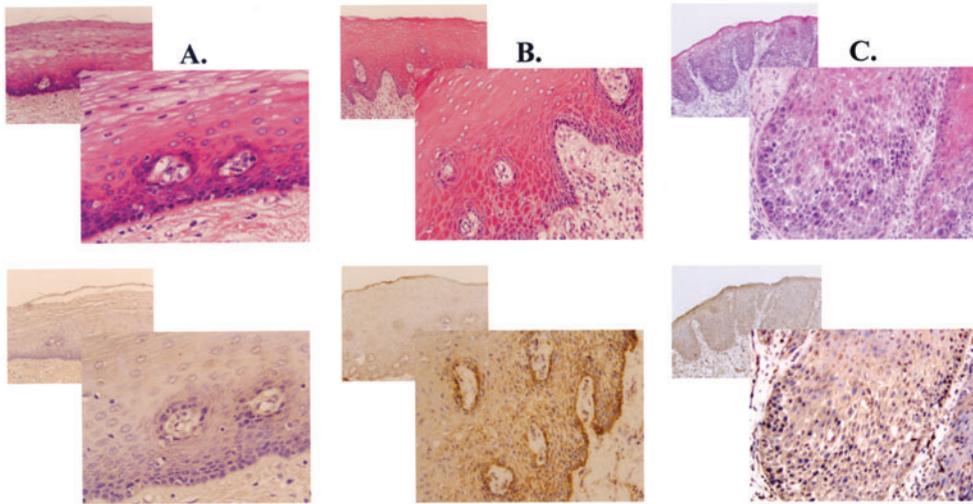


Fig. 1 Immunohistochemical studies of H&E stain and p-AKT antibody expression in each region of human tongue (original magnification $\times 40$, $\times 200$)

A, Normal Tissue (NT). B, Epithelial Dysplasia (mild, Dys). C, Early Cancer (ECa). Few p-AKT-positive cells were observed (A). Many of them were observed among basal layers, parabasal layers and part of spinous layers (B). They were partially observed among basal cell layers and spinous layers (C). They were higher in Dys than in ECa or NT.

lates the inositol ring of phosphatidyl-ID-myo-inositol (PI), structural components of the cell membrane, at the 3-position. Activation of PI3K by growth factor or other stimulation generates PI(3,4)P₂ and PI(3,4,5)P₃. These phosphatidylinositol lipids act as second messengers. PI(3,4,5)P₃ in particular, causes activation of various proteins containing a PH domain. Through activation of the PH domain-containing proteins AKT and PDK1, the AKT upstream kinase, PI3K transmits signals related to cell survival and proliferation¹¹. PI3K is classified into class I, II and III based on its structure.

PI3K Class III positively regulates autophagosome formation by forming a complex with p150, Ser/Thr protein kinase⁸). It also phosphorylates the inositol ring of PI at the 3-position to produce PI(3)P. PI(3)P is known to act as a signal in intracellular vesicle transport. Furthermore, 3-methyladenine, which has long been known to inhibit autophagosome formation, has been shown to inhibit class III PI3K activity, demonstrating that class III PI3K is essential for autophagy²¹). Our study's most interesting finding was related to

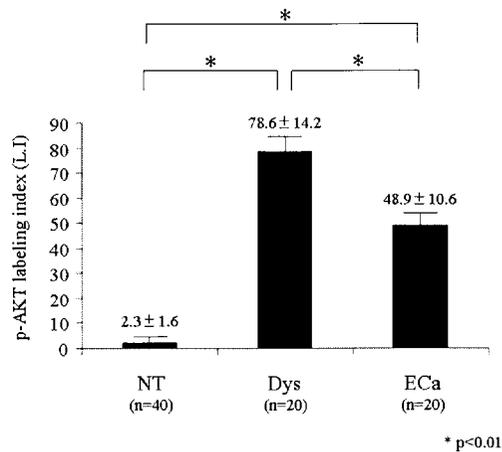


Fig. 2 Averages of p-AKT labeling index in each lesion (Mean \pm SD)

the expression of the PI3K class III gene: the expression ratio was 2.5 and 11 times greater than in normal tissue in ECa and Dys, respectively. It has been reported that activation of the PI3K signal pathway is closely related to the malignancy of human malignant tumors^{10,13,16,17,30}), and it is known that this signal

pathway is activated at a relatively early stage in oral, colon, and lung carcinogenesis^{1,14,25}. An evolutionary conserved mechanism in all eukaryotes, autophagy enables cells to undergo renewal and adapt to nutrient starvation. Autophagy dysfunction is known to be connected with cancer in higher order organisms²⁶. The class I PI3K signaling pathway induces cellular proliferation and suppression of apoptosis and is reported to be closely involved in carcinogenesis³¹ and the PI3K class III gene was associated with carcinogenesis.

The high expression of BCL2-related protein A1 that is commonly detected in Dys and ECa may be related to carcinogenesis because BCL2 has been reported to be expressed at a high level in dysplasia and in squamous cell carcinoma of the head and neck region^{24,26}. The signal intensities of keratin 19, cadherin 3 type 1, and keratin 13 in Dys were about 3 times those in the normal tissue. These genes may be related to carcinogenesis, since, on gene analysis, they have been detected at higher signal intensities in lung cancer tissues than in normal tissues³. Keratin 14 and matrix metalloproteinase 3 genes may also be cancer-related, since on genetic analysis they showed high signal intensities in lung cancer tissues³. The signal intensities of those in ECa were about 4 times those in normal tissue.

Comparing the overexpressed and under-expressed genes of Dys and ECa, most of the detected genes were consistent. Many of the genes that were noted to have increased or inhibited expressions in ECa may have already been expressed at similar levels in Dys. This is consistent with the finding that accumulation of altered gene expression leads to the development of head and neck cancers²⁹. Thus, the genes detected in this study may act in the process of transition from normal tissue to carcinogenesis.

2. Immunohistochemistry using p-AKT antibody

It has recently been reported that various factors involved in the PI3K-AKT pathway activated downstream of growth factors induce cancer cell abnormalities⁵. AKT plays a cen-

tral role in the promotion of cell proliferation and survival, and p-AKT is involved in the development of multiorgan cancers. It has also been reported that overexpression of p-AKT promotes carcinogenesis in oral cancers²⁹. p-AKT-positive cells were absent in NT. In Dys, many p-AKT-positive cells were noted in the basal and parabasal cell layers and occasionally in the spinous layer.

The AKT-mediated cell proliferation and survival signaling pathway has been proposed as an effective target in the development of anticancer drugs. Reasons for this include²⁷:

- a) Mutations or deficiency of the tumor suppressor gene product PTEN are observed in many cancers.
- b) Gene amplification of PI3K and AKT2/AKT3 is reported in many cancers.
- c) This pathway does not play a major role in normal cells.

Moreover, recent studies have reported mutations in the PH domain of AKT1 in breast, colon and ovarian cancer⁶. Due to these mutations, AKT1 is constantly localized at the cell membrane and activated. In this way, AKT is closely involved in malignant transformation.

In immunohistochemical studies, p-AKT positive cells were detected at a high frequency in dysplasia of the lung, head, and neck^{1,29}. On the other hand, in early cancer lesions located in these areas, the expression level decreased compared to in dysplasia, although positive cells were still present^{1,29}. In particular, a tongue immunohistochemical study using p-AKT noted that cytoplasmic p-AKT expression was found in premalignant lesions, and that the majority of lesions showed cytoplasmic staining; however, only a few cells showed nuclear staining in squamous cell carcinoma¹⁵. Even though p-AKT is detected in very few cells of the basal and parabasal cell layers in normal tissue, in dysplasia, p-AKT positivity is present in the nuclei and cytoplasm of many cells in the basal cell, parabasal cell, and spinous layers¹. In ECa, we found p-AKT-positive cells in the basal cell layer and the spinous layer; many of the cells had cytoplasmic staining and a few cells had

positive nuclear staining. In Dys and ECa, the region with the positive cells was almost always consistent with the region of the target cells collected by laser microdissection at the time of mRNA extraction. Similar findings have been reported in studies dealing with digestive organ carcinogenesis, such as gastric cancer¹⁾; this suggests that activation of the PI3K-AKT signal pathway is closely related to oral precancerous regions, and that genetic mutation is generated at a relatively early stage of cancer.

On gene analysis using cDNA microarray, the PI3K class III gene was expressed in dysplasia and early cancer; PI3K class III gene expression was high in dysplasia. Immunohistochemistry using an antibody against p-AKT, a component of the PI3K-AKT signal pathway, revealed positive cells in dysplasia and early cancer; p-AKT expression was high in dysplasia. These findings suggest that activation of the PI3K-AKT signal pathway is involved in oral carcinogenesis. Further study is needed to elucidate the mechanism of oral carcinogenesis in cases of dysplasia and early cancer.

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