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Gene expression analysis by cDNA microarray in oral squamous cell carcinoma

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BACKGROUND: Oral squamous cell carcinoma (OSCC) is common type of human cancer, but little is known about the molecular mechanisms deciding on this malignancy. Comprehensive gene expression profiling is essential for understanding OSCC.

METHODS: cDNA microarray was used to analyze expression patterns of 16 617 genes in nine OSCC patients.

RESULTS: Forty-seven genes with altered expression among all cases were extracted. The ontology of these 47 genes was classified into 10 categories. To validate the microarray data, the expression of genes, including *TGFBI*, *FADD* and *DUSP1* was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). By hierarchical clustering analysis, the nine cases were divided into two clusters.

CONCLUSIONS: The 47 genes are suggested as having a functional significance in oral squamous cell carcinogenesis. It is also suggested that the gene expression patterns by hierarchical clustering analysis can represent degrees of differentiation. The postoperative recovery was uneventful and patients free from tumor after surgery. In the future, on the occasion when the time comes that the number of cases accumulated for microarray increases and each case is observed more over a long-term, these data of 5-year survival rate will be added. Thereby, it will become possible to represent the malignancy of OSCC by these gene expression patterns.

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Introduction

Oral cancer is one of the most common types of human cancer in the world (1). Among them, oral squamous cell carcinoma (OSCC) occurs with the highest frequency. Nevertheless, factors deciding on its clinical characteristics including carcinogenesis, development, progression, invasion, and metastasis have not been elucidated as of yet. This cancer is a highly variable disease with multiple heterogeneous genetic and epigenetic changes associated with various types of biologic and clinical behavior. The alteration of genes has been traditionally revealed by the use of cytogenetics, immunohistochemistry, or molecular approaches based on one or a few genes that changed the expression of many genes such as oncogenes and tumor suppressor genes which have been associated with oral carcinogenesis (2, 3). Comprehensive and exhaustive expression studies of a great number of genes, including functionally unknown genes, are essential to understanding the complexity and polymorphisms of OSCC.

The final deciphering of the complete sequencing of the human genome, together with the improvement of high throughput technologies, is causing a fundamental transformation in cancer research (4). Microarray analysis is a powerful new tool for extensively studying the molecular basis of interactions on a scale previously unattainable with conventional analysis methods (5, 6). This technology makes it possible to examine the expression of tens of thousands of genes simultaneously, and promises to lead to improvements in developing rational approaches to therapeutics, patient-tailored therapeutics, as well as to improvements in more correct diagnosis and prognosis (7–9).

We surveyed the expression of cancer-related genes in OSCC tissues by cDNA microarray analysis to identify the molecular mechanism for this malignancy and to predict aggressive tumor behavior. Forty-seven genes with altered expression among nine OSCC cases were extracted. In hierarchical clustering analysis, the nine

OSCC cases were divided into two groups by their gene expression patterns.

Materials and methods

Patients

The study subjects included 14 patients with histopathologically defined OSCC. All of these patients, except three, were males with an average age of 57.2 years. Their clinical and pathological data including tissue origin are listed in Table 1.

Isolation of total RNA from clinical tissue samples

With the patients' informed consent, OSCC tissue samples were obtained from the surgical specimens of 14 patients undergoing surgery at the Tokyo Medical and Dental University Hospital. Pair-wised control tissue samples were also obtained from oral mucosa surrounding each tumor of the same surgical specimens. All tissue samples were immediately put into RNA Later (Qiagen, Tokyo, Japan) for protection of the RNA. Total RNA was extracted from each sample using RNeasy mini Kit (Qiagen) according to the manufacture's protocol treating with DNase (Qiagen). Its quality and concentration were checked by 2% agarose gel electrophoresis and a spectrophotometer.

Microarray hybridization of cDNA chips

Total RNA from nine pair-wised surgical specimens (case number 1–9) was used for cDNA microarray analysis. T7-based RNA amplification and labeling were carried out using RNA Transcript SureLABEL Core Kit (Takara, Shiga, Japan), Cy3-UTP and Cy5-UTP (Amersham Biosciences Corp, NJ, USA) according to the manufacture's protocol. Labeled probes were mixed in a proportion of one-to-one with formamide and a hybridization buffer (12X SSC, 0.4% SDS, 10X Denhardt's solution, 0.2 mg/ml denatured salmon sperm DNA), and applied to a pair of chips of IntelliGene HS Human Expression CHIP (Takara). These chips include 16 617 human cDNA fragments and 66 housekeeping genes. The complete gene list is listed on the website, http://www.takara-bio.co.jp/.

Table 1 Clinical and pathological data

Case	*Sex*	*Age*	*Primary site*	*TN*	*Differentiation*
1	M	62	Buccal mucosa	T1N0	Well
2	M	56	Lower gingiva	T2N0	Moderately
3	M	55	Upper gingiva	T2N0	Moderately
4	M	50	Tongue	T1N0	Moderately
5	M	59	Tongue	T2N0	Moderately
6	M	50	Tongue	T2N0	Well
7	F	66	Upper gingiva	T2N0	Well
8	M	45	Lower gingiva	T1N0	Well
9	F	50	Lower gingiva	T2N0	Well
10	M	76	Tongue	T2N0	Moderately
11	M	70	Upper gingiva	T2N0	Well
12	F	66	Tongue	T2N0	Well
13	M	66	Lower gingiva	T2N0	Well
14	M	30	Tongue	T2N0	Well

After hybridization for 12 h at 70 °C, these microarray chips were washed in 2X SSC and 0.2% SDS for 10 min at 65 °C three times, washed in 0.05X SSC for 1 min at room temperature, and then scanned using GMS 418 Array Scanner (Genetic MicroSystems Inc., MA, USA). The scanned Cy3 and Cy5 images were imported into array image analysis software ImaGene version 3.04 (BioDiscovery, CA, USA). Cy3 and Cy5 fluorescent signals were normalized by global normalization. The intensities of corresponding spots were subsequently converted into ratios of the intensity for OSCC to the intensity for control oral mucosa. Fluorescence intensities differing by at least a 2.0-fold induction or a 0.5-fold repression between OSCC and control tissues were defined as a potentially different gene expression.

Semi-quantitative RT-PCR

One microgram of total RNA from each of the 14 pair-wise samples was used as the template for reverse transcription using 1st Strand cDNA Synthesis Kit for reverse transcriptase-polymerase chain reaction (RT-PCR) (Roche Diagnostics, Tokyo, Japan). The cDNA mix was subsequently used as a template for *GAPDH* (housekeeping gene) and several gene-specific products. RT-PCR was carried out using TaKaRa Ex Taq (Takara) and specific primers as follows: *GAPDH* sense 5′-TGCCTCCTGCACCACCAACTGC-3′ and *GAPDH* antisense 5′-AATGCCAGCCCCAGCGTCAAAG-3′: *TGFBI* sense 5′-CTGAATTCTGTATTCAAAGA-3′ and *TGFBI* antisense 5′-CTTCAAGCTAATGCTTCATC-3′: *FADD* sense 5′-CTCAGGTCCTGCCAGATGAAC-3′ and *FADD* antisense 5′-GGACGCTTCGGAGGTAGATG-3′: *DUSP1* sense 5′-TGAGGACTAATCGAGTCAAG-3′ and *DUSP1* antisense 5′-GAATGTGCTGAGTTCAGCAA-3′. The amplification cycle for each gene fragment was in the linear range. The PCR products were then fractionated by 2% agarose gel electrophoresis followed by photography. In nine cases, the image data processing software, imageJ, was used to digitize the intensity of each PCR product, and these values were subsequently converted into ratios of the intensity for OSCC tissue to the intensity for control oral mucosa tissue.

Hierarchical clustering analysis

Consistently occurring with at least five of nine cases, fluorescence intensity differing by 2.0-fold or more or by half or less between OSCC and control tissues was chosen for hierarchical clustering analysis. Following the log-transformation of the mean ratio of tumor tissue vs. pair-wise control tissue (T/C) for each gene, we then used the clustering analysis software Expression Profiler (http://ep.ebi.ac.uk/EP/EPCLUST/), a web-based platform for microarray gene expression and other functional genomics-related data analysis, to perform hierarchical clustering analysis. The resulting expression map was visualized with Treeview using the average-linkage clustering algorithms in the same software package. Those genes that were increased in tumor tissues were indicated by a red color, while those that

were decreased in tumor tissues were indicated by a green color. A black color indicated that those genes were not changed between pair-wise OSCC and control oral mucosa.

Results

Differential expression of genes in OSCC

To investigate genes involving oral squamous cell carcinogenesis, we used cDNA microarray hybridization followed by colorimetric detection to analyze the differential expression pattern of 16 617 genes among nine OSCC tissue samples. Forty-seven genes were picked up either with a 2.0-fold induction or with a 0.5-fold repression consistently with the nine OSCC samples relative to their control samples. The ontology of these 47 genes was further classified into 10 categories, denoting adhesion, apoptosis, biosynthesis, cell cycle, growth, metabolism, signal transduction, transcription, others and unknown. Twenty-eight of these were consistently induced at least 2.0-fold (Table 2), while 19 of them were consistently repressed at least 0.5-fold (Table 3) with all OSCC samples.

Validation of microarray data by RT-PCR

To confirm these gene expression data identified by cDNA microarray analysis, the same total RNA used in the microarray were used for semi-quantitative RT-PCR. Two consistently up-regulated genes within the nine cases, *TGFBI* and *FADD*, and one consistently down-regulated gene, *DUSP1*, were chosen for confirmation. Consistently, with the cDNA microarray analysis, the up-regulation of *TGFBI* and *FADD* genes was also observed in most OSCC samples (Fig. 1). *DUSP1* gene was also consistently repressed in most OSCC samples (Fig. 2). Additionally, to confirm the expression pattern of *TGFBI*, *FADD* and *DUSP1*, total RNA isolated from different subset of clinical specimens (case number 10–14) was harvested for RT-PCR. Consistent with case 1–9, the up and down-regulation of *TGFBI*, *FADD* and *DUSP1* was also observed in different OSCC samples (Fig. 3) The results of RT-PCR were in accord with those of cDNA microarray analysis, suggesting that the data obtained by the microarray gene expression analysis were valid and that these genes could potentiality serve as cancer-related genes.

Table 2 Genes showing consistently up-regulated

Category	*Gene*	*Accession number*	*Average fold changes*
Adhesion	*CDH3*	NM_001793.2	5.2
Adhesion	*TGFBI*	NM_000358.1	8.3
Apoptosis	*FADD*	NM_003824.2	4.3
Apoptosis	*LGALS1*	NM_002305.2	3.8
Biosynthesis	*C20orf3*	NM_020531.1	2.8
Cell cycle	*DKC1*	NM_001363.2	2.8
Cell cycle	*SH3BP4*	NM_014521.1	2.9
Growth	*PLAU*	NM_002658.1	8.3
Growth	*TFG*	NM_006070.2	2.1
Metabolism	*BRS3*	NM_001727.1	2.8
Metabolism	*FLJ13855*	NM_023079.2	2.4
Transcription	*EN1*	NM_001426.2	2.8
Transcription	*HSF1*	NM_005526.1	2.6
Transcription	*HMGB1*	NM_002128.2	2.2
Transcription	*MCM4*	XM_030274.6	5.7
Transcription	*RFX5*	NM_000449.2	2.9
Transcription	*TEAD4*	NM_003213.1	2.3
Other	*ARPC1B*	NM_005720.2	3.5
Other	*DSS1*	NM_006304.1	3.4
Other	*IFI30*	NM_006332.3	3.2
Other	*NXN*	NM_022463.2	2.2
Unknown	*CAV1*	NM_001753.3	3.6
Unknown	*FLJ13031*	NM_024688.1	2.3
Unknown	*GPCR41*	NM_024531.1	2.1
Unknown	*LAPTM4B*	NM_018407.2	3.2
Unknown	*LYRIC*	XM_043070.10	2.4
Unknown	*MGC2477*	NM_024099.1	2.2
Unknown	*MGC45714*	NM_152464.1	2.5

Table 3 Genes showing consistently down-regulated

Category	*Gene*	*Accession number*	*Average fold changes*
Apoptosis	*MAL*	NM_002371.2	0.16
Biosynthesis	*IMPDH2*	NM_000884.1	0.37
Biosynthesis	*MRPL16*	NM_017840.2	0.41
Biosynthesis	*RPL15*	NM_002948.2	0.32
Cell cycle	*DUSP1*	NM_004417.2	0.33
Metabolism	*ALDH9A1*	NM_000696.2	0.31
Metabolism	*DPYSL2*	NM_001386.3	0.26
Metabolism	*H11*	NM_014365.1	0.28
Metabolism	*MGLL*	NM_007283.2	0.31
Metabolism	*PAM*	NM_015057.1	0.37
Transcription	*FOS*	NM_005252.2	0.37
Transcription	*NOTCH1*	NM_017617.2	0.34
Transcription	*SF1*	NM_004630.1	0.34
Other	*GPX3*	NM_002084.2	0.22
Other	*IK*	NM_006083.2	0.41
Other	*VAV3*	NM_006113.3	0.27
Unknown	*GLTSCR2*	NM_015710.2	0.37
Unknown	*LLGL2*	NM_004524.1	0.31
Unknown	*TXNIP*	NM_006472.1	0.29

Results of hierarchical clustering analysis

Fluorescence intensity differing by 2.0-fold or more or by half or less between OSCC and control tissues was chosen as a potentially different gene expression in at least five of nine cases. Six thousands-one hundred and seven genes were chosen, and hierarchical clustering analysis was used to find similarities between the nine OSCC cases by grouping the expression patterns of these genes. The nine cases were divided into each cluster by their gene expression patterns. These clusters were discussed as to whether they had relevance to clinical and pathological data, including tumor site, TN classification, degree of differentiation, and lymph node metastasis. It was revealed that the nine cases were able to be divided into two clusters, one cluster included four of five cases of well differentiated types, and the other included three of four cases of moderately differentiated types (Fig. 4).

Discussion

The number of genes selected either with a 2.0-fold induction or with a 0.5-fold repression consistently

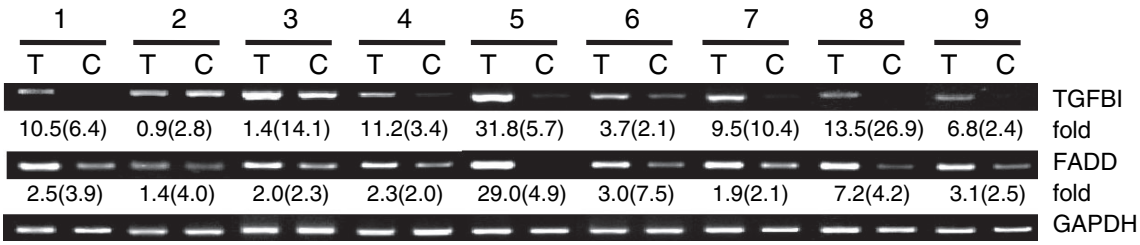

Figure 1 Validation of the expression patterns of *TGFBI* and *FADD* by semi-quantitative RT-PCR. The same total RNA used for cDNA microarray analysis was used. *GAPDH* was used as a loading control. Consistent with the cDNA microarray data, the expression of *TGFBI* and *FADD* genes were up-regulated in most OSCC tissues. Arabic numerals represent fold changes of each PCR product by imageJ, and numerals in parentheses represent fold changes of microarray data by ImaGene. T, tumor tissue; C, control tissue in each sample.

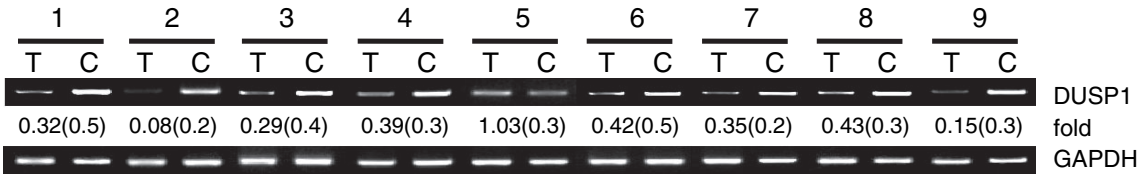

Figure 2 Validation of the expression pattern of *DUSP1* by semi-quantitative RT-PCR. The expression of *DUSP1* gene was down-regulated in most OSCC tissues. Arabic numerals represent fold changes of each PCR product by imageJ, and numerals in parentheses represent fold changes of microarray data by ImaGene. T, tumor tissue; C, control tissue in each sample.

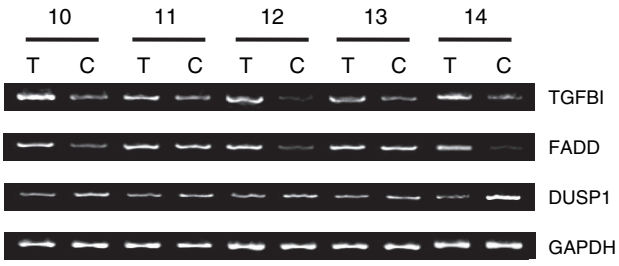

Figure 3 Validation of the expression pattern of *TGFBI*, *FADD* and *DUSP1* by semi-quantitative RT-PCR. Total RNA isolated from case 10–14 was used. The expression of *TGFBI*, *FADD* and *DUSP1* was consistent with case 1–9.

within nine OSCC cases by our cDNA microarray analysis consisted of only 0.3% of the total genes on the microarray chip. Even so, because the expression of these 47 genes was consistently changed with OSCC, they may have relevance to the malignant alteration of oral mucosal epithelial cells. Furthermore, it is strongly suggested that several genes included in these 47 genes are deciding factors of oral squamous cell carcinogenesis. Although 37 of these 47 genes have been identified by their characteristics and functions, the exact nature of the remaining 10 genes was previously unknown. These genes have the potential of playing significant roles in elucidating the molecular mechanisms of the carcinogenesis and malignant behavior. We propose that these genes might provide intriguing insight into OSCC progression.

TGFBI gene was initially identified as a novel gene that was induced by TGF-β in a human adenocarcinoma cell line derived from the lung (10). It encodes for a secreted extracellular matrix (ECM) protein, which is thought to act on ECM composition and the negative regulation of cell adhesion (11). Additionally, *TGFBI* missense mutations were identified in families affected with human autosomal dominant corneal dystrophies (12). Interestingly, *TGFBI* was previously shown to be one of the up-regulated genes in primary colorectal cancers (13), primary pancreatic cancer (14), and esophageal squamous cell carcinoma cell line (15). Such as in these cancers, *TGFBI* was also up-regulated consistently with the nine OSCC cases in our studies. Because *TGFBI* is thought to be involved in cell attachment to the ECM, thereby influencing cell adhesion, it is possible that an increased level of *TGFBI* gene expression in OSCC may influence the invasion and lymph node metastasis of cancer cells.

The Fas-associated death-domain (FADD) protein, which is recruited to the receptor through its carboxy-terminal 'death domain' (DD) and amino-terminal 'death effecter domain' (DED), is a key adapter protein in death receptor-induced apoptosis (16). Although Fas and *FADD* have been predominantly recognized as apoptosis inducers, there is increasing evidence for additional apoptosis-independent functions, including induction to proliferation in T cells and fibroblasts, hepatocyte regeneration, chemokine production, dendritic cell regulation (17), neurite outgrowth (18) and NFκB activation (19). However, the molecular mechanisms of Fas and *FADD* signaling in most of these processes are poorly understood. FADD protein has a domain that regulates cell proliferation independent of its role in receptor-mediated cell death, so it balances cell proliferation and apoptosis (20). Our results show that the overexpression of *FADD* gene was observed in OSCC. The cancer cells may induce apoptosis through overexpression of *FADD* gene for maintenance of homeostasis, although, the apoptosis signal was interrupted by another molecules. However at least six genes,

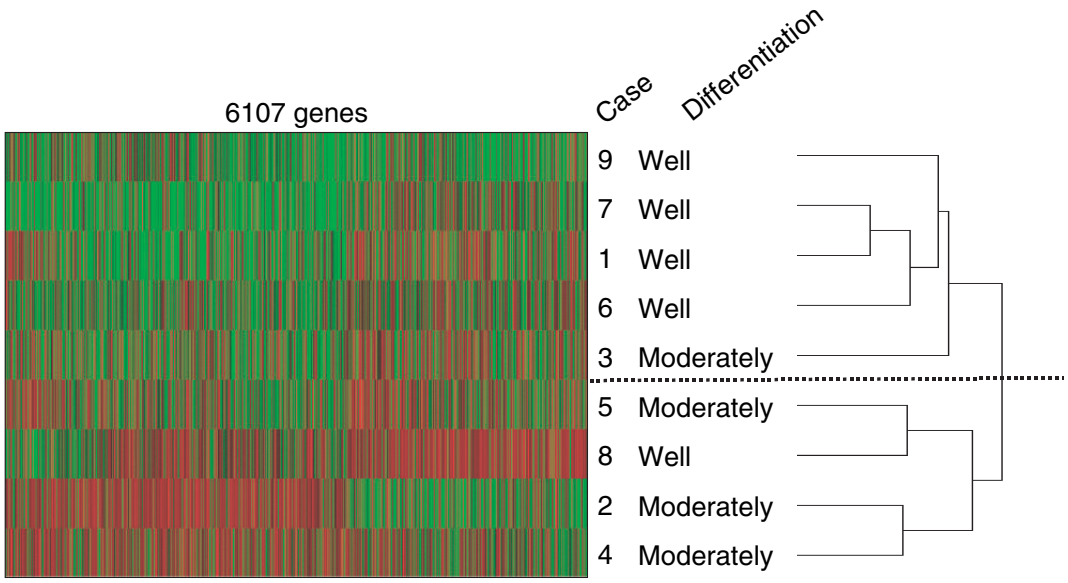

Figure 4 Hierarchical clustering of the nine OSCC cases in the expression data of the chosen 6107 genes. The results were visualized by Treeview. The nine cases were divided into two clusters by the expression patterns of the 6107 genes. One cluster included mostly cases of well differentiated types, and the other included mostly cases of moderately differentiated types. Case numbers and their clinical and pathological data are based on Table 1.

Caspase-1, -3, -7, -8, -9 and Apaf-1 genes, with altered expression were not extracted in this study.

DUSP1/MKP1/CL100/PTPN10, a dual-specificity phosphatase for tyrosine and threonin, specifically inactivates mitogen-activated protein kinase and suppresses its activation by ras (21). *DUSP1* gene is a transcriptional target of tumor suppressor p53, inducing cell cycle arrest or apoptosis (22). Furthermore, the expression of *DUSP1* gene was induced by the introduction of exogenous PTEN, a tumor suppressor, into endometrial cancer cell lines (23). It was reported that the expression of *DUSP1* is decreased in primary ovarian tumors compared with corresponding normal tissues (24). Therefore, in view of our results from the microarray analysis and RT-PCR experiments, which showed that the expression of *DUSP1* gene is decreased in OSCC, we consider this gene to be a candidate for tumor-suppression, mediating PTEN signaling pathways in OSCC.

The nine OSCC cases were divided into two clusters by hierarchical clustering analysis. One cluster included mostly cases of well differentiated types, and the other included mostly cases of moderately differentiated types. Hence, these results suggested that the expression patterns of the 6107 genes selected for this hierarchical clustering analysis could represent a degree of differentiation of OSCC. In general, the poorer cancer cells differentiate, and the higher become malignant. Briefly, it is proposed that the moderately differentiated group may be the higher malignancy group. Therefore, this result shows that it may be possible to represent the malignancy of OSCC and divided it into several groups by gene expression patterns. According to follow-up data, the postoperative recovery was uneventful and all nine patients free from tumor 15–18 months after surgery. In the future, on the occasion when the time comes that the number of cases accumulated for microarray increases and each case is observed over a long-term, these data of 5-year survival rate will be added. Thereby, it will become possible to represent the malignancy of OSCC by these gene expression patterns.

In conclusion, we extracted several candidate genes that had the potential of being linked with oral squamous cell carcinogenesis. Moreover, we suggest that it may be possible to represent not only the degree of differentiation, but also the malignancy of OSCC by these gene expression patterns. Further studies determining the functional significance of unknown genes in OSCC will carry forward cancer research. These candidate genes could be new diagnostic tumor makers. In the future, if appropriate gene diagnostic makers are established together with the ability to predict prognosis by gene expression profiling data, it will be possible to exercise preoperative gene diagnosis of OSCC. Furthermore, among classically indistinguishable tumors, these studies have allowed the classification of new clinically and biologically important subclasses, during the first medical examination may represent different diseases requiring different strategies. We have obtained gene expression profiling data that have the potential to become basic data in the understanding of OSCC.

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