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Glucose metabolism changes during the development and progression of oral tongue squamous cell carcinomas

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Abstract. Previous studies have revealed several genes involved in the carcinogenesis of oral cancer. However, the detailed mechanisms underlying this process are poorly understood. Previously, we established a database cataloging the transcriptional progression profile of oral carcinogenesis and identified several candidate genes with continuously increasing or decreasing expression, which specifically promote the transition of oral premalignant lesions to invasive carcinomas. In this study, using our microarray database, we attempted to determine significant genes that may contribute to metabolic alterations during oral carcinogenesis. After performing a literature survey, we focused on 15 candidate genes associated with glucose metabolism changes, particularly the tri-carboxylic acid cycle, and investigated the mRNA-expression status of these genes with our database. Only the solute carrier family 2 member 1 gene (also known as GLUT1), showed significantly increased mRNA expression during oral tumorigenesis. Immunohistochemical analysis confirmed that GLUT1 protein expression significantly increased during oral carcinogenesis. In addition, tumors with high expression of this protein significantly correlated with nodal status (P=0.002). Kaplan-Meier survival curves clearly demonstrated the adverse impact of high GLUT1 protein expression on disease-free survival (P=0.004). GLUT1 mRNA and protein expression increased in the order of normal mucosal tissues, epithelial dysplastic lesions and invasive carcinomas. Therefore, metabolic alterations, especially in glucose metabolism, occurred at the very early stage of development of oral malignancies. In addition, GLUT1 played a significant role in oral cancer, acquiring a malignant phenotype.

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Introduction

Head and neck cancer is the eighth most common cancer worldwide. Oral cancers account for approximately one-half of head and neck cancers. In 2018, approximately 345,900 new cases of oral cancer were documented and 177,400 deaths from the disease occurred worldwide (1). In Japan, it is estimated that over 9,000 new cases of head and neck cancer occurred in 2015. Among them, over 2,000 new oral cancers were added to the clinical statistics registry, 90% of which were diagnosed histologically as squamous cell carcinoma (Japan Society for Head and Neck Cancer, 2015). Advances in surgical techniques, radiotherapy, and chemotherapy have improved the extent of organ preservation and the overall quality of life and have decreased morbidity. However, a better understanding of the molecular mechanisms underlying the transitions from normal epithelium to pre-malignancy to invasive oral carcinoma is still necessary for improving the long-term survival of affected patients.

To examine the mechanisms underlying this process in detail, we established a transcriptional progression database for oral tumorigenesis in identical oral cancer samples using laser microdissection and expression microarray analysis. Then, we examined genes that were differentially expressed in normal tissues compared with oral dysplastic lesions (ODLs) and in ODLs compared with invasive carcinomas and identified 15 candidate genes with continuously increasing or decreasing expression during oral carcinogenesis (2). Furthermore, several candidate genes that specifically contribute to the transitioning of ODLs to invasive carcinomas were found using this database (3).

Cancer metabolism is clearly distinct from normal cellular metabolism. Many cancer cells exhibit a specific type of irregular metabolism characterized by a high dependence

on glycolysis to meet their higher energy requirements (4). This is called the 'Warburg effect,' which depends on aerobic glycolysis and is characterized by cancer phenotypes such as a high glycolytic rate and elevated lactate production under normoxia (5,6). Because tumor cells are often exposed to hypoxia under physiological conditions, their sustained hypoxic metabolism may be a direct cause of the Warburg effect; however, the underlying mechanism remains incompletely understood. Glucose metabolism changes support the acquisition and maintenance of malignant properties. Because some altered metabolic features are observed in many types of malignant cells, reprogrammed metabolism is a characteristic common to cancer cells and is considered a very significant alteration contributing to the development and maintenance of malignant phenotypes. Regarding oral cancer, such reprogrammed metabolism is also regarded as a crucial factor for oral carcinogenesis and is associated with radiotherapy and chemotherapy resistance, as well as tumor recurrence (7). Evidence suggests that various metabolic changes occurring in cancer cells, glycolysis, mitochondrial oxidative phosphorylation, and glutaminolysis play particularly important roles in tumor metabolism (8). Understanding metabolic changes occurring in cancer cells has uncovered remarkable activities in specific pathways activated by tumor cells that support these key functions. However, it has not been elucidated how and when metabolic changes occur during carcinogenesis in oral cancer. In this study, we aimed to identify significant metabolic alterations during oral carcinogenesis using our transcriptional progression database.

Patients and methods

Patients and tissue samples. All clinical and histopathological data were reviewed from medical records of the Department of Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (Tokyo, Japan). All clinical samples were obtained from patients with oral tongue squamous cell carcinoma (OTSCC) or oral tongue epithelial dysplasia (OTED) who had undergone surgical excision as a primary treatment at our department between 2010 and 2014. The protocols used in this study were reviewed and approved by the Research Ethics Committee of the Faculty of Dentistry of the Tokyo Medical and Dental University (approval no. D2015-534). Written informed consent forms were obtained from all patients in accordance with institutional guidelines. Clinical staging was defined according to the Union for International Cancer Control TNM classification system (https://www.uicc.org/). Tumors were classified histopathologically as being poorly, moderately, or well differentiated, and epithelial dysplastic lesions were classified histopathologically as being low- or high-grade according to World Health Organization criteria (9). Disease-free survival (DFS) was measured from the time of initial examination to the time of local, regional, or distant recurrence of the disease or the time of last follow-up. Overall survival (OS) was measured from the time of initial examination to the time of death or last follow-up.

Profiling analyses using microarray data. Microarray samples of invasive tumor, adjacent dysplastic lesions and noncancerous normal tissue were collected from 11 patients with primary OTSCCs. None of these patients received preoperative treatment. Before Laser microdissection (LMD), an oral pathologist determined the area of invasive tumor, adjacent dysplastic lesion and noncancerous normal tissue on all samples. Cancer tissue for LMD was immediately cut to 3 mm thick sections, while excluding the center of the tumor for pathological diagnosis and embedded in Tissue-Tek OCT compound medium (Sakura, Tokyo, Japan) after resection. The sections were then fixed in liquid nitrogen and stored at -80˚C. Frozen sections, 9 μm thick, were cut from the frozen samples and mounted onto a foil-coated glass slide, membrane slide (Leica Microsystems, Wezlar, Germany). Frozen sections were fixed in 70% ethanol for 30 sec and stained with hematoxylin and eosin before dehydration (5 sec each in 70, 95 and 100% ethanol). After air-drying, the sections were laser microdissected using AS LMD (Leica) (2).

Microarray data of the transcriptional progression profiles obtained during oral carcinogenesis were previously deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE35261 (2). In this study, we aimed to identify genes whose expression increased in the order of normal mucosal tissues, dysplastic lesions, and invasive tumors. First, we selected 15 candidate genes related to the tri-carboxylic acid (TCA) cycle using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.kegg.jp). Fold-changes were calculated using the ratios of geometric means of gene-expression levels between each tissue. We further selected 15 candidate genes related to metabolic control mechanisms that were differentially expressed in normal mucosal tissues and dysplastic lesions compared with invasive tumors using the Wilcoxon signed-rank test, with a significance level of 0.005. Among these, genes with >3-fold upregulation in invasive tumors were selected. To focus on genes involved in carcinogenesis, we selected genes with average expression levels in tumor tissues of at least 100 units (3). Furthermore, comparison of gene expression levels among normal mucosal tissues, dysplastic lesions, and invasive tumors were performed by the Friedman test, followed by the Wilcoxon signed-rank test adjusted by the Bonferroni correction (P=0.010). Analysis of the gene-expression data was performed using R statistical software, version 3.3.2 (http://www.r-project.org).

Immunohistochemical (IHC) analysis. IHC analysis was used to confirm GLUT1 protein expression. Formalin-fixed, paraffin-embedded (FFPE) specimens collected from the Department of Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University were used for IHC analysis. GLUT1 expression was assessed by IHC staining in 65 cases of OTED, 110 cases of OTSCC, and 20 cases of normal tongue mucosal tissue. A histologic normal part of resected specimen of cancer was used as normal tissues.

Paraffin blocks were sectioned at a thickness of 4 μm. Initially, the sections were deparaffinized and rehydrated, and then heated in 10 mM sodium citrate buffer (pH 6) for 15 min at 121˚C in an autoclave for antigen retrieval. Next, sections were immersed in 3% hydrogen peroxide for 20 min at room temperature to inhibit endogenous peroxidases. Subsequently, the sections were incubated at room temperature for 1 h with a primary mouse monoclonal antibody against

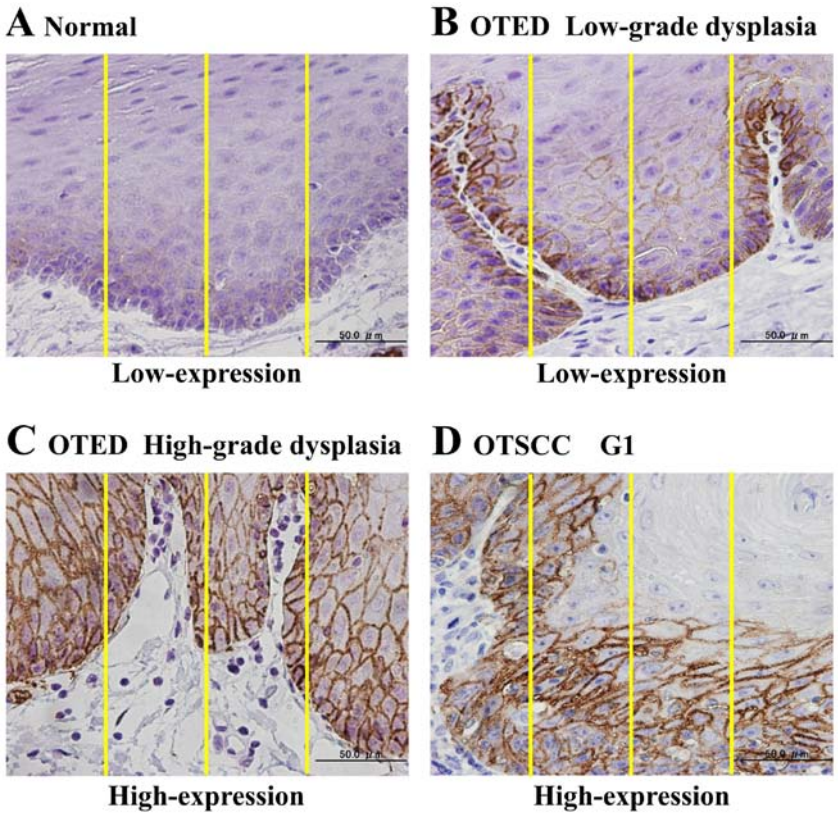

Figure 1. Examples of the immunohistochemical analysis method used to evaluate GLUT1 expression. (A) Normal mucosal tissue. Following evaluation, the expression area was assigned a score of 2, and the staining intensity was assigned a score of 1. The expression intensity was calculated by multiplying the two scores, resulting in an overall score of 2 (low GLUT1 expression). (B) OTED, low-grade dysplasia. The expression area was assigned a score of 3, and the staining intensity was assigned a score of 2. The expression intensity was calculated by multiplying the two scores, resulting an overall score of 6 (low GLUT1 expression). (C) OTED, high-grade dysplasia. The expression area was assigned a score of 4, and the staining intensity was assigned a score of 3. Therefore, the expression intensity was 12, corresponding to high GLUT1 expression. (D) OTSCC G1 (well-differentiated type). The expression area was assigned a value of 4, and the staining intensity was assigned a value of 3. Therefore, the expression intensity was 12, corresponding to high GLUT1 expression. GLUT1, glucose transporter type 1, erythrocyte/brain; OTED, oral tongue epithelial dysplasia; OTSCC, oral tongue squamous cell carcinoma.

GLUT1 (ab40084; Abcam, Cambridge, UK) at a 1:200 dilution and then for 1 h at room temperature with a secondary antibody using the EnVision™ + Dual Link System-HRP Kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions. Coloration was conducted with the 3, 3'-diaminobenzidine substrate. All slides were assembled, counterstained with hematoxylin, and evaluated under light microscopy. GLUT1 (stained brown) was expressed on the cell membranes.

We examined each section at a low magnification to identify areas with highest GLUT1 expression in cancer cells and then selected 3 fields in each case. Three microphotographs were taken in each case at a high magnification (400x) to score the proportion of positive cells and their staining intensity, as described below. The percentages of positive cells were scored as follows: 1 (0% to ≤25%), 2 (>25% to ≤50%), 3 (>50% to ≤75%), 4 (>75%). Similarly, the staining intensities of positive tumors were scored as follows: 1 (no staining or weak staining), 2 (moderate staining), or 3 (high staining). These two scores were averaged, and the expression intensity was calculated by multiplying both scores to yield a final score, as follows: (1-6) low-expression or (7-12) high-expression, as described previously (10,11). Examples of the above evaluation method are shown in Fig. 1. Evaluation of the immunostaining was conducted by three independent observers including oral pathologists, who were blinded to the clinical data.

Statistical analysis. With the IHC assay data, two-tailed Fisher's exact test was applied to analyze differences in expression intensities in normal tongue mucosal tissues and OTEDs, compared with OTSCCs, and the results were compared with the clinicopathological factors. DFS and OS were calculated by the Kaplan-Meier method, and statistical significance was determined by the log-rank test. Multivariate DFS and OS analyses were performed using the Cox proportional hazards model. $P<0.05$ was considered to indicate a statistically significant difference. These statistical analyses were performed using SPSS software, version 15.0 J (SPSS, Inc., Chicago, IL, USA).

Results

Selection of genes that specifically contributed to metabolic changes during oral carcinogenesis. We first attempted to select genes that may play important roles in metabolic control mechanisms. We searched genes associated with the TCA cycle using the KEGG pathway database. We chose 15 candidate genes related to the TCA cycle. For example, solute carrier family 2 member (SLC2), L-lactate dehydrogenase (LDH), pyruvate dehydrogenase kinase isozyme (PDK), pyruvate dehydrogenase (PDH), and hypoxia-inducible factor 1 (HIF-1) were selected (Table I).

Table I. Identification of 15 candidate genes that are associated with the tri-carboxylic acid cycle.

Gene symbol	Gene Bank accession no.	Function
SLC2A1	NM_006516.1	D-glucose transmembrane transporter activity
LDHC	NM_002301.2	L-lactate dehydrogenase activity
LDHAL6B	NM_033195.1	L-lactate dehydrogenase activity
LDHA	NM_005566.1	L-lactate dehydrogenase activity
PDK2	NM_002611.3	Pyruvate dehydrogenase (acetyl-transferring) kinase activity
PDK3	NM_005391.1	Pyruvate dehydrogenase (acetyl-transferring) kinase activity
PDK1	NM_002610.3	Pyruvate dehydrogenase (acetyl-transferring) kinase activity
LDHAL6A	NM_144972.3	L-lactate dehydrogenase activity
PDHA1	NM_000284.1	Pyruvate dehydrogenase (acetyl-transferring) activity
HIF1A	NM_001530.2	DNA binding transcription factor activity
PDHA2	NM_005390.3	Pyruvate dehydrogenase (acetyl-transferring) activity
PDK4	NM_002612.3	Pyruvate dehydrogenase (acetyl-transferring) kinase activity
SLC2A3	NM_006931.1	D-glucose transmembrane transporter activity
PDHB	NM_000925.1	Pyruvate dehydrogenase (acetyl-transferring) activity
LDHB	NM_002300.3	L-lactate dehydrogenase activity

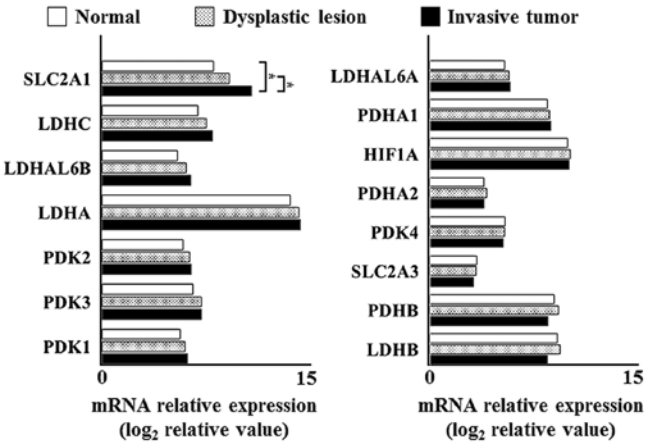

Figure 2. mRNA-expression statuses of the 15 candidate genes were examined in normal mucosal tissue, dysplastic lesions, and invasive cancer using our expression array database (http://www.ncbi.nlm.nih.gov/geo under accession no. GSE35261). Only one gene (SLC2A1) demonstrated significantly increased mRNA expression during oral carcinogenesis. SLC2A1 additionally demonstrated a significant expression difference between normal mucosal tissues or dysplastic lesions versus invasive tumors, using the Wilcoxon signed-rank test. *P<0.005.

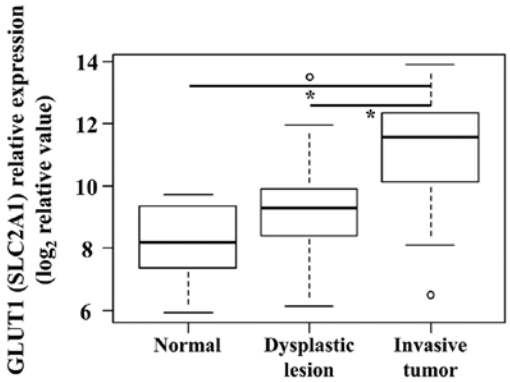

Figure 3. SLC2A1 mRNA expression significantly differed in normal mucosal tissues and dysplastic lesions versus invasive tumors, as determined using Fisher's exact test. *P<0.005.

Then, we examined the mRNA expression status of each candidate gene in normal mucosal tissue, dysplastic lesions, and invasive cancer tissues using our expression array database to identify genes showing significant expression differences between normal mucosal tissues or dysplastic lesions versus invasive tumors, using the Wilcoxon signed-rank test with a significance level of 0.005 (Fig. 2). Only SLC2A1 expression was significantly different in normal mucosal tissues and dysplastic lesions, compared with invasive tumors, as determined using Fisher's exact test with a significance level of 0.005 (Fig. 3). In addition, the expression levels of SLC2A1 showed significant difference among normal mucosal tissues, dysplastic lesions, and invasive tumors. (P=0.009 by Friedman test). Moreover, we performed hierarchical clustering analysis for each of the 15 candidate genes and confirmed that the mRNA-expression status increased in order of normal mucosal tissues, epithelial dysplastic lesions, and invasive carcinomas (Fig. 4).

IHC analyses. The SLC2A1 gene provides instruction for producing a protein called the glucose transporter protein type 1 (GLUT1). To confirm the mRNA expression status of SLC2A1 during oral tumorigenesis, GLUT1 protein-expression status was investigated by performing IHC staining with 110 OTSCC samples, 65 OTED samples, and 20 normal samples. In the normal mucosa, GLUT1 protein expression was detected predominantly on the cell membrane and was expressed only in the basal and parabasal layers (Fig. 5A), and all 20 cases (100%) showed low-expression. Overall, the area positive for GLUT1 expression was very narrow, and the staining intensity ranged from no staining to weak staining. In addition, the OTED staining range had expanded to include the spinous layer (Fig. 5B and C), with 14 samples (21.5%) showing high-expression and 51 samples (78.5%) showed

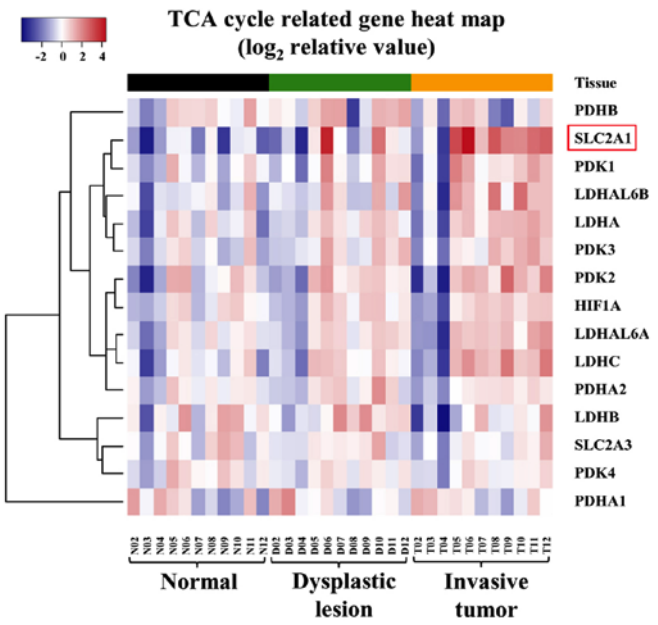

Figure 4. Hierarchical clustering analysis of mRNA-expression levels for each of 15 candidate genes. The analysis indicated that mRNA expression increased in order of normal mucosal tissues, epithelial dysplastic lesions and invasive carcinomas. TCA, tri-carboxylic acid cycle.

low-expression. Overall the expression area was limited to epithelial dysplasia lesions, and the staining intensity was weak to moderate. In contrast, in the invasive OTSCCs, the GLUT1 protein was expressed at the periphery of cancer nests (Fig. 5D-F), with 82 samples (74.5%) showing high-expression and 28 samples (25.5%) exhibiting low-expression. Overall the expression area included the cancer nests and invading tissues, and the staining intensity was moderate to high. GLUT1 protein-expression levels were significantly different between normal mucosa or OTEDs versus OTSCCs, as determined using Fisher's exact test with a significance level of $P<0.001$ (Table II), indicating that GLUT1 may play a significant role in progression from normal mucosa or precancerous lesions to invasive cancer.

Clinicopathological significance of GLUT1 protein expression in OTSCCs. The correlation between GLUT1 protein expression and the clinicopathological features of the 110 OTSCC samples are summarized in Table III. No significant association was observed between GLUT1 protein expression and age, sex, cellular differentiation, the mode of invasion, and local recurrence. However, high-expression of GLUT1 correlated significantly with the nodal status ($P=0.002$). Although no patients with nodal metastatic disease had tumors with low GLUT1 expression, 21 of 82 patients (25.6%) with tumors showed high- expression had nodal metastases. Kaplan-Meier survival curves clearly demonstrated the adverse impact of high GLUT1 expression on DFS ($P=0.004$). However, no significant association was found between GLUT1 expression and OS ($P=0.090$; Fig. 6).

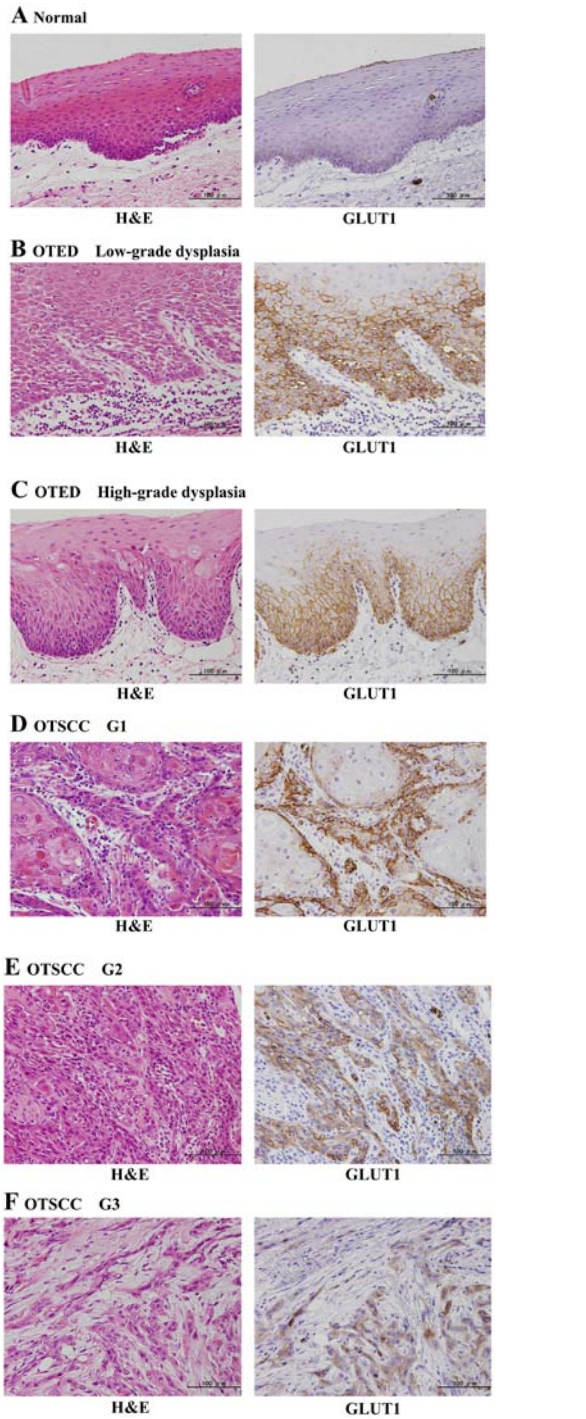

Figure 5. HE and IHC staining analysis of GLUT1 expression in normal mucosal tissues, OTEDs and OTSCCs. IHC analysis using an anti-GLUT1 mouse monoclonal antibody confirmed that GLUT1 expression was higher in OTSCC cells compared with normal mucosal tissues and OTEDs. (A) Low GLUT1 expression in normal mucosal tissue. (B) High GLUT1 expression in low-grade OTED. (C) High GLUT1 expression of high-grade OTED. (D) High GLUT1 expression of OTSCC G1 (well-differentiated type). (E) High GLUT1 expression in OTSCC G2 (moderate-differentiated type). (F) Low GLUT1 expression in OTSCC G3 (poorly-differentiated type). HE, hematoxylin and eosin; IHC, immunohistochemical; GLUT1, glucose transporter type 1, erythrocyte/brain; OTED, oral tongue epithelial dysplasia; OTSCC, oral tongue squamous cell carcinoma.

Discussion

Cancer metabolism is one of the oldest areas of research in cancer biology, predating the discovery of oncogenes and tumor-suppressor genes by a half century. The field is based on the principles that metabolic activities are altered in cancer cells relative to normal cells and that these alterations

Table II. Immunohistochemical staining for GLUT1 protein expression status in normal mucosal tissues, OTEDs and OTSCCs.

Histologic status	Low-expression (%)	High-expression (%)
Normal mucosal tissues	20/20 (100)	0/20 (0)[a]
OTEDs	51/65 (78.5)	14/65 (21.5)[a]
OTSCCs	28/110 (25.5)	82/110 (74.5)
Normal mucosal tissues	20/20 (100)	0/20 (0)
OTEDs		
Low-grade	29/36 (80.6)	7/36 (19.4)
High-grade	22/29 (75.9)	7/29 (24.1)
OTSCCs		
G1	20/76 (26.3)	56/76 (73.7)
G2	5/21 (23.8)	16/21 (76.2)
G3	3/13 (23.1)	10/13 (76.9)

[a]P<0.001 vs. OTSCCs. OTED, oral tongue epithelial dysplasia; OTSCC, oral tongue squamous cell carcinoma; GLUT1, glucose transporter type 1, erythrocyte/brain.

support the acquisition and maintenance of malignant properties. Tumors reprogram pathways of nutrient acquisition and metabolism to meet the bioenergetic, biosynthetic, and redox demands of malignant cells. These reprogrammed activities are recognized as hallmarks of cancer, and recent work has uncovered remarkable flexibility in the specific pathways activated by tumor cells to support these key functions (12). Although metabolic alterations were thought to have an important role in the carcinogenic process, no metabolism-related genes have been implicated in oral squamous cell tumorigenesis. In the present study, we analyzed metabolic changes arising during oral carcinogenesis. We examined the mRNA-expression statuses of 15 candidate genes related to the TCA cycle using our expression array database to search for genes showing significant expression changes during this process. As a result, we identified that only GLUT1 expression at both the mRNA and protein levels was significantly elevated during oral tumorigenesis, suggesting GLUT1 may be the most important metabolism-related genes that promote malignant transformation of the oral mucosa.

GLUT proteins are encoded by the SLC2 genes and are members of the major facilitator superfamily of membrane transporters. Humans express 14 different GLUT proteins. These GLUT proteins can be categorized into three classes according to their sequence similarity: Class 1 (GLUTs 1-4, and 14), class 2 (GLUTs 5, 7, 9, and 11), and class 3 (GLUTs 6, 8, 10, 12, and 13/HMIT). GLUTs comprise a family of transmembrane proteins that mediate the transport of glucose across cellular membranes. The GLUT proteins are mainly distributed as follows: GLUT1/erythrocyte and brain, GLUT2/liver and islet of Langerhans, GLUT3/brain and testes, GLUT4/adipose tissue and skeletal and cardiac muscle, GLUT5/small intestine and kidney, GLUT6/brain and spleen, GLUT7/small intestine and colon, GLUT8/testes and brain, GLUT9/kidney and liver, GLUT10/heart and lungs, GLUT11/heart and muscle, GLUT12/heart and prostate, GLUT13/HMIT/brain and adipose tissue, GLUT14/testes (13). Among them, GLUT1 was the first of the family of facilitative GLUT proteins to be cloned. Although GLUT1 expression in normal human tissues is limited, it is expressed at higher levels in erythrocytes, as well as brain, cartilage, retinal, and placental tissue. Moreover, it is widely overexpressed in many kinds of human malignancies, including hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, endometrial, ovarian, cervical, and oral SCCs (14).

The data generated in this study demonstrated that in normal mucosal tissues, the GLUT1 protein was expressed predominantly on the cell membrane, but only in the basal and parabasal layers, and weak staining was found with all 20 normal specimens. These observations were consistent with previous reports (15), suggesting that the expression status in the normal mucosa seems to respond to a hypoxic environment. In contrast, in the OTEDs, GLUT1 expression was strong throughout the entire dysplastic area. GLUT1 expression occurred in the basal area of mildly dysplastic epithelium tissue, the basal and suprabasal areas in moderately dysplastic epithelium tissue, and in all areas of highly dysplastic epithelium tissue. Thus, GLUT1 expression area depended on the extent of epithelial dysplasia. These findings also agree with a previous report and similar findings were observed in another malignancy, such as cervical cancer (16,17). Recent findings indicated that the metabolic alteration of cancer cells is more a consequence of the activation of proto-oncogenes (e.g., Myc), transcription factors (e.g., HIF-1), and signaling pathways (e.g., PI3K), as well as the inactivation of tumor-suppressor genes (e.g., p53), rather than the primary generation of much needed energy (18). Considering these possibilities, changes in GLUT1 expression of the dysplastic epithelium are more likely caused, not only by responses to a hypoxic and nutrient-poor environment, but also by accumulation of numerous genetic abnormalities. Further investigation was required to clarify the mechanism of enhanced GLUT1 expression associated with malignant transformation.

On the other hand, in the invasive OTSCCs, the GLUT1 protein was expressed at the periphery of cancer nests and was absent from the center of more differentiated tumor islands. In addition, the GLUT1-expression intensity in invasive OTSCCs was significantly stronger than that of normal mucosa and OTEDs. GLUT1 overexpression was also observed in various malignant tumors, including non-small cell lung cancer, colorectal cancer, breast cancer, and gastric cancer (14). The upregulation of GLUTs has been reported in numerous cancer types due to perturbations in gene expression or protein re-localization or stabilization (19) and might be a critical event for cancer cells that reside in a microenvironment with a limited glucose supply (4). These findings indicated that GLUT1 upregulation is not lacking during the malignant transformation process in many kinds of human malignancies. An important question is why GLUT1 upregulation is indispensable for many types of cancer cells. Tumor cells are known to have accelerated metabolic rates and high glucose and energy demands in a nutrient-poor environment, due to their rapid proliferation. Therefore, it seems that cancer cells necessarily undergo

Table III. Clinicopathological parameters of 110 OTSCCs and correlation with GLUT1 protein expression status.

Clincopathological parameter	Total number (%)	GLUT1 protein expression		P-value[a]
		Low-expression	High-expression	
Age, years				
<60	44 (40.0)	10	34	0.659
≥60	66 (60.0)	18	48	
Sex				
Male	55 (50.0)	13	42	0.827
Female	55 (50.0)	15	40	
Cellular differentiation				
Well to moderate	97 (69.1)	25	72	1.000
Poor	13 (11.8)	3	10	
Mode of invasion (YK)				
1-3	89 (80.9)	25	64	0.268
4C-4D	21 (19.1)	3	18	
Nodal status[b]				
Metastasis	21 (19.1)	0	21	0.002
No metastasis	89 (80.9)	28	61	
Local recurrence				
Positive	4 (3.6)	0	4	0.571
Negative	106 (96.7)	28	78	

[a]By two-tailed Fisher exact test. [b]Histopathologic diagnosis. OTSCC, oral tongue squamous cell carcinoma; GLUT1, glucose transporter type 1, erythrocyte/brain.

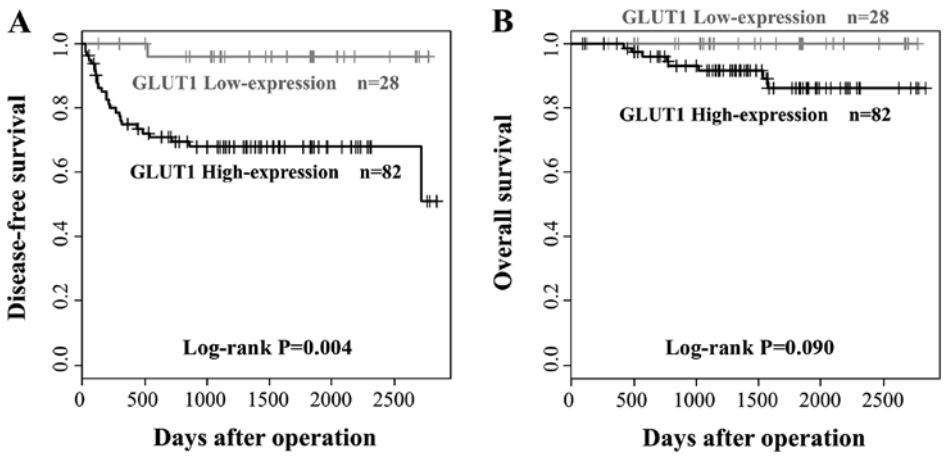

Figure 6. Kaplan-Meier survival curves demonstrated the adverse impact of high GLUT1 expression on DFS (P=0.004). However, no significant association was identified between GLUT1 expression and OS (P=0.090). Kaplan-Meier plots for (A) disease-free survival according to the GLUT1 protein-expression status and (B) overall survival according to the GLUT1 protein-expression status. GLUT1, glucose transporter type 1, erythrocyte/brain.

a marked transformation of their metabolism. Glycolysis generates ATP with lower efficiency, but at a faster rate, than oxidative phosphorylation. The enhanced rate of ATP generation has been postulated to be beneficial for rapid proliferating cells. However, several recent findings have identified mitochondria as the major source of cellular ATP in most cancer cell lines and tissues (18,20). Rather, cancer cells have been found to benefit from the production of glycolytic intermediates by altering their glucose metabolism (18). Moreover, high-level glucose metabolism causes a large amount of lactate secretion into the extracellular space. The subsequent accumulation of extracellular lactate may create a tumor microenvironment favorable for tumor cell migration, angiogenesis, and the immunological escape of tumors. Consequently, increased GLUT1 expression may be essential for ensuring energy production, accelerating cell growth, and preparing the microenvironment for malignant transformation and tumor progression.

Many previous reports revealed GLUT1 overexpression as a prognostic indictor in OSCC and that it was also significantly associated with metastases and other clinical factors (21). Recent prisma-compliant meta-analysis of the prognostic value of GLUT1 expression in OSCC also showed that GLUT1 overexpression was associated with aggressive clinical features and worsened OS in OSCC (22). In this study, we examined the GLUT1-expression status for 110 OTSCCs, which were divided into high- and low-expression groups and examined for correlations with clinicopathological factors. Cervical lymph node metastasis was significantly more frequent in the high GLUT1-expression group than in the low GLUT1-expression group (P=0.002). Moreover, although no significant effect of the GLUT1-expressions level on OS was found, the DFS rate in the high GLUT1-expression group was significantly lower than that of the low GLUT1-expression group, as determined by Kaplan-Meier survival analysis (P=0.004). These observations suggested that the GLUT1-expression status correlated significantly with clinical aggressiveness and the metastatic ability of cancer cells.

Why do cancer cells with high GLUT1 expression show aggressive behavior? Tumor cells with high GLUT1 expression actively transport extracellular glucose and obtain great energy for rapid proliferation, and then the increased lactic acid produced is transported from tumor cells into the extracellular space. Lactic acid efflux leads to acidosis of the tumor microenvironment, which can drive tumor growth and metastasis. Increased lactate levels promote the emergence of an immune-permissive microenvironment by attenuating dendritic and T cell activation and monocyte migration (23-25). In addition, lactate stimulates the polarization of resident macrophages to a so-called M2 state, which plays a role in immunosuppression (26,27). Furthermore, lactate accumulation is instrumental in promoting angiogenesis. Lactate also enhances the stabilization of *HIF-1α*, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and phosphoinositide 3-kinase (PI-3K) signaling in endothelial cells, and induces secretion of the proangiogenic vascular endothelial growth factor (VEGF) from tumor-associated stromal cells (28-31). Increased levels of lactate also stimulate hyaluronic acid production by fibroblasts, which may contribute to tumor invasiveness (32). Presumably, high GLUT1 expression in tumor cells is clinically aggressive feature because of the greater activity (described above) than in cells with low GLUT1 expression. However, further studies on the correlation between GLUT1-expression status and clinical aggressiveness are needed.

In conclusion, we sought to determine significant genes that may contribute to metabolic alterations during oral carcinogenesis and determined that only GLUT1 expression was significantly elevated at both the mRNA and protein levels during this process. These findings suggest that GLUT1 serves a crucial role in the initiation and progression of OSCC and that elevated GLUT1 expression is an early critical event in the development of invasive OSCCs. Moreover, regarding OSCCs, tumors with high GLUT1 expression were significantly associated with the presence of cervical lymph node metastases. Therefore, the GLUT1-expression status might be used both as a diagnostic tool for the early detection of pre-neoplastic lesions, as a biomarker for treatment escalation, and as an independent prognostic marker for OSCC patients. Further studies are needed to evaluate the prognostic and diagnostic potentials of GLUT1 and address its potential role in oral carcinogenesis.

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Availability of data and materials

The data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

The study was conceived by KN, YM, TY and NU. Collection of samples and the laboratory preparations were conducted by KN. The manuscript was drafted by KN. The statistical analysis was performed by KM, KK, MT, KT and JS. All authors were involved in the preparation and revision of the manuscript.

Ethics approval and consent to participate

The protocols used in this study were reviewed and approved by the Research Ethics Committee of the Faculty of Dentistry of the Tokyo Medical and Dental University (approval no. D2015-534; Tokyo, Japan). Written informed consent forms were obtained from all patients in accordance with institutional guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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