Glucose and glutamine metabolism in oral squamous cell carcinoma: insight from a quantitative metabolomic approach

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Objective. To characterize the metabolic system of oral squamous cell carcinoma (OSCC) by metabolome analysis.

Study Design. The metabolome profiles, including the Embden-Meyerhof-Parnas pathway (EMPP), the pentose phosphate pathway, the tricarboxylic acid cycle (TCAC), and amino acids, were obtained from OSCC and its surrounding normal tissues (32 patients) using capillary electrophoresis and a time-of-flight mass spectrometer.

Results. Enhancement of glucose consumption and lactate production (Warburg effect) was observed in OSCC tissues. The decrease of glucose along with the decrease of the downstream intermediates in the EMPP suggests that incorporated glucose is mainly consumed for biosynthesis. Glutamine consumption with the increase of the intermediates in the last half of the TCAC suggests the involvement of glutaminolysis, in which glutamine is converted to lactate via the last half of the TCAC.

Conclusions. It is suggested that OSCC tissues show the Warburg effect, which stems from the combined enhancement of glucose consumption and glutaminolysis. (Oral Surg Oral Med Oral Pathol Oral Radiol 2014;118:218-225)

It is becoming more important to comprehensively understand the fundamental biofunction of cancer cells, based on genome,1 transcriptome,2 and proteome3 analyses. Genome analysis has found that genetic mutations associated with tumorigenesis are highly variable and complicated. Thousands of point mutations, translocations, amplifications, and deletions of genes are reported to be involved in cancer initiation and promotion.4 In addition, it has been found that mutations of cancer-associated genes alter many of the major signaling pathways and result in oncogenesis. These findings have led to difficulty in targeting specific signaling molecules as a practical chemotherapeutic strategy.5

On the other hand, it is becoming clear that, despite various genetic mutations and oncogenic signaling pathways, tumor cells commonly enhance their metabolic activity to compensate for the energy required for rapid and persistent growth.6 Moreover, metabolic flux and metabolites regulate enzymes, transcription factors, and gene expression.6,7 These observations have therefore led to the idea that comprehensive analysis of metabolites, namely metabolome analysis, in addition to genome, transcriptome, and proteome analyses, is a useful basis to understand tumor cellular function.

In the 1920s, Otto Warburg observed lactate accumulation during glucose metabolism in ascites cancer cells even in the presence of abundant oxygen8 and hypothesized that cancer cells have a defect in mitochondria that leads to impaired aerobic respiration and a subsequent dependency on glycolytic metabolism.9 This phenomenon is known as the Warburg effect or aerobic glycolysis, being a common metabolic characteristic of cancer cells. However, the low efficiency of adenosine triphosphate (ATP) production in aerobic glycolysis does not meet the energy requirement for rapid proliferation of cancer cells. In the 1960s, it was reported that cancer cells can use not only glucose but also glutamine as an additional energy source.10,11 Since the late 1990s, metabolome analysis has been developed as an ultimate tool for the comprehensive identification and quantification of metabolites in biologic systems and is one of the most powerful approaches to metabolism research. The progress of metabolome analysis has revealed various additional metabolic characteristics, such as glutamine degradation to lactate via the tricarboxylic cycle.
acid cycle (TCAC) (glutaminolysis),\textsuperscript{12} glutamine reduction to acetyl coenzyme A (acetyl CoA) via the reverse TCAC,\textsuperscript{13,14} and fumarate respiration,\textsuperscript{15-17} depending on the type of cancer. Contrary to Warburg’s hypothesis,\textsuperscript{8,9} recent studies also found that mitochondrial function was not impaired in most cancer cells.\textsuperscript{18} Furthermore, organ-specific differences in metabolic pathways were reported. For example, the metabolome profile of the TCAC was different between colon and stomach cancer tissues, despite their histologic resemblance.\textsuperscript{19} On the basis of these findings, it is expected that oral squamous cell carcinoma (OSCC), the most frequent cancer in the orofacial region,\textsuperscript{20} may possess a specific metabolic pathway.

In the present study, therefore, we aimed to characterize metabolic systems in OSCC by metabolome analysis of the main metabolic pathways, including the Embden-Meyerhof-Parnas pathway (EMPP), the pentose phosphate pathway (PPP), the TCAC, and amino acids in clinical specimens of OSCC.

**MATERIALS AND METHODS**

**Sample collection and metabolite extraction**

All experiments were conducted according to the study protocol approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, and patient consent was obtained. OSCC tissue and its surrounding normal tissue were collected from 32 patients with cancer during tumor excision (Table I). Samples of the excised tissues were weighed (about 30 mg each), frozen immediately in liquid nitrogen, and stored at −80°C until metabolite extraction.

To extract metabolites, preweighed deep-frozen samples were crushed by a cell disruptor (MS-100R; Tomy, Japan) at 4000 rpm for 5 minutes at 2°C in the presence of zirconium beads and 500 μL methanol containing internal standards (10 mmol/L each of methionine sulfone and 5,2-(N-morpholino)-ethane-sulfonic acid). The homogenized solution was mixed with chloroform and Milli-Q water in a volume ratio of 5:5:2 using a cell disruptor at 2000 rpm for 100 seconds and centrifuged at 4600 × g for 5 minutes at 4°C. The aqueous layer (200 μL) was transferred to a 5-kDa-cutoff ultrafilter tip (Ultrafree-MC-PLHCC-HMT; Human Metabolome Technologies Inc, Japan) and centrifuged at 9100 × g for 4 hours at 4°C to remove large molecules. The filtrate was dried for 4 to 5 hours by a vacuum centrifugal dryer (Eyela Centrifugal Evaporator CVE-3100; Tokyo Rikakikai Co, Japan), resuspended in 50 μL Milli-Q water containing internal standards (Internal standard solution 3; 10 mmol/L each of 3-amino-pyrididine, N,N-diethyl-2-phenylacetamide, trimersate, and 3-hydroxynaphthalene-2,7-disulfonic acid; Human Metabolome Technologies), and stored at −80°C until metabolome analysis.

**Metabolome analysis**

Metabolome analysis, except for glucose, was conducted by capillary electrophoresis with time-of-flight mass spectrometry (CE-TOFMS) using a CE machine (G1600AX; Agilent Technologies, Waldbronn, Germany) equipped with a time-of-flight mass spectrometer (G1969A; Agilent Technologies). CE is capable of separating ionic small molecules precisely. The majority of human metabolites are separated by CE, because 80% to 90% of the metabolites are ionic small molecules such as phosphorylated sugars, carbonic acid, and amino acids. It is also necessary to identify and quantify these separated metabolites in terms of mass. TOFMS has an advantage of accurate mass analysis. CE-TOFMS is thus suitable for quantifying metabolites.\textsuperscript{21-23} A fused silica capillary (H3305-2002; Human Metabolome Technologies) was used for separation of metabolites in CE. The applied voltage was set at +30 kV when the electrospray ionization was operated in the negative ion mode or +27 kV in the positive ion mode, and the capillary voltage was set at 3.5 kV in the negative ion mode and 4 kV in the positive ion mode. The flow rate of heated dry nitrogen gas (300°C) was maintained at 7 L/min. All standard metabolites and chemicals used were of analytical or reagent grade. Metabolites separated by CE were mixed with sheath liquid (H3302-1020; Human Metabolome Technologies, Japan) and stored at −80°C until CE-TOFMS analysis.
Metabolome Technologies), which provides the electric contact as well as the appropriate flow and solvent composition for optimal ionization of metabolites, and continuously sent to TOFMS for mass analysis. Obtained raw data were processed by calculation software (Mass Hunter Workstation Software Qualitative Analysis; Agilent Technologies) for identification and quantification of metabolites. All target metabolites were identified by matching their migration times with those of the standard compounds determined by CE and according to mass-to-charge ratio (m/z) values (physical quantity in the electrodynamics of charged particles) determined by MS. Processed data were exported for further statistical analysis.

Glucose was quantified enzymatically by a kit (LabAssay Glucose, Wako, Japan). The mixture of the sample (1.6 μL) and color reagent (240 μL) was incubated at 37°C for 5 minutes, and the absorbance of the test sample and standard solution with a blank solution as the control was measured at 520 nm (Infinite F200 PRO, Tecan, Salzburg, Austria).

Statistics

The concentration of metabolites in each sample was normalized using the weight of sample tissues; 64 samples were prepared, and 67 metabolites were quantified twice in each sample. Differences in the amounts of metabolites between the cancer and the normal tissue were analyzed by the Wilcoxon signed rank test. The P value was adjusted by the Hochberg correction for multiple comparisons from .05 to .00114.

RESULTS

Glucose metabolism in OSCC tissues

The concentrations of glucose, 3-phosphoglycerate (3PG), and 2-phosphoglycerate (2PG) in OSCC tissues were significantly lower than those in normal tissues (Figure 1, A). Lactate (see Figure 1, B), known to be derived from pyruvate, was significantly higher in OSCC tissues.
(see Figure 1, C) showed no significant difference between OSCC and normal tissues. Fumarate and malate in the last half of TCAC (see Figure 1, D) were significantly higher in OSCC tissues.

Amino acid metabolism in OSCC tissues
In OSCC tissues, the highest amino acid was glutamate, followed by glutamine, alanine, lysine, and aspartate (Figure 2). On the other hand, in normal tissues, the highest was glutamine, followed by glutamate, alanine, glycine, lysine, and serine. Glutamate, aspartate, glycine, proline, cysteine, hydroxyproline, creatinine, and putrescine in OSCC tissues were significantly higher than in normal tissues, whereas creatine in OSCC tissues was significantly lower.

Energy charge and redox status in OSCC tissues
In OSCC tissues, the amounts of adenosine monophosphate (AMP), guanosine triphosphate (GTP), guanosine diphosphate (GDP), and guanosine monophosphate (GMP) were significantly higher than in normal tissues, whereas ATP and adenosine diphosphate (ADP) showed no significant difference (see Figure 1, E). Adenylate energy charge, calculated by \[\frac{[(ATP)+1/2(ADP)]}{[(ATP)+(ADP)+(AMP)]}\] as an index of the intracellular energy state, was significantly lower in OSCC tissues (Table II). Guanylate energy charge, \[\frac{[(GTP)+1/2(GDP)]}{[(GTP)+(GDP)+(GMP)]}\], compatible with adenylate energy charge, also showed a significant decrease. The concentrations of reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide (NAD\(^+\)), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and nicotinamide adenine dinucleotide phosphate (NADP\(^+\)), coenzymes of dehydrogenases, functioning as intracellular reduction/oxidation reactions and representing the intracellular redox status, showed no significant differences between OSCC and normal tissues (see Table II). In addition, reduced glutathione and oxidized glutathione, which also indicate the intracellular redox status, showed no significant differences.
DISCUSSION

Glucose metabolism in OSCC tissues

There was a large variation in the amount of metabolite among samples (see Figure 1, A-E). Similar variation was also observed in amino acids and other metabolites, as described in subsequent paragraphs (see Figure 2). This large variation is possibly due to small sample size, site-specific difference in tissue structure, and the coexistence of cancer tissue and normal tissue. The contamination of oral microorganisms may also be involved, as reported previously.25-27

The decrease of glucose (see Figure 1, A) indicates that OSCC tissues consume glucose and convert it to G6P (glucose 6-phosphate) efficiently, as reported previously in other types of cancer cells.24 It was reported that glucose transporter 1 (GLUT1)28 and hexokinase,29 which transport glucose and phosphorylate it to G6P, were overexpressed in cultured cancer cells. This might be the case in OSCC tissues. The lactate concentration of OSCC tissues was considerably higher than that of their corresponding normal counterparts (see Figure 1, B). If the Warburg effect is defined as the combination of overconsumption of glucose and accumulation of lactate, it was also observed in OSCC tissues.

OSCC tissues, however, contained a nearly equal or lower amount of intermediates in the EMPP than their corresponding normal counterparts (see Figure 1, A). G6P is not only a metabolic intermediate of the EMPP but also an initial substrate for the PPP, suggesting that G6P partly flows in the PPP in OSCC tissues. This metabolic shift could be beneficial for OSCC tissues, because the PPP supplies reducing power for fatty acid synthesis and pentose phosphates for nucleotide synthesis. The amounts of AMP, GTP, GDP, and GMP in OSCC tissues were significantly higher than in normal tissues (see Figure 1, E), supporting this hypothesis. Similarly, the significant decrease of 3PG and 2PG in the middle part of the EMPP (see Figure 1, A) suggests that these metabolic intermediates are used as precursors for amino acids.12,30 Overall, these findings suggest that glucose can be used as a precursor for biosynthesis in OSCC tissues, which may explain the rapid and persistent growth of OSCC tissues. Contrary to the depletion of metabolic intermediates in the middle part of the EMPP, lactate, an end product of the EMPP, was increased in OSCC tissues (see Figure 1, B), suggesting the possibility that lactate can be derived from other metabolic pathways, as discussed in the next section.

In the TCAC of both cancer and normal tissues, the amounts of cis-aconitate, isocitrate, α-ketoglutarate, and succinyl CoA were low, whereas those of citrate, succinate, fumarate, and malate were high (see Figure 2). These trends have also been observed in other cancer and normal tissues such as the stomach,19,31 colon,19 lung, and prostate,32 probably owing to the enzymatic equilibrium of the TCAC. In colon cancer tissues, the concentration of succinate was the highest, followed by fumarate and malate, suggesting that colon cancer bypasses the EMPP to succinate via malate and fumarate (fumarate respiration),15-17 a method that nematodes possess and use in an anaerobic environment.33 In OSCC tissues, the concentrations of fumarate and malate were also significantly higher than in normal tissues; however, succinate was lower than fumarate and malate (see Figure 1, D). This observation suggests that instead of fumarate respiration, alternative metabolic pathways may be involved.

Table II. Energy charge and redox status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cancer</th>
<th>Normal</th>
<th>Wilcoxon signed rank test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate energy charge</td>
<td>0.51 ± 0.17</td>
<td>0.64 ± 0.17</td>
<td>0.00000004</td>
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<tr>
<td>Guanylate energy charge</td>
<td>0.44 ± 0.17</td>
<td>0.53 ± 0.20</td>
<td>0.00104</td>
</tr>
<tr>
<td>Concentration (nmol/mg tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>0.03 ± 0.04</td>
<td>0.02 ± 0.03</td>
<td>0.085</td>
</tr>
<tr>
<td>NADPH</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.02</td>
<td>0.875</td>
</tr>
<tr>
<td>NADPH/NADP</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.818</td>
</tr>
<tr>
<td>Glutathione (reduced)</td>
<td>2.31 ± 3.54</td>
<td>1.41 ± 1.46</td>
<td>0.114</td>
</tr>
<tr>
<td>Glutathione (oxidized)</td>
<td>0.78 ± 0.75</td>
<td>0.79 ± 0.65</td>
<td>0.417</td>
</tr>
<tr>
<td>FAD</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.02</td>
<td>0.0000005 *</td>
</tr>
</tbody>
</table>

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; GMP, guanosine monophosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide.

* p < 0.00114.

1 Adenylate energy charge was calculated as (ATP+1/2ADP)/(ATP+ADP+AMP).
2 Guanylate energy charge was calculated as (GTP+1/2GDP)/(GTP+GDP+GMP).
Amino acid metabolism in OSCC tissues

Glutamate was the highest in OSCC tissues, whereas glutamine was the highest in normal tissues (see Figure 2). This reverse association indicates that the conversion of glutamine to glutamate was enhanced in OSCC tissues and suggests that glutamine can be used as an additional energy source. In cervical and breast cancers, the decrease in glutamine was reported to stem from overexpression of glutamine transporters, increase of glutaminase activity, and deactivation of glutamine synthetase. These phenomena might also occur in OSCC tissues. Glutamate formed from glutamine can further be converted to α-ketoglutarate by the catalysis of glutamate dehydrogenase or glutamate oxaloacetate transaminase. In the latter reaction, oxaloacetate can be transaminated to aspartate. The significant increase of aspartate in OSCC tissues (see Figure 2) might be partly derived from this reaction.

The increase of fumarate and malate in the last half of the TCAC in OSCC tissues (see Figure 1, D) suggests the inflow of amino acids, such as conversion of glutamate to α-ketoglutarate, into the TCAC. Furthermore, the increases of lactate (see Figure 1, B) suggest that malate can be converted to pyruvate by malic enzyme (NAD⁺-dependent malate decarboxylase, EC 1.1.1.39, and NADP⁺-dependent malate decarboxylase, EC 1.1.1.40) and finally to lactate by lactate dehydrogenase. The metabolic pathway from glutamine to lactate has been known as glutaminolysis (Figure 3). Somashekar et al. reported an increase of amino acids in OSCC clinical specimens as detected using nuclear magnetic resonance and suggested the possibility of glutaminolysis; however, metabolic intermediates in the TCAC were not measured in their study. To our knowledge, the present study is the first to directly suggest the involvement of glutaminolysis in OSCC tissues based on metabolome analysis of the central carbon metabolism and amino acid metabolism.

Glutaminolysis is thought to be advantageous because the reducing power (yielding NADH, NADPH, and reduced flavin adenine dinucleotide [FADH₂]) derived from the pathway (see Figure 3) can be used for ATP production through oxidative phosphorylation and biosynthesis such as lipid synthesis, and these metabolic intermediates can be supplied as carbon frames for rapid proliferation of cancer cells. It is reported that at the normal concentration of oxygen, glutaminolysis was enhanced in human glioma line SF188 cells. On the other hand, at the low oxygen concentration of 1% to 3%, glutamine was metabolized to α-ketoglutarate and then converted into isocitrate, citrate, and finally acetyl CoA through the reverse direction of the TCAC (reductive TCAC) (see Figure 3). In the present study, however, the metabolic intermediates in the first half of the TCAC were nearly equal to those of normal oral tissues, declining the function of the reductive
In squamous epithelium, blood supply seems to be poor, and therefore the oxygen concentration might be low, but the tissue is exposed to air, and oxygen is continuously supplied from the outside environment. The absence of any significant difference in the redox status between OSCC and normal tissues (see Table II) also supports the similarity in oxygen concentration.

Given the aforementioned information, it is proposed that the accumulation of lactate in OSCC tissues was caused by enhancement of glutaminolysis rather than by enhancement of the EMPP. Moreover, acidification in a microenvironment due to lactate accumulation may create a potentially favorable microenvironment for cancer cells to proliferate, as it promotes the liberation of peptides and amino acids from extracellular matrices.

### Glucose and glutamine as energy substrates in OSCC tissue

Glucose and glutamine are 2 of the most abundant nutrients in plasma, and together they account for most carbon and nitrogen metabolism in mammalian cells. It is reported that the concentrations of glucose and glutamine were similar in normal tissues of the stomach and colon, whereas in cancer tissues glutamine concentration was 3 to 10 times higher. However, the present study found that the glutamine concentration in both cancer and normal oral tissues was 7 to 11 times higher than that of glucose (see Figure 1, A, and Figure 2). In addition, the concentrations of most amino acids in oral tissues detected in the present study (see Figure 2) are basically higher than those reported in other tissues, such as the stomach, colon, muscle, and blood, suggesting that oral tissues have high protein turnover in their nature. The increase of most amino acids in OSCC tissues (see Figure 2) may be due to protein degradation through the activation of matrix metalloproteinases, often found in cancer tissues. The significant increase in glycine, proline, and hydroxyproline in OSCC tissues suggests collagen degradation, as reported in previous studies.

Proliferating cancer cells have to activate energy generation for the biosyntheses of nucleotides, protein, and lipids. It is well known that ATP-generating (catabolic) pathways are restrained in cells with a high energy charge. Namely, the consumption of ATP and the resultant low adenylate energy charge enhance the reactions for energy production and catabolism. The significant low adenylate energy charge in OSCC tissues (see Table II) suggests the high consumption of ATP and the enhancement of catabolic reactions. Decreased guanulate energy charge (see Table II) might reflect the decreased adenylate energy charge, because guanulate energy charge is compatible with adenylate energy charge.

### CONCLUSION

The present study indicated that, in OSCC tissues, the glucose consumption was enhanced and the glutamine metabolism (glutaminolysis) was activated. It is suggested that metabolic intermediates of the EMPP are partly used for biosynthesis through the PPP and other related pathways, whereas glutaminolysis can provide lactate via the last half of the TCAC and pyruvate. In OSCC tissues, the Warburg effect, that is, aerobic glucose consumption and lactate production, seems to stem from the combination of enhancement of glucose consumption and glutaminolysis. The new knowledge about metabolism specific to OSCC may provide new strategies to diagnose OSCC and to control its clinical behavior (e.g., more effective antineoplastic agents based on metabolism). For instance, metabolome profiling regarding glucose consumption and glutaminolysis may be a useful indicator for cancer diagnosis. Development of novel cancer therapeutic agents may also be significantly promising and effective to repress glucose consumption and glutaminolysis and to normalize the metabolic properties of cancer cells. Further intensive study is needed for these clinical applications.

### REFERENCES


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