Serum and Tissue Metabolomics of Head and Neck Cancer

KOICHIRO YONEZAWA[1], SHIN NISHIUMII[2], JUNKO KITAMOTO-MATSUDA[1], TAKESHI FUJITA[1], KOICHI MORIMOTO[1], DAISUKE YAMASHITA[1], MIKI SAITO[1], NAOKI OTSUKI[1], YASUHIRO IRINO[3], MASAKAZU SHINOHARA[3], MASARU YOSHIDA[2,3,4] and KEN-ICHI NIBU[1,5]

[1]Department of Otolaryngology-Head and Neck Surgery, Divisions of [2]Gastroenterology and [4]Metabolomics Research and [3]The Integrated Center for Mass Spectrometry, Graduate School of Medicine, Kobe University, Kobe, Japan

Abstract. *Background: No reliable clinical markers to diagnose early stage-disease and predict its prognosis have yet been found for squamous cell carcinoma of the head and neck (HNSCC). Materials and Methods: In the present study, the metabolomic analysis of serum and tissue samples obtained from patients with HNSCC was performed using gas chromatography/mass spectrometry. Results: In serum, levels of several metabolites related to the glycolytic pathway, such as glucose, were higher in patients with HNSCC, and the levels of several amino acids were lower. In contrast to sera, the levels of many metabolites related to the glycolytic pathway appeared to be lower in tumor tissues of HNSCC than in non-tumorous tissues, and the levels of several amino acids, such as valine, thyrosine, serine and methionine, were higher. Conclusion: Our results demonstrate that changes in metabolite patterns are useful in assessing the clinical characteristics of HNSCC, and will hopefully lead to the establishment of novel diagnostic tools.*

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common type of cancer worldwide (1). Despite improvements in diagnosis and treatment, the survival rates of patients with advanced HNSCC have not improved due to the high proportion of patients with advanced diseases and the frequent relapse. Biomarkers allowing early detection of primary HNSCC or relapse may aid in improving clinical outcome. It was reported that the sensitivity of the squamous cell carcinoma antigen (SCC-Ag), which is the most reliable tumor marker for HNSCC, is 43% at most. This reliability is not as high in the clinical field (2). Recently, Ki67, p53, collagen XVII, and EGFR have been demonstrated as candidate biomarkers for HNSCC, with sensitivity of 63%, 54.3%, 78.3%, and 82.6%, respectively (3). However, these candidates have not actually been used as biomarkers with high reliability for diagnosis in clinical practice.

The 'omics' technologies including genomics, proteomics and metabolomics, have evolved rapidly. In particular, focus has been given to metabolomics, developing its analytical techniques and the high possibility that the metabolome is a summary manifestation of all the other upstream omics profiles. Sreekumar *et al*. revealed that the urine level of sarcosine was significantly higher in patients with prostate cancer compared with healthy individuals *via* metabolomics, suggesting that sarcosine is a potential candidate for future development of biomarkers for early disease detection (4). Hirayama *et al*. also reported that multiple metabolite levels, including of glycolytic intermediates, amino acids, several tricarboxylic acid (TCA) and urea cycle intermediates, and nucleosides, were higher in tumor tissues compared with the normal tissues in patients with colorectal cancer (5). In our previous studies, it was also demonstrated that alterations of serum metabolome were observed in the patients with esophageal (6), stomach (6), colorectal (6, 7), pancreatic (8) and lung cancers (9). Thus, the pathogenesis of cancer seems to be intimately related to alterations in metabolome (10). To identify reliable clinical biomarkers in diagnosing a disease at an early stage and to predict its prognosis, metabolomic analysis of tumor and non-tumorous tissues obtained from patients with HNSCC was performed using gas chromatography/mass spectrometry (GC/MS) in the present study. The differences between pre- and postoperative serum metabolome were then evaluated.

Materials and Methods

Participants. This study was approved by the Ethics Committee at Kobe University Graduate School of Medicine (# 720). The human samples were used in accordance with the guidelines of Kobe

Correspondence to: Ken-ichi Nibu, MD, Ph.D., Professor and Chairman. Department of Otolaryngology-Head and Neck Surgery, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan. Tel: +81 783826021, Fax: +81 783826039, e-mail: nibu@med.kobe-u.ac.jp

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University Hospital. Written informed consent was obtained from all participants. Twenty-five patients with HNSCC were included in the present study. Characteristics of the participants are summarized in Tables I and II. Of these patients, serum samples were collected from 17 with HNSCC at Kobe University Hospital from July 2009 to December 2010. Samples of tumor tissue and the surrounding non-tumorous tissue were obtained from 19 patients with HNSCC who had surgical treatment. Pathological diagnoses were performed for all patients with HNSCC.

Sample collection and preparation. Sample collection and preparation were performed with a few modifications according to our previous report (8). The collected blood was immediately centrifuged at 3,000 ×*g* for 10 min at 4˚C, and the serum was transferred to a clean tube and stored at –80˚C until use. To extract low-molecular-weight metabolites, 50 μl of serum was mixed with 250 μl of a solvent mixture (MeOH:H_2O:$CHCl_3$=2.5:1:1) containing 10 μl of 0.5 mg/ml 2-isopropylmalic acid (Sigma - Aldrich, Tokyo, Japan) dissolved in distilled water as an internal standard, and then the solution was shaken at 1,200 rpm for 30 min at 37˚C before being centrifuged at 16,000 ×*g* for 3 min at 4˚C. Two hundred twenty-five microliters of the supernatant obtained was transferred to a clean tube, and 200 μl of distilled water was added to the tube. After being mixed, the solution was centrifuged at 16,000 ×*g* for 3 min at 4˚C, and 250 μl of the resultant supernatant was transferred to a clean tube before being lyophilized using a freeze dryer (FDU-1200, Tokyo Rikakikai, Tokyo, Japan). For oximation, 40 μl of 20 mg/ml methoxyamine hydrochloride (Sigma - Aldrich) dissolved in pyridine was mixed with each lyophilized sample before being shaken at 1,200 rpm for 90 min at 30˚C. Next, 20 μl of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) (GL Science, Tokyo, Japan) was added for derivatization, and the mixture was incubated at 1,200 rpm for 30 min at 37˚C. The mixture was then centrifuged at 16,000 ×*g* for 5 min at 4˚C, and the resultant supernatant was subjected to GC/MS measurement.

Tissue collection and preparation. The tumor and non-tumorous tissues were cut into small pieces from the excised specimen, transferred to a clean tube, and were immediately stored at –80˚C until use. Non-tumorous tissues were cut from areas that were sufficiently far from the tumor region. Twenty milligrams of tumor tissue were transferred to a clean tube and homogenized in 1,500 μl of a solvent mixture (MeOH:H2O:CHCl3=2.5:1:1). Then, 10 μl of 0.5 mg/ml 2-isopropylmalic acid (Sigma–Aldrich) were added to each tube, and the mixture was mixed well. The mixture was subsequently shaken at 1,200 rpm overnight at 37˚C, before being centrifuged at 16,000 ×*g* for 3 min at 4˚C. One thousand microliters of the obtained supernatant were collected in a clean tube, and 1,000 μl of distilled water were added to the collected supernatant before being centrifuged at 16,000 ×*g* for 3 min at 4˚C. Subsequently, 1500 μl of the resultant supernatant were collected into a clean tube, and 500 μl of $CHCl_3$ were added to the collected supernatant before being centrifuged at 16,000 ×*g* for 3 min at 4˚C. The upper layer obtained was lyophilized using a freeze dryer before oximation and derivatization. Oximation and the subsequent derivatization were performed as described for serum, and the resultant solution was subjected to GC/MS analysis.

GC/MS analysis. According to our previous report (11), GC/MS analysis was performed using a GCMS-QP2010 Ultra (Shimadzu Co., Kyoto, Japan) with a fused silica capillary column (CP-SIL 8 CB low bleed/MS; 30 m × 0.25 mm inner diameter, film thickness 0.25 μm; Agilent Technologies Inc., Palo Alto, CA, USA). The front inlet temperature was 230˚C. The helium gas flow rate through the column was 39.0 cm/s. The column temperature was held at 80˚C for 2 min and then raised by 15˚C/min to 330˚C and held there for 6 min. The transfer line and ion-source temperatures were 250˚C and 200˚C, respectively. Twenty scans per second were recorded over the mass range 85-500 *m/z* using the Advanced Scanning Speed Protocol (ASSP, Shimadzu Co.).

Data processing. Data processing was performed according to previous reports (11, 12). Briefly, MS data were exported in the netCDF format. Peak detection and alignment were performed using the MetAlign software (Wageningen UR, the Netherlands (11). The resulting data were exported in CSV- file format and analyzed with an in-house analytical software (AIoutput). This software enables peak identification and quantification using the in-house metabolites library. For semi-quantification, the peak height of each ion was calculated and normalized using the peak height of 2-isopropylmalic acid as an internal standard. In GC/MS analysis, there is the possibility that multiple peaks for a metabolite are detected. In this case, the peaks most reflecting the level of a metabolite were adopted for semi-quantitative evaluation.

Statistics. In the serum analysis, Bartlett's test was used to verify the homogeneity of variances. The statistical difference was determined by Student's *t*- or Welch's *t*-test for relative two-armed comparison or by paired-*t* or Wilcoxon test for non-relative two-armed comparison according to the homogeneity of variances. Regarding tissue analysis, after using Bartlett's test to verify the homogeneity of variances, the statistical difference was determined by the paired Student's *t*-test. In all analyses, a *p*-value of less than 0.05 was accepted as indicating statistical significance. All analyses were carried out using JMP Version 9 (SAS Institute Inc., Cary, NC, USA).

Results

Subject characteristics. The metabolomics analysis of sera and tissues was performed to establish a novel diagnostic approach for HNSCC. Sera were obtained from 17 patients with HNSCC: eight cases of hypopharyngeal cancers, seven oral cancers, one oropharyngeal cancer, and one cervical esophageal cancer. The tissues were obtained from 19 patients: eight cases of hypopharyngeal cancers, seven oral cancers, two laryngeal cancers, one oropharyngeal cancer, and one cervical esophageal cancer. These sera and tissues were subjected to GC/MS measurements. Four patients with oral cancer and one patient with cervical esophageal had relapse within 12 months after surgery. Eight patients did not have any remarkable complication. Four patients had hypertension, four patients had diabetes, three had other types of tumors that were completely cured before this study, one had alcoholic hepatitis, and one had hepatitis B. The other eight patients did not have any remarkable diseases other than HNSCC. The clinical characteristics of the patients are summarized in Tables I and II. Disease in all

Table I. *Clinical characteristics of patients with head neck cancer for serum analyses (n=17).*

Characteristic	No. of patients	No. of relapses
Gender		
Female	1	0
Male	16	5
Primary site		
Oral cavity	7	4
Oropharynx	1	0
Hypopharynx	8	0
Larynx	0	0
Cervical esophagus	1	1
Stage*		
I	2	0
II	5	3
III	1	1
IV	9	1

*According to the TNM Classification of Malignant Tumours 6th Edition (UICC).

Table II. *Clinical characteristics of patients with head and neck cancer for tissue analyses (n=19).*

Characteristic	No. of patients	No. of relapses
Gender		
Female	2	0
Male	17	5
Primary site		
Oral cavity	7	4
Oropharynx	1	0
Hypopharynx	8	0
Larynx	2	0
Cervical esophagus	1	1
Stage*		
I	1	0
II	6	3
III	3	1
IV	9	1

*According to the TNM Classification of Malignant Tumours 6th Edition (UICC).

patients was diagnosed by biopsy. Each cancer was classified using the sixth edition of the TNM Classification of Malignant Tumours (12). None of the patients had been treated with therapeutic agents, radiotherapy, or other therapies before this study. Blood samples were obtained before the treatment and three months after surgery. Four patients with hypopharyngeal cancer were treated with postoperative chemoradiotherapy, and three patients with hypopharyngeal cancer were treated with postoperative radiotherapy.

Table III. *The list of Significantly changed metabolites in the sera of head and neck cancer patients: Values of the peak intensity at three months after surgery divided by the preoperative peak intensity (Pre) were used for statistical analysis to correct for individual differences. According to the variance, p-values were calculated with Student's or Welch's t-test. Post 3M R, three months after surgery in patients with relapse. Post 3M, three months after surgery in patients without relapse.*

	Fold induction		
Compound	Post 3M R/Pre	Post 3M/Pre	*p*-Value
Lysine	0.6943	0.9633	0.010
Glucose	1.208	0.9190	0.011
Ribose	1.810	1.116	0.012
Fructose	1.701	0.8961	0.012
Tagatose	2.063	1.096	0.014
α-Sorbopyranose	1.610	0.8825	0.018
Hippurate	0.6060	1.907	0.021
Trans-4-Hydroxy-L-proline	0.7578	1.793	0.028
4-Hydroxymandelate	0.5739	3.609	0.029

Table IV. *Significantly changed metabolites in the sera of patients with oral cancer: Values of the peak intensity at three months after the surgery. Divided by the preoperative peak intensity (Pre) were used for statistical analysis to correct for individual differences. According to the variance, p-values were calculated with Student's or Welch's t-test. Post 3M R, three months after surgery in patients with relapse; Post 3M, three months after surgery in patients without relapse.*

	Fold induction		
Compound	Post 3M R/Pre	Post 3M/Pre	*p*-Value
Glucose	1.170	0.7600	0.003
Methionine	0.6286	0.9672	0.013
Ribulose	1.489	0.3953	0.016
Ketoisoleucine	0.9094	1.170	0.016

Serum metabolite profiling of HNSCC. A total of 112 metabolites were detected in serum (data not shown). The values of the peak intensity at three months after surgery divided by the preoperative peak intensity were used for the statistical analysis to correct the individual differences.

As shown in Table III, the levels of the following nine metabolites were significantly changed in sera of the patients who had disease relapse compared to those who did not have relapse: glucose, ribose, fructose, and tagatose (or psicose) significantly increased; and lysine, hippurate, trans-4-hydroxy-L-proline, and 4-hydroxymandelate significantly decreased ($p<0.05$ by Student's t or Welch's t-test). As shown in Table IV, the levels of the following four metabolites were significantly changed in sera of the oral

Table V. *Significantly changed metabolites in tumor tissues of patients with head and neck cancer: The data represent the relative values of the normalized peak intensity of tumor tissues against that of normal tissues. According to Variance, p-values were calculated with paired-t- or Wilcoxon test.*

Compound	Fold induction	*p*-Value
Coniferyl aldehyde	0.546	<0.0001
Pipecolic acid	0.550	<0.0001
3-Hydroxyisovaleric acid	0.557	<0.0001
Malonic acid	0.558	<0.0001
Phosphate	1.740	<0.0001
Inosine	1.877	<0.0001
2-Aminoethanol	2.741	<0.0001
Hypoxanthine	3.405	<0.0001
Putrescine	4.233	<0.0001
Homoserine	4.488	<0.0001
2-Dehydro-D-gluconate	5.150	<.0001
Uracil	6.229	<0.0001
1,3-Propanediamine	1.130	<0.001
Cysteine Sulfonic acid	1.719	<0.001
Oxalate	1.736	<0.001
Citrulline	2.133	<0.001
Glyceraldehyde	2.342	<0.001
Phenylalanine	2.510	<0.001
Valine	2.562	<0.001
Xylitol	2.564	<0.001
Threonine	2.569	<0.001
Tyrosine	2.951	<0.001
Glycine	3.218	<0.001
Proline	3.312	<0.001
Nicotinamide	3.356	<0.001
Histidine	3.950	<0.001
Aspartic acid	3.963	<0.001
Glutamic acid	4.199	<0.001
Asparagine	5.518	<0.001
Lysine	2.368	0.002
Serine	2.168	0.004
Trans-4-hydroxy-L-proline	2.795	0.004
Cytosine	0.427	0.005
2'-Deoxyuridine	0.706	0.005
Methionine	2.219	0.005
Glucose	0.180	0.006
Glucarate	1.673	0.006
O-Phosphoethanolamine	2.213	0.007
Glycerol	1.384	0.009
Glycolic acid	0.538	0.009
Pyroglutamic acid	1.734	0.010
α-Sorbopyranose	0.464	0.011
Sarcosine	2.057	0.012
Cystathionine	3.100	0.012
Fructose-6-phosphate	0.700	0.012
Fructose	0.432	0.013
2-Hydroxypyridine	0.711	0.013
Cytosine	1.043	0.015
Ornithine	2.463	0.018
Trehalose	2.748	0.018
S-Benzyl-L-Cysteine	0.669	0.020
Alanine	1.647	0.020
Melibiose	0.181	0.029
β-N-Methyl-amino-L-alanine	0.706	0.038
Ribose	1.554	0.040
Taurine	2.012	0.044

Table VI. *Significantly changed metabolites in tumor tissues of patients with oral cancer: The values represent the relative values of the normalized peak intensity of tumor tissues against that of normal tissues. According to the variance, p-values were calculated with paired-t- or Wilcoxon test.*

Compound	Fold induction	*p*-Value
Glycerol	1.482	<0.001
Xylitol	2.793	<0.001
2-Aminoethanol	3.452	<0.001
Nicotinamide	3.912	<0.001
Oxalate	1.959	0.001
Homoserine	2.568	0.002
Uracil	6.188	0.002
Putrescine	4.596	0.003
Inosine	2.144	0.003
Glycine	3.849	0.003
Hypoxanthine	3.457	0.003
Valine	2.542	0.004
Aspartic acid	5.504	0.005
Glutamic acid	5.945	0.005
Proline	3.168	0.006
3-Hydroxyisovaleric acid	0.419	0.007
Ascorbic acid	0.419	0.007
Coniferyl aldehyde	0.419	0.007
Malonic acid	0.419	0.007
Pipecolic acid	0.419	0.007
S-Benzyl-L-cysteine	0.445	0.007
Asparagine	4.677	0.007
Sarcosine	2.383	0.008
Threonine	2.754	0.008
Phosphate	2.110	0.009
Glyceraldehyde	2.188	0.010
Pyroglutamic acid	2.082	0.011
Acetoacetic acid	1.904	0.012
O-Phosphoethanolamine	4.046	0.013
2-Aminobutyric acid	1.873	0.019
Glucose	0.134	0.021
Tyrosine	2.684	0.022
Trans-4-Hydroxy-L-proline	2.455	0.023
Alanine	2.419	0.029
Phenylalanine	2.463	0.029
Serine	2.381	0.032
Methionine	2.186	0.033
Mannitol	2.375	0.033
Histidine	2.512	0.040

cancer patients who had relapse compared to patients with oral cancer who did not have disease relapse: glucose and ribulose significantly increased; methionine and ketoisoleucine significantly decreased ($p<0.05$ by Student's *t*- or Welch's *t*-test).

Tissue metabolite profiling of HNSCC. A total of 109 metabolites were detected in the tissues (data not shown). In the tissues obtained from the 19 patients, the levels of 41 out of the 109 metabolites significantly increased in the tumor

tissues compared with the non-tumorous tissues, while those of 15 of the 109 metabolites significantly decreased ($p<0.05$ by paired-*t*- or Wilcoxon test). In the tissues of seven patients with oral cancer, the levels of 32 out of the 109 metabolites were significantly increased in the tumor tissues compared with the non-tumor tissues, and the levels of 7 of the 109 metabolites significantly decreased ($p<0.05$ by paired-t- or Wilcoxon test) (Tables V and VI).

Discussion

In this study, sera from the 17 patients with HNSCC, as well as the surgically-resected tumors and the surrounding normal tissues from the 19 patients with HNSCC, were subjected to GC/MS-based metabolomics to investigate whether the pathogenesis of HNSCC leads to alterations in the levels of low-molecular-weight metabolites and, furthermore, to examine whether the targeted metabolites are useful for diagnosing HNSCC. As shown in Table III, the serum levels of metabolites related to the glycolytic pathway, namely, glucose1, ribose, and fructose, were revealed to be higher in the patients who had disease relapse than those who did not. The levels of amino acids lysine and *trans*-4-hydroxy-L-proline were lower in the patients who had disease relapse than those who did not. The serum levels of metabolites related to the glycolytic pathway, that is, glucose and ribulose, were higher in patients with relapse of oral cancer than those without relapse (Table IV), and the levels of amino acids, in particular, methionine and ketoisoleucine, were lower in patients with relapse of oral cancer than those without relapse (Table IV). In a previous study, the increase in the serum levels of metabolites related to the glycolytic pathway in patients with oral cancer was reported *via* Nuclear Magnetic Resonance (NMR)-based metabolomics, suggesting that alterations of these metabolites in sera of patients after surgical treatment for HNSCC may contribute to early detection of recurrence (14).

In contrast to the sera, the tissue levels of many metabolites related to the glycolytic pathway were lower in the tumor tissues than in the non-tumorous tissues (Tables V and VI), whereas the levels of phenylalanine, valine, threonine, tyrosine, glycine, proline, histidine, aspartic acid, glutamic acid, asparagine, lysine, serine, methionine, and alanine, were higher in the tumor tissues than in the non-tumorous tissues (Tables V and VI). In a previous report, Hirayama *et al*. revealed a lower level of glucose and higher levels of many amino acids in tumor tissues than in normal tissues from patients with colon and stomach cancer (5); our results are consistent with this. Most cancer cells depend on aerobic glycolysis rather than oxidative phosphorylation for energy production (15). Cancer cells also use glutamine as a major source of energy (16), and glutamine metabolism allows tumor cells to sustain TCA cycle activity during tumor proliferation. Hirayama *et al*. indicated that cancer cells deplete glucose in the hypovascular microenvironment (5) and the amino acid levels are higher in tumors through the degradation of the extracellular matrix (17) and the autophagic degradation of pre-existing intracellular proteins (18). In *in vitro* experiments, HNSCC tumor cells were dependent on glucose but not on glutamine for energy production and survival (19). Therefore, the levels of glucose and amino acids in HNSCC may reflect this uniquely contrasting behavior.

In conclusion, our results demonstrate that changes in the metabolite pattern are useful in assessing the clinical characteristics of HNSCC. To establish a novel diagnostic tool, these results should be validated in a large prospective cohort. Comparison with metabolomics of healthy individual should also be conducted.

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