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Amino acids signatures of distance-related surgical margins of oral squamous cell carcinoma

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ABSTRACT

Background: Histological assessment of resected margins has some drawbacks. We therefore aimed to identify a panel of metabolic markers for evaluating the surgical margins of oral squamous cell carcinoma during surgery.

Methods: A total of 28 case of OSCC samples were enrolled in the study. Gas chromatography-mass spectrometry based untargeted metabolic analysis was employed to acquire the metabolic perturbation of the distance-related surgical margins in the development group. The acquired MS data were then subjected to univariate and multivariate analysis by MetaboAnalyst. Ultra–high performance liquid chromatography–tandem mass spectrometerbased targeted metabolomics for quantitative analysis of the validation group was performed to verify the results of the development group. Another 60 OSCC patients with dysplastic surgical margins were used to further validate the results of the development group by immunohistochemical examination of key enzyme expression, and correlate them with clinicopathological parameters and clinical outcomes.

Findings: We finally identified 4 amino acids as negative margin markers, and 6 amino acids as dysplastic margin markers. IHC analysis showed that asparagine synthetase positive expression in dysplastic surgical margins and its higher expression was correlated with tumor recurrence and local relapse-free survival.

Interpretations: We developed a panel of metabolic molecular markers to supplement the evaluation of negative and dysplastic margins.

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Research in context

Evidence before this study

Obtaining negative surgical margins during surgery has always been the pursuit of tumor surgeons. In current clinical practice, histological assessment of the resected margins is the gold standard for determining the status of surgical margin of oral squamous cell carcinoma (OSCC). However, histological evaluation has significant deficiencies, such as the limitation of the thickness of slide, and other reasons, resulting the higher local recurrence (LR) although the margin status was negative. Many studies have demonstrated metabolic reprogramming in cancer or precancerous cells. Therefore, we aimed to identify a panel of metabolic molecular markers for evaluating the surgical margins of OSCC during the surgery.

Added value of this study

In this study, we first elucidated the characteristics of molecular changes in OSCC at different distances from surgical margins in terms of amino acid metabolism. We developed 4 amino acids as negative margin markers, and 6 amino acids for dysplastic margin markers. We further examined asparagine synthetase (aspartic acid metabolic key enzyme) expression in 60 OSCC samples with dysplastic margins by IHC analysis, which showed that ASNS positive expression in dysplastic surgical margins was correlated with tumor recurrence and local relapse-free survival (RFS). These results indicate that the amino acid markers at the surgical margin have positive clinical significance.

Implications of all the available evidence

Evaluation of surgical margins at the molecular level is a promising new molecular diagnostic method, which can effectively supplement the traditional pathological evaluation. The report of molecular margin analysis in OSCC stems from 1953, the observation that histologically normal tissue harbors clonal populations of cells with premalignant change, namely field cancerization. However, most studies on molecular margin were restricted to single gene or protein, and lacked molecular combinations that effectively predicted the margin status. In this study, molecular markers of the surgical margin of oral squamous cell carcinoma were determined at the level of amino acid metabolism. It provides a theoretical basis for the development of amino acid diagnostic reagents and the improvement of margin evaluation.

1. Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common head and neck cancers, with 6 million deaths worldwide every year [1]. Tobacco (smoked or chewed), alcohol consumption, and human papillomavirus infection are the most important risk factors for OSCC [2]. Although OSCC management has been greatly improved, only a minor improvement in OSCC survival has been obtained over the last 30 years, partly due to the difficulty in obtaining 'clear' resection margins. The pathological margin is an important prognostic factor for relapse-free survival (RFS) of oral cancer patients treated with primary surgery, because tumor cells or dysplastic epithelia that remain in the margins may lead to the local recurrence (LR) of OSCC [3–6]. Our team previously reported that patients with mild dysplasia margins who did not undergo re-excision had a worse prognosis than those with negative margins [7]. Therefore, an overarching goal of oncologic surgery is to resect tumors with histological tumor-free margins, as adequate surgical resection is essential for good local control and improved prognosis. Paradoxically, excising dysplasia area adjacent to the cancer impairs the life quality of patients. Additionally, the LR rates still hover from 10% to 30% even if the histological surgical margins are 'clear' [8]. Therefore, determining the molecular markers of margins is especially crucial to supplement the pathological diagnosis.

One of the most striking features of cancer cells is metabolic reprogramming, where cancer cells alter metabolic pathways to adapt to environmental stress and meet their growth need. For example, many cancers display an increasing demand for specific amino acids and become dependent on either an exogenous supply or upregulated de novo synthesis. Moreover, targeting the 'addicted' amino acids showed promising clinical applications [9,10]. Therefore, a better understanding of the dysregulated amino acids and related pathways that control OSCC progression is essential for developing diagnostic and prognostic predictors.

Recently, many tumor tissues and biological fluid samples have been studied in metabolic research [11,12]. A list of metabolites has been identified to distinguish normal and tumor samples [13]. However, the metabolic study of OSCC margin has not been reported. Therefore, our purpose was to characterize the amino acids that clearly delineate OSCC margins and to supplement the clinicopathological diagnosis.

2. Materials and methods

2.1. Patients and tumor specimens

Our study was approved by the medical ethics committee of the Nanjing stomatology hospital. A total of 28 cases (each case including Tumor, Margin-1, Margin-2 and Margin-3) were recruited in the study. 8 of the 28 cases were used for developing markers (Development group), and another 20 cases (Validation group) were used to validate the results of the Development group. Two adjacent lumps of tissues (containing tumor and the longest margin, Fig. 1**A**) were collected from every patient. One was used for pathological assessment, while the other was used for mass spectrometry (MS) analysis. All samples were divided into four groups: Tumor, Margin-1 (0–0.5 cm from tumor margin), Margin-2 (0.5–1 cm from tumor margin), Margin-3 (>1 cm from tumor margin) **(**Fig. 1**B).** All tissues were frozen in liquid nitrogen and stored at −80 °C before sample preparation for gas chromatography–mass spectrometry (GC–MS) or ultra–high performance liquid chromatography - tandem mass spectrometer (UHPLC-MS/MS). Tumor, Margin-1, Margin-2 and Margin-3 samples were analyzed by GC–MS or UHPLC-MS/MS **(**Fig. 1**C).** The clinical details of the 28 patients are shown in **Supplemental** Table 1. The HE-stained samples were evaluated by professional pathologists. The Tumor group contained 80% tumor cells. The Margin-1 group contained dysplastic epithelium, while the Margin-2 and Margin-3 groups were histologically negative margins. And the Margin-3 group can be identified as normal tissues **(**Fig. 1**B).**

2.2. Gas chromatography-mass spectrometry untargeted analysis

GC–MS experiment is under the guidance of professor Tong Xie (Nanjing University of Chinese Medicine). Materials and reagents for GC–MS analysis were prepared according to previous method. GC–MS analysis was performed by Trace 1310 Gas Chromatograph equipped with an AS 1310 auto sampler, which connected a TSQ 8000 triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA). See references for specific steps [14].

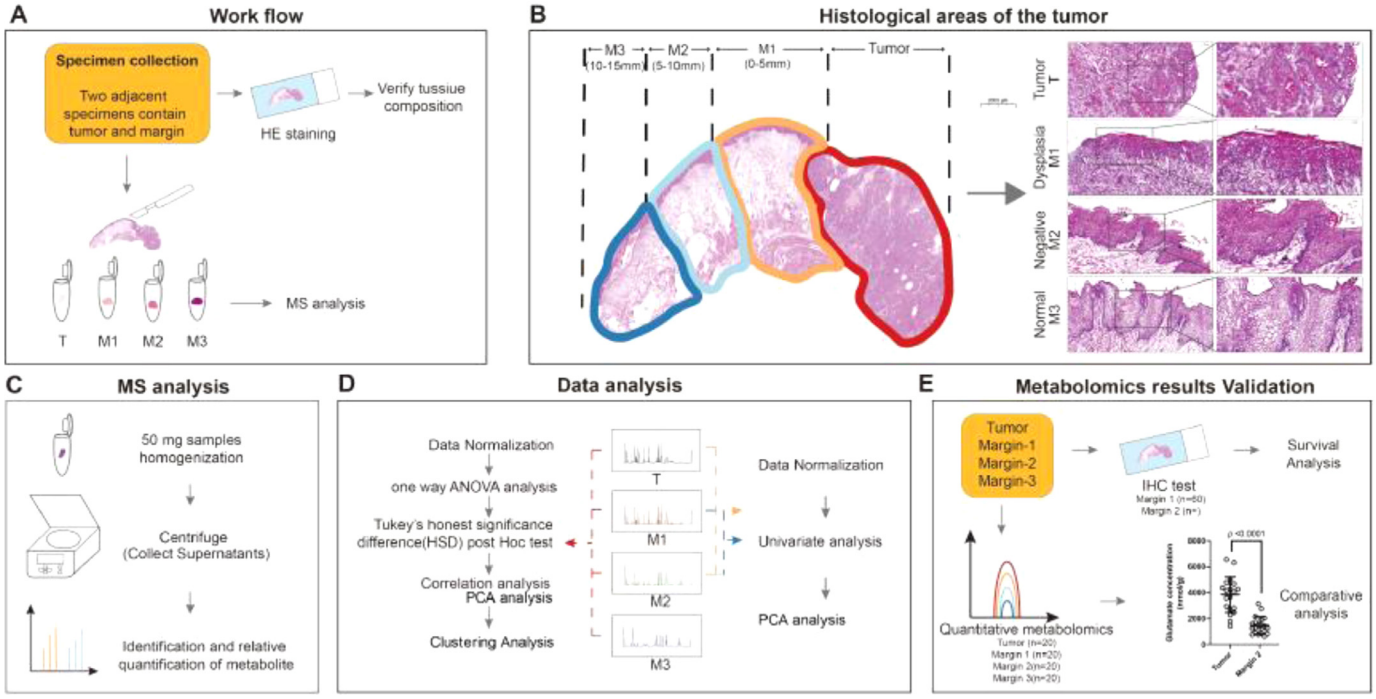

Fig. 1. The overall design of this experiment. **A** Work flow. **B** HE staining of the whole tissue which were divided into four parts: Tumor (T), Margin-1 (Dysplasia), Margin-2 (Negative), Margin-3 (Normal). **C** MS analysis. **D** Data analysis. **E** Validation, including validating in 20 patients using targeted quantitative analysis and IHC stain of ASNS in 60 OSCC patients with dysplastic margins.

Table 1
Metabolic overall analysis among the four groups (Tumor, Margin-1, Margin-2 and Magin-3) by one way ANOVA and Tukey's honest significance difference (HSD) post Hoc test analysis.

		TvsM1		TvsM2		TvsM3		M1vsM2		M1vsM3		M2vsM3	
No.	Metaboli te.name	log2FC	P	log2FC	P	log2FC	P	log2FC	P	log2FC	P	log2FC	P
1	glutamate	0.044	0.999	0.897	0.011	0.885	0.012	0.853	0.018	0.841	0.019	-0.012	0.999
2	alanine	-0.021	0.999	0.826	0.033	0.639	0.102	0.847	0.026	0.661	0.083	-0.186	0.953
3	glutamine	-0.751	0.481	2.909	0.278	2.398	0.334	3.661	0.013	3.151	0.018	-0.511	0.999
4	proline	-0.105	0.975	0.98	0.051	0.679	0.187	1.085	0.019	0.784	0.084	-0.3	0.916
5	serine	-0.047	0.996	0.807	0.054	0.656	0.123	0.854	0.034	0.703	0.081	-0.151	0.978
6	spartic aci	-0.1	0.981	0.972	0.072	0.716	0.198	1.073	0.031	0.816	0.097	-0.256	0.953
7	lysine	-0.263	0.89	1.509	0.118	0.818	0.424	1.773	0.025	1.082	0.131	-0.691	0.867
8	ornithine	0.176	0.936	1.151	0.044	0.797	0.163	0.975	0.148	0.621	0.413	-0.354	0.92

Firstly, Performed by One way ANOVA threshold p<0.05, then Tukey's honest significance difference(HSD) post Hoc test threshold P <0.05 and Fold change>1.5 or <0.667.
P value:Tukey's honest significance difference(HSD) post Hoc test. FC: fold change

2.3. UHPLC-MS/MS targeted quantitative analysis

An Agilent 1290 Infinity II series UHPLC System (Agilent Technologies), equipped with a Waters ACQUITY UPLC BEH Amide column (100 × 2.1 mm, 1.7 μm) carried UHPLC separation. 1% formic acid in water construct mobile phase A, and 1% formic acid in acetonitrile construct mobile phase B. The temperature of the column was set to 35 °C. The temperature of auto-sampler temperature was set to 4 °C and the injection volume was set to 1 μL. We applied Agilent 6460 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an AJS electrospray ionization (AJS-ESI) interface for metabolite assay development. Through the standard of each metabolite, the optimal MRM parameters of the target metabolites are obtained. MRM data acquisition and processing were performed by Agilent MassHunter Work Station Software (B.08.00, Agilent Technologies).

2.4. Histopathological examination of tissues

Tumor and surgical margin specimens were fixed with 10% formalin, and then hematoxylin-eosin (HE) staining was performed to identify the cellular components. The cell morphology and tissue composition were observed under microscope (Olympus, Tokyo, Japan).

2.5. Immunohistochemical examination

The specific steps of immunohistochemistry are described in our previous paper [15]. A total of 60 OSCC patients with dysplastic surgical margins were used to further validate the results of the development group by immunohistochemical stain of asparagine synthetase (ASNS) (Proteintech, China), and correlate with clinicopathological parameters and clinical outcomes by SPSS 18.0 soft-

ware package. Relationship between ASNS expression and prognosis was performed by GraphPad Prism 8 software package.

2.6. Metabolomics data processing

After removing the artificial peaks due to derivatization, we obtained raw data files from GC–MS by searching against NIST 2014 standard mass spectral databases (Thermo Scientific, Waltham, MA). The peak area of each metabolite was calculated by Xcalibur 2.2 by normalizing to the internal standard. Specific concentrations of individual metabolites were obtained by UHPLC-MS/MS analysis (nmol/g).

2.7. Statistical analysis

Our metabolomic statistical analyses were performed using the MetaboAnalyst, which offers a variety of methods commonly used in metabolomic data analyses. The differential amino acids were first selected using students' *t*-test with fold change >1.5 and *p*-value <0.05. Then, the diagnostic performances of the differential metabolites were evaluated by receiver operating characteristic (ROC) curve analysis. The metabolic variance between the multiple groups (Tumor,Margin-1,Margin-2 and Margin-3) were analyzed using one way ANOVA and Tukey's honest significance difference (HSD) post Hoc test.

In development group, the substantial experimental data were normalized by the total spectral intensity and additionally Pareto scaled (for multivariate analysis). Univariate analysis was by ANOVA, *t*-test with application of a False-Discovery Rate (FDR) adjusted *p*-value of 0.05. Multivariate analysis was via unsupervised Principal Component Analysis (PCA) followed by Partial Least Squares Discriminant Analysis (PLS-DA) validated via leave one out cross-validation to automatically determine optimal number of components as well as the quality of the model (described in terms of accuracy). Hierarchical cluster analysis was carried out using pheatmap package.

Validation group the resulting lower-limits of detection and quantitation (LLODs and LLOQs) which the LLODs ranged from 2.44 to 156.25 nmol/L and the LLOQs ranged from 4.88 to 312.50 nmol/L for all the analytes. Correlation coefficients (R2) of regression fitting were above 0.9963 for all the analytes, indicating a good quantitative relationship between the MS responses and the analyte concentrations, which was satisfying for targeted metabolomics analysis. Analytical recoveries and relative standard deviations of the (quality control) QC samples, with 8 technical replicates. The recoveries determined were 92.8%–111.3% for all the analytes, with all the (relative standard deviations) RSDs below 5.7% ($n = 8$). The difference of quantitative analysis results between the two groups was analyzed by GraphPad Prism 8 (*t*-test, $p < 0.05$).

3. Results

3.1. Clinical characteristics of the OSCC patients in this study

The overall flow chart of the study is shown in Fig. 1**.** A total of 28 cases (each case including Tumor, Margin-1, Margin-2, and Margin-3 sections) were included in the study, eight of which were used to identify the markers (development group) and the other 20 to validate the results of the development group (validation group). The patients were divided into four groups (Tumor, Margin-1, Margin-2, and Margin-3) according to the margin distance and analyzed by GC–MS or UHPLC-MS/MS **(**Fig. 1**C).** The clinical details of the 28 patients are shown in **Supplemental Table 1**. The HE-stained samples were evaluated by pathologists. The Tumor group contained 80% tumor cells. The Margin-1 group contained dysplastic epithelium, while the Margin-2 and Margin-3 groups were histologically negative. The Margin-3 group can be identified as normal tissue **(**Fig. 1**B).**

3.2. Untargeted metabolomic analyses of the distance-related margins in development group

In order to identify the metabolic profiles of the distance-related margins, we performed GC–MS based untargeted analysis **(**Fig. 2**A).** The identified metabolites were mainly amino acids, followed by carbohydrates. Amino acid metabolic reprogramming was reported to occur in tumor cells, so we focused on OSCC dysregulated amino acid metabolism [16]. Initially, an unsupervised cluster analysis of all metabolites was performed using the k-means clustering method**,** which did not show a clear clustering **(Supplementary Fig. 1)**. However, when we selected the 8 amino acids that were the most significantly differentiated (filtrated by frequency: appeared in more than 50% of samples in at least one group of samples, p value < 0.05 and fold change >1.5 or <0.667) out of 244 named entities among the four groups (Tumor, Margin-1, Margin-2, and Margin-3), the four groups were interestingly clustered into two levels (Fig. 2**B).** Tumor and Margin-1 groups were clustered together, while Margin-2 and Margin-3 groups were clustered together. The clustering results were consistent with the pathological diagnosis of Tumor, Margin-1, Margin-2, and Margin-3.

3.3. Identification of four amino acids as potential negative surgical margin biomarkers using targeted metabolomic analyses

As obtaining negative surgical margins is important for tumor surgery, we investigated the amino acid biomarkers associated with negative margins. Margin-2 was identified as the negative surgical margin by two pathologists **(**Fig. 1**B).** We then tried to identify biomarkers that distinguished the negative margins from tumors by comparing the Tumor and Margin-2 groups in the development group ($n = 8$). All metabolites were first analyzed using a volcano plot with fold change>1.5 or <0.667 and p value <0.05. There were 36 out of 244 named entities with fold change >1.5 or <0.667 and p value <0.05, including 16 amino acids (fold change >1.5 and p value <0.05) (Fig. 2**C, Supplementary Table 2**). Additionally, the sPLS-DA analysis based on the 16 amino acids achieved great separation between the Tumor and Margin-2 groups **(**Fig. 2**D).** We then tested the ability of the 16 amino acids to distinguish Tumor from Margin-2 using ROC curve analysis. 10 amino acid markers (AUC > 0.90) displayed high sensitivity and specificity **(**Fig. 3**A-J)**, and another 6 amino acid markers (AUC > 0.80) displayed moderate sensitivity and specificity in the development group (**Supplementary Table 3**).

Next, we examined the ability of 10 markers (AUC > 0.90) to predict the negative surgical margin (Margin-2) using UHPLC-MS/MS based targeted quantitative analysis in the validation group ($n = 20$). The results showed 9 out of 10 were consistent with development group, which had significant differences ($p < 0.05$) between Tumor and Margin-2 **(**Fig. 4**A-I,** Table 2**).** Moreover, the concentration of these 9 amino acids showed an increasing trend from normal tissue (M3) to tumor (T) **(Supplementary Fig. 4A-I).** We tested the ability of these 9 amino acids to distinguish Tumor from Margin-2 by ROC curve analysis in the validation group, four out of nine amino acid markers (aspartic acid, glutamate, proline, valine) displayed high sensitivity and specificity **(**AUC > 0.90) for diagnosing negative margins (Table 2**).** These 4 amino acids (aspartic acid, glutamate, proline, valine) were analyzed by binary logistic regression in Glmnet package which obtained optimal sensitivity and specificity **(**AUC = 0.98, Fig. 4**J).** Therefore, the combination of the 4 amino acids can accurately predict negative surgical margins.

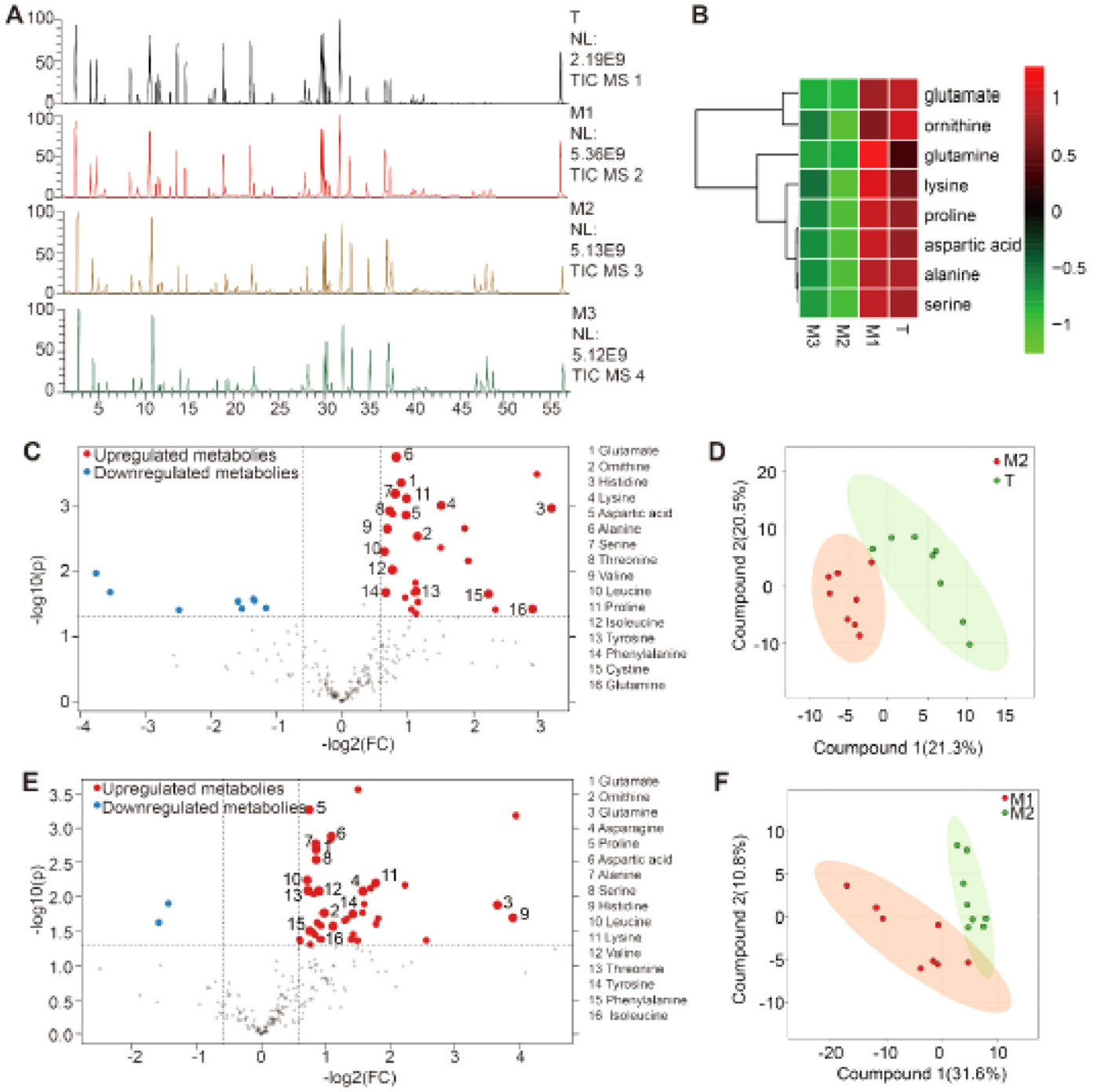

Fig. 2. Differential metabolites for negative and dysplasia margin. **A** Representative mass spectra of Tumor (T), Margin-1 (M1), Margin-2 (M2), Margin-3 (M3). **B** The heat map of the 8 amino acids levels between Tumor, Margin-1, Margin-2 and Margin-3. **C** Volcano plot of Tumor and Margin-2. **D** PLS-DA models based on the 16 amino acids for Tumor and Margin-2. **E** Volcano plots of Margin-1 and Margin-2. **F** PLS-DA models based on the 16 amino acids for Margin-3 and Margin-2.

3.4. Six amino acids were identified as potential dysplastic surgical margin biomarkers using targeted metabolic analyses

Dysplastic epithelium identified during surgery has been proved as an unsafe margin. Therefore, it is becoming increasingly urgent to find specific biomarkers of dysplastic surgical margin. In this study, we first compared metabolic levels between Margin-2 and Margin-1 in the development group ($n = 8$). Margin-1 was confirmed as a dysplastic margin by pathologists. All metabolites were first analyzed by a volcano plot with fold change>1.5 or < 0.667 and p-value<0.05.There were 44 out of 244 named entities with fold changes > 1.5 or < 0.66 and p < 0.05, including 16 amino acids (fold changes > 1.5 and p<0.05) (Fig. 2**E, Supplementary Table 4**). Additionally, the sPLS-DA analysis based on the 16 amino acids revealed a noteworthy difference between the Margin-1 group and Margin-2 group (Fig. 2**F**). Similarly, when the individual amino acids were tested for the ability to detect Margin-1 based on ROC curve analysis, 9 amino acids (AUC > 0.90) displayed a good sensitivity and specificity (Fig. 5**A-I**).

We also tested the ability of these 9 amino acids (AUC > 0.90) to predict the dysplastic surgical margin (Margin-1) by UHPLC-MS/MS based targeted quantitative analysis in the validation group

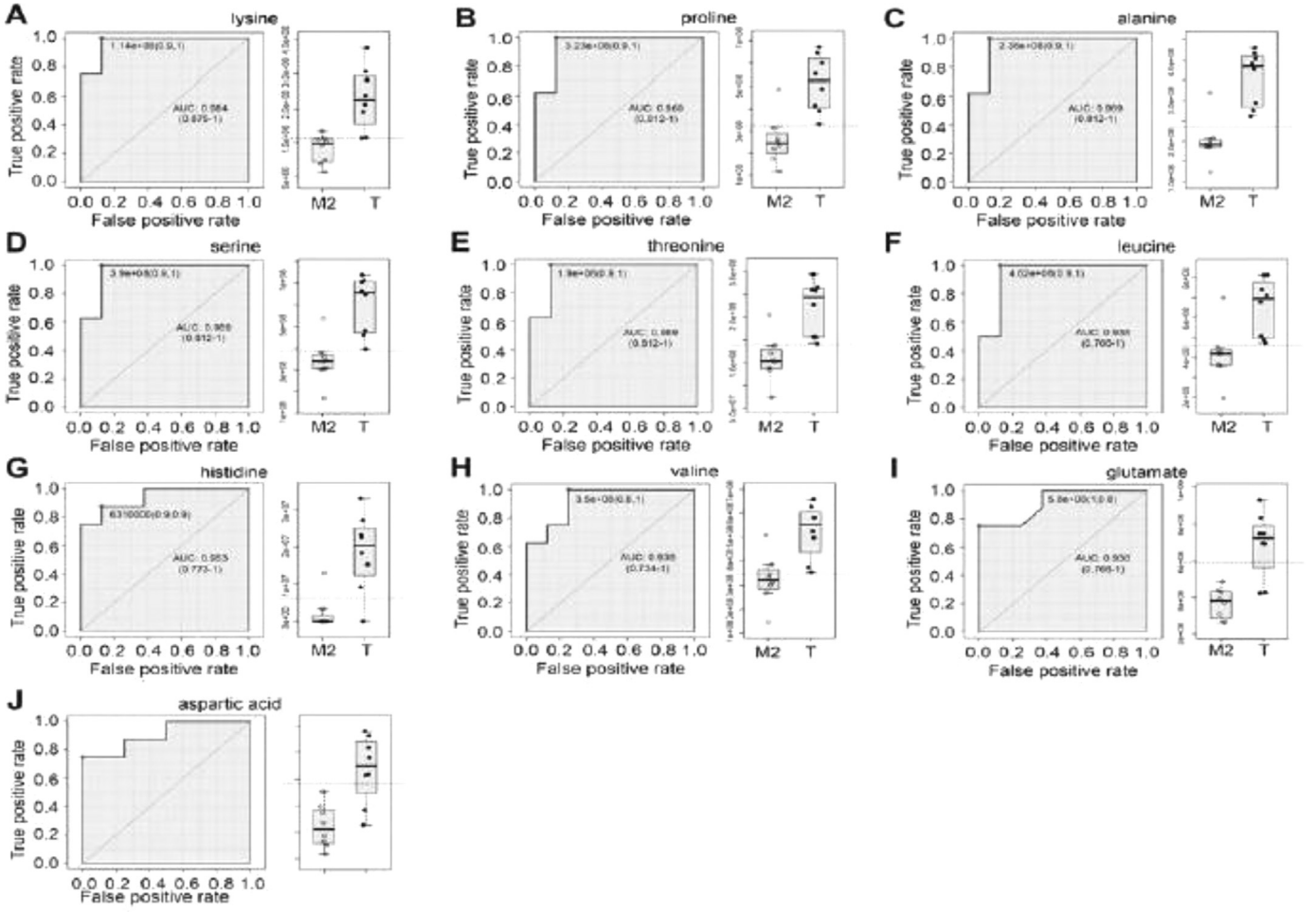

Fig. 3. Receiver operating characteristic (ROC) curves for lysine, proline, alanine, serine, threonine, leucine, histidine, valine, glutamate, aspartic acid, (A-J) between Tumor and Margin-2 in development group.

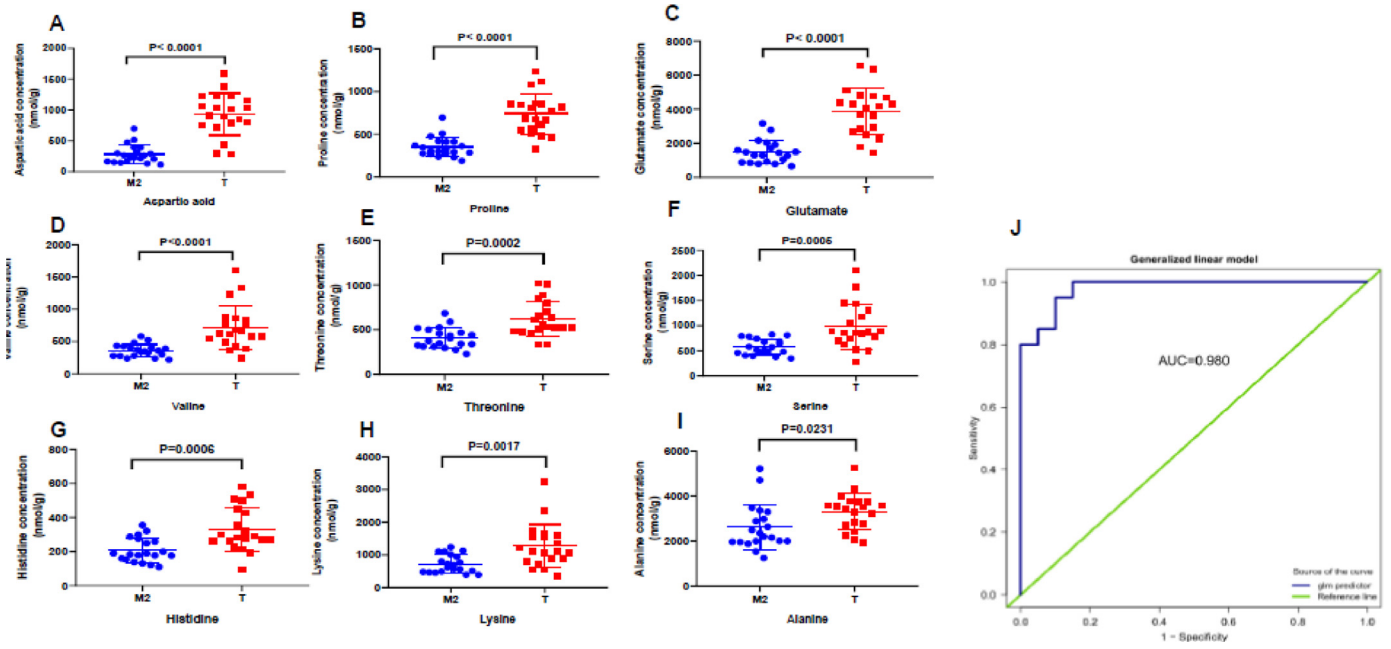

Fig. 4. UHPLC-MS/MS based quantitative analysis of negative margin markers in validation group. **A-I** The levels of the aspartic acids, proline, glutamate, valine, threonine, serine, histidine, lysine, alanine, between Tumor and Margin-2. The results showed 9 out of 10 were consistent with development group, which had significant differences ($P < 0.05$) between tumor and Margin-2. **J** Four amino acids (aspartic acid, glutamate, proline, valine. AUC > 0.90, *t*-test $P < 0.0001$) in validation group were performed by binary logistic regression in Glmnet package, obtain optimal sensitivity and specificity (AUC = 0.98).

Table 2
The list of amino acids as negative surgical margin (Margin-2) markers by ROC curve analysis (AUC > 0.90).

Metabolite	AUC values		Agreement	Test power
	Development group (n=8)	Validation group (n=20)		
lysine	0.97	0.79	yes	0.98
proline	0.95	0.94	yes	0.99
alanine	0.95	0.75	yes	0.88
serine	0.95	0.85	yes	0.99
threonine	0.95	0.84	yes	0.99
histidine	0.94	0.81	yes	0.99
valine	0.92	0.9	yes	0.99
glutamate	0.91	0.95	yes	0.99
aspartic acid	0.91	0.96	yes	0.99
leucine	0.94	not detected	not detected	not detected

ROC:Receiver operating characteristic; Agreement: metabolite in both the development group and the validation group was statistically significant (yes, $p < 0.05$ in t test).
Leucine was not detected by UHPLC-MS/MS in validation group.
Test power performed by R.stats package (threshold > 0.80) in validation group.

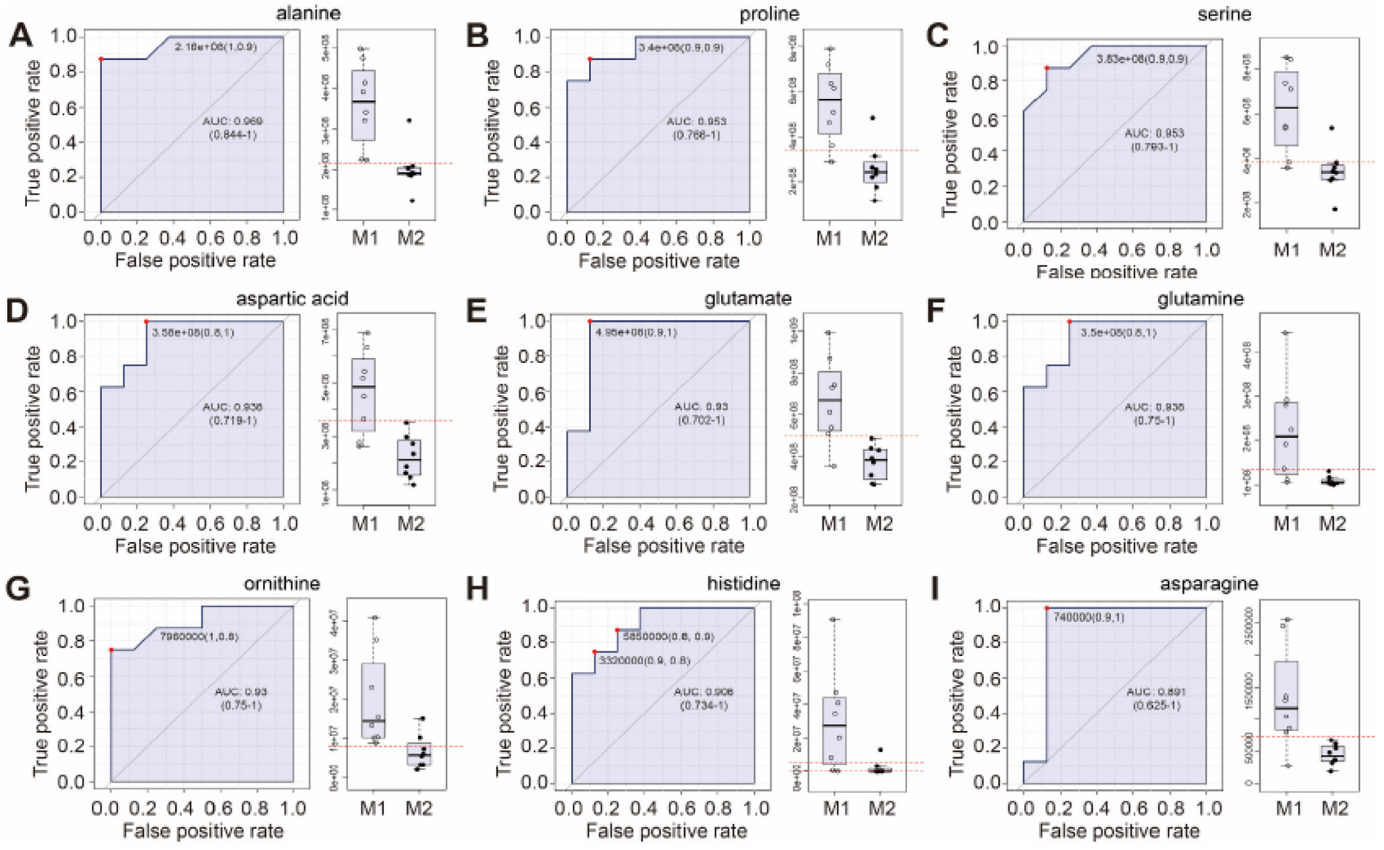

Fig. 5. ROC curves for proline, alanine, serine, aspartic acid, glutamate, glutamine, ornithine, histidine, asparagine, (A-I) between Margin-1 and Margin-2 in development group.

($n = 20$). The results showed 6 out of 9 were consistent with the development group, which had significant differences ($p < 0.05$) between Margin-1 and Margin-2 (Fig. 6**A-F,** Table 3). Moreover, the concentration of these 6 amino acids showed an increasing trend from normal tissue (Margin-3) to tumor (Tumor) **(Supplementary Fig. 4).**

We also tested the ability of these six amino acids to distinguish Margin-1 from Margin-2 by ROC curve analysis in the validation group. All the six amino acids displayed moderate sensitivity and specificity **(**AUC: 0.65–0.76) (Table 3**).** When all the 6 amino acids were evaluated by binary logistic regression in the Glmnet package, an optimal sensitivity and specificity were obtained **(**AUC = 0.81, Fig. 6**G).** Therefore, these six amino acid combinations have a higher ability to predict dysplastic margins than an individual amino acid and may be potential biomarker panel for OSCC dysplastic margins.

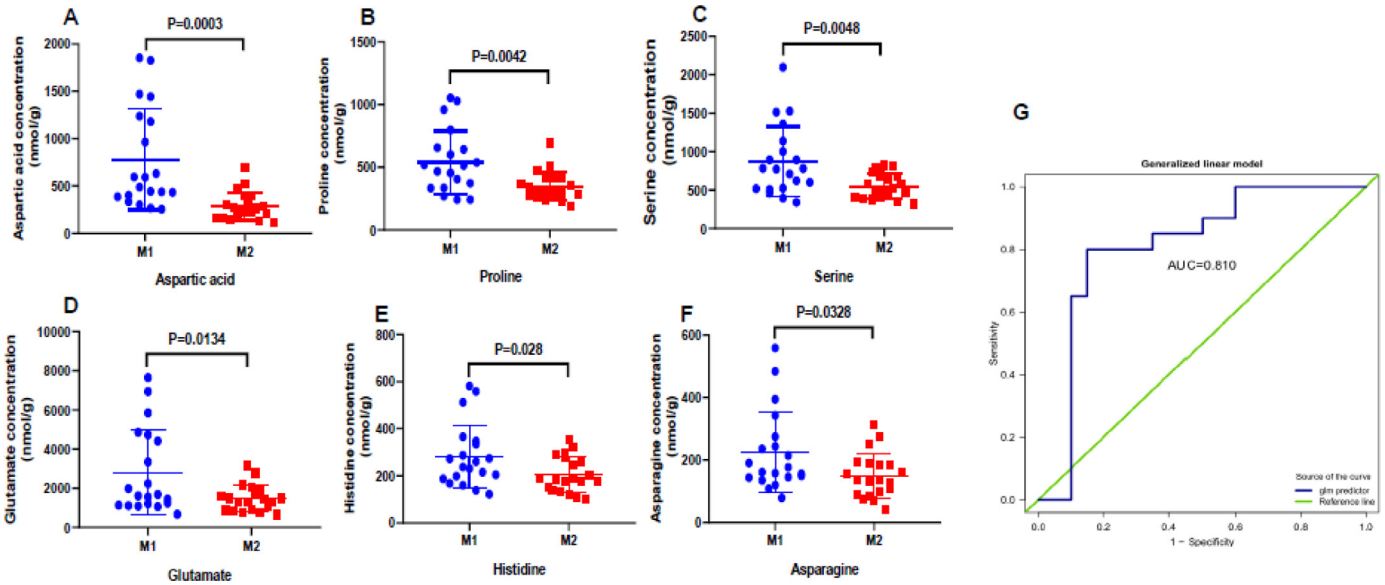

Fig. 6. UHPLC-MS/MS quantitative analysis of dysplastic margin biomarkers (AUC > 0.90) identified by in validation group. A–F The levels of the aspartic acids, proline, serine, glutamate, histidine, asparagine, between Margin-1 and Margin-2. The results showed 6 out of 9 were consistent with development group, which had significant differences ($P < 0.05$) between Margin-1 and Margin-2. G All of 6 amino acids were performed by binary logistic regression in Glmnet package, a significantly improved sensitivity and specificity.

Table 3
The list of amino acids as dysplastic surgical margin (Margin-1) biomarkers by ROC curve analysis (AUC > 0.90).

	AUC values			
Metabolite	**Development group (n=8)**	**Validation group (n=20)**	**Agreement**	**Test power**
aspartic acid	0.92	0.76	yes	0.97
proline	0.94	0.75	yes	0.97
glutamate	0.92	0.67	yes	0.92
asparagine	0.91	0.66	yes	0.8
histidine	0.91	0.66	yes	0.8
serine	0.94	0.66	yes	0.87
ornithine	0.91	0.66	no	0.56
alanine	0.96	0.59	no	0.41
glutamine	0.92	0.53	no	0.15

ROC:Receiver operating characteristic; Agreement: metabolite in both the development group and the validation group was statistically significant (yes, p<0.05 in t test)
Test power performed by R.stats package (threshold > 0.80) in validation group.

3.5. High expression of ASNS in dysplastic surgical margins predicted poor clinical outcomes

Targeted quantitative analysis showed that asparagine could be used as a potential biomarker for dysplastic margins. Moreover, aspartic acid and asparagine levels gradually increased from normal tissue to tumor indicating their important role controlling OSCC progression (Fig. 7**A, B**). Additionally, pathway enrichment analysis of tumors by KEGG showed that aspartic acid metabolism was active in tumor tissues (Fig. 7**C**). In order to elucidate the clinical significance of asparagine as potential biomarkers for dysplastic margin, we then examined the ASNS expression levels in dysplastic margin tissues by IHC analysis (Fig. 7**D**) and further explored its prognostic value (Fig. 7**G**). Results showed that the expression of ASNS in dysplasia margins was positively correlated with tumor recurrence ($p < 0.05$) (Table 4) and the expression levels of ASNS increased gradually with the deterioration of hyperplasia (Fig. 7**E**). We also found that high ASNS expression predicted poor local RFS (Fig. 7**F**). These results suggested that asparagine and its key enzyme ASNS may be involved in the malignant transformation of the oral epithelium and the relevant mechanism of malignant transformation deserves further study.

4. Discussion

The goal of oncological surgery is to completely remove all cancerous tissue or the “dangerous” tissues. To achieve this, surgeons usually resect surrounding tissues beyond the gross tumors to ensure the complete removal of cancer cells that will invade into the adjacent tissues [1]. Practically, because of the unique anatomical site, the resection range is limited during oral cancer surgery in order to preserve the structures and function of the organs. Therefore, surgical margins are assessed by pathologists to determine whether tumor cells are present. Traditionally, surgeons and pathologists have classified surgical margins as involved margins (margin ≤ 1 mm), close margins (margin 1–5 mm), or clear margins (margin > 5 mm) [5]. However, even if the histological surgical margins are ‘negative’, patients still have a risk of relapses [17].

Current approaches to determining tumor boundaries rely heavily on the histopathologic analysis of frozen sections. Addition-

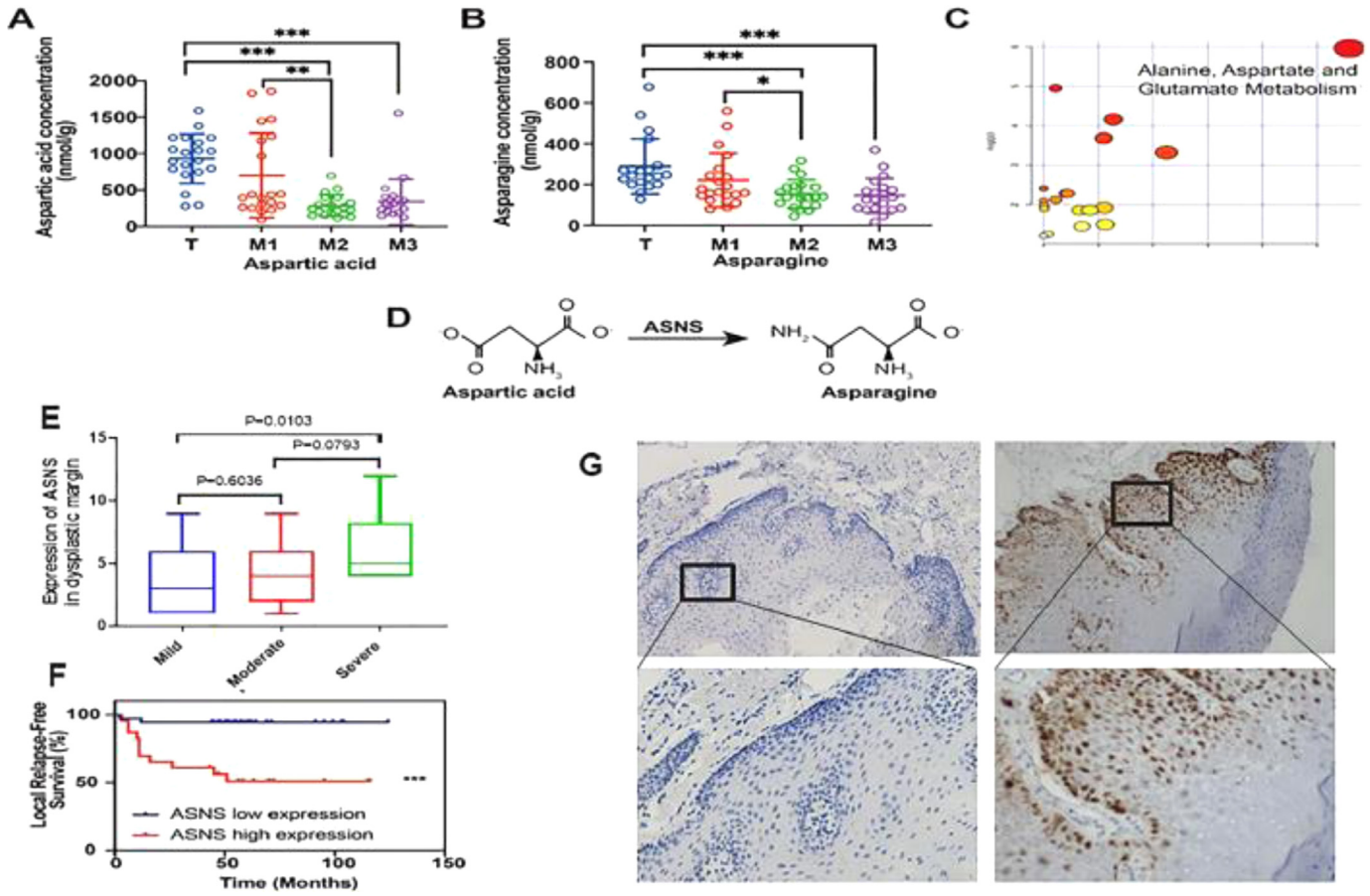

Fig. 7. ASNS expression in dysplastic surgical margins and its clinical significance. Aspartic acid (**A**) and asparagine (**B**) concentration in Tumor, Margin-1, Margin-2 and Margin-3 by UHPLC-MS/MS quantitative analysis. **C** Pathway enrichment analysis of OSCC (tumor vs normal) by KEGG. **D** Schematic diagram of aspartic acid metabolism: ASNS catalyzes the synthesis of asparagine from aspartic acid. **E** Box plot of IHC scores of ASNS in mild, moderate and severe surgical margin. **F** Kaplan Meier curves for local relapse free survival between ASNS low and high expression **G** The representative IHC images of ASNS low and high expression in mild dysplastic surgical margin. **** for $p < 0.0001$, *** for $p < 0.001$, ** for $p < 0.01$, * for $p < 0.05$.

ally, alternative techniques for margin assessment include Mohs, non-fluorescent dyes, fluorescent dyes, auto-fluorescent imaging, narrow-band imaging, confocal microscopy, and high-resolution microendoscopy [18]. Although there are many ways to evaluate the OSCC surgical margins, strategies for assessing the completeness of resection developed slowly. Therefore, introducing the concept of the 'molecular surgical margin' as a supplement to the traditional pathological surgical margin may represent an advance.

Previous studies have shown that post-surgery recurrence of OSCC develops from genetically altered fields around the primary tumors [19,20].

Molecular margin analysis of OSCC was first reported in 1953, with the observation that histologically normal tissue harbors clonal populations of cells with premalignant changes, namely field cancerization [21]. Since then, with the further understanding of the genetic mechanism of malignant transformation of normal margin tissues, a number of potential clinical markers have been found. At present, molecular margin markers mainly include two aspects, i.e. genetic mutations (p53, loss-of-heterozygosity [LOH] markers) and epigenetic markers (e.g., methylation profiles and protein expression) [22–26]. The carcinogenic effects of these markers have been well elucidated, with some markers showing specific impacts on patients' prognosis. We previously reported that P53 expression in dysplastic surgical margins correlated with early OSCC recurrence [27]. However, most studies on molecular margin were restricted to a single gene or protein and lacked molecular combinations that effectively predicted the margin status.

In this study, we studied the metabolic markers of distance-related margins with different histopathological status based on the GC–MS untargeted and UHPLC-MS/MS targeted metabolic analysis. Global metabolic analysis of the development group revealed that 16 out of 244 named entities among the four groups (Tumor, Margin-1, Margin-2, and Margin-3) were significantly differentiated, including 8 amino acids. Clinically, the evaluation of negative margins is usually determined by pathologists based on the morphology of epithelial cells and lacks a molecular diagnostic basis. Therefore, it is helpful to develop a marker for negative surgical margins to improve the accuracy of clinical diagnosis. We developed nine markers as negative margin markers (AUC > 0.90, fold change>1.5, and $p < 0.05$) by combining GC–MS untargeted and UHPLC-MS/MS targeted metabolomic methods. The combination of these metabolites can effectively distinguish the negative margin and supplement the traditional margin assessment.

Dysplastic epithelium is not a safe margin for a surgical procedure, post-surgery LR is often indicated by the presence of residual cancer or dysplastic cells in the OSCC surgical margins [27,28]. However, the relationship between the degree of dysplasia and tumor recurrence has not been fully elucidated, especially lack of the molecular markers to predict OSCC recurrence for patients with dysplastic surgical margin. In our study, we identified six markers as dysplastic margin markers by combining GC–MS untargeted and UHPLC-MS/MS targeted metabolic methods. We further validated

Table 4
The correlation of ASNS expression in dysplasia margin with clinical characteristics.

Characteristics	ASNS expression		P
	High (n=22)	Low (n=38)	
Age			
< 60	10	16	0.801
≥ 60	12	22	
Sex			
Male	13	20	0.789
Female	9	18	
Smoking history			
Yes	9	14	0.788
No	13	24	
Histological grade			
Well differentiated	16	4	0.149
Poorly differentiated	6	34	
Recurrence			
Yes	9	4	0.009
No	13	34	
Grade of dysplasia			
Mild	13	23	0.913
Moderate	5	11	
Severe	4	4	

that high ASNS (asparagine synthetase) expression in dysplastic surgical margins was positively correlated with tumor recurrence and predicted poor prognosis. ASNS expression levels increased gradually with the deterioration of hyperplasia. These results suggested that aspartic acid and asparagine and asparagine synthetase are valuable markers for dysplastic surgical margins. Moreover, the concentration of these 9 amino acids (negative margin markers) and 6 amino acids (dysplastic margin markers) showed an increasing trend from normal tissue (Margin-3) to tumor (Tumor), indicating these molecules are associated with the development of OSCC, further confirming the significance of these molecules.

As oral cavity is size limited and only the patients whose samples contained margins larger than 1.5 cm were selected, the sample collection process is difficult and only 28 patients were enrolled in this study. Due to the limited size of the patients, our study is a preliminary study and the accuracy of the diagnostic values of our identified panels remained to be verified. In the near future, we will conduct a multi-center clinical study and enroll more clinical cases to further consolidate the amino acid markers of surgical margins.

In conclusion, we developed a panel of metabolites based on the GC–MS untargeted and UHPLC-MS/MS targeted metabolic method to evaluate the negative and dysplastic margins. We then validated that ASNS expression in dysplastic surgical margins, which was found to be a predictor for tumor recurrence for OSCC patients. Our results will complement traditional surgical margin evaluation and may improve the accuracy of clinical diagnosis.

Author contributions

XHY, QGH and YHN performed Investigation and Project administration for the study. SQH, XFH and LZ supply Resources and Software for the study. XHY, LD, YF, SW carried out Data curation and Formal analysis. XXZ and YJ performed Methodology and Supervision. YHN performed Validation and Visualization the data. XHY wrote the Writing-original draft, and XXZ, YHN, QGH performed Writing-review & editing. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2019.10.005.

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