Lactylome analyses suggest systematic lysine-lactylated substrates in oral squamous cell carcinoma under normoxia and hypoxia

Fan Song  
Sun Yat-sen University

Chen Hou  
Sun Yat-sen University

Jianfeng Liang  
Sun Yat-sen University

Hongshi Cai  
Sun Yat-sen University

Guoli Tian  
Sun Yat-sen University

Yaoqi Jiang  
Sun Yat-sen University

Ziyi Wang  
Sun Yat-sen University

Danqi Qiu  
Sun Yat-sen University

Jinsong Hou (houjs@mail.sysu.edu.cn)  
Sun Yat-sen University

Research Article

Keywords: oral squamous cell carcinoma, post-translational modifications, lysine lactylation, RNA splicing, ribosome pathway, glycolysis pathway

Posted Date: May 11th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2901339/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background

Intracellular lactate is shown to drive a novel type of post-translational modification (PTM), lysine lactylation (Kla), which has been confirmed to affect the malignant progression of tumors such as hepatocellular carcinoma (HCC) and gastric cancer. However, the systemic lactylome profiling of oral squamous cell carcinoma (OSCC) is still unclear.

Methods

In this study, we utilized liquid chromatography-tandem mass spectrometry (LC-MS/MS) to conduct the quantitative lactylome analyses in OSCC cell line under normoxia and hypoxia. Then, bioinformatics analyses were applied to reveal the conserved motif sequences and enrichment pathways. What’s more, Immunoprecipitation and western blotting verified the results of lactylome.

Results

The integrative lactylome and proteome analyses identified 1011 Kla sites within 532 proteins and 1197 Kla sites within 608 proteins in SCC25 cells under normoxic and hypoxic environments, respectively. Among these lactylated proteins, histones accounted for only a small fraction, suggesting the presence of Kla modification in large number of non-histones proteins. Notably, Kla prefers to enrich in spliceosome, ribosome and glycolysis/gluconeogenesis pathway in both normoxic and hypoxic cultures. Compared with normoxia, 231 differentially lactylated proteins with 334 differentially lactylated sites were detected under hypoxia, which were mainly associated with glycolysis/gluconeogenesis pathway by KEGG analysis. Importantly, we verified the presence of lactylation in spliceosomal proteins SF3A1 and hnRNPA1 as well as the glycolytic enzyme PFKP.

Conclusion

Our study is the first report to elucidate the lactylome and its biological function in OSCC, which deepens our understanding of the mechanisms underlying OSCC progression and provides a novel strategy for targeted therapy for OSCC.

1 Introduction

Oral squamous cell carcinoma (OSCC), accounting for approximately 95% of all oral cancers, ranks among the top 15 most prevalent cancers globally[1]. OSCC is characterized by local invasion, recurrence and early cervical lymph node metastasis, posing a considerable threat to patients’ health and lives, with an unsatisfactory long-term survival rate[2–4]. In recent years, the application of proteogenomics has greatly broadened our insights into the pathogenesis and progression of OSCC[5–7], which emphasizes the importance of tumor-driven protein expression patterns and sheds light on the identification of promising therapeutic and prognostic targets.
Post-translational modifications (PTMs) trigger rapid protein functional transformations to different extracellular signals by regulating enzymatic activity, protein degradation, protein interactions and so on[8, 9]. Dysregulation of PTMs has been proved to influence various physiopathological processes, such as metabolic reprogramming[10, 11], malignant cell proliferation[12] and cell survival[13]. Common types of PTMs include phosphorylation, ubiquitination, acetylation, methylation and glycosylation[14, 15]. In 2019, a novel type of PTM was identified as histone lysine lactylation (Kla), derived from cellular lactate, the end-product of glycolysis (Fig. 1A)[16]. Histone lactylation triggered reparative genes transcription in hypoxia and bacterial challenges to promote immune homeostasis, which highlights the role of lactate as epigenetic regulator beyond metabolic byproduct or energy source. Acetyltransferase p300 has been proved to introduce Kla, while histone deacetylases HDAC1/2/3 and SIRT1/2/3 act as delactylases[16, 17].

Recent studies have confirmed the regulatory roles of histone lactylation in inflammation, somatic cell reprogramming as well as oncogenesis[18–20]. Glis1 has been proved to induce epigenetic remodeling and pluripotency in somatic cells through the coordination of histone acetylation and lactylation[21]. Yu et al. reported that the lactylation of H3K18 contributed to tumorigenesis by promoting YTHDF2 expression in ocular melanoma[22]. Yang et al. discovered a positive feedback loop between histone lactylation and PDGFRβ signaling, which drove the malignant progression of clear cell renal cell carcinoma[23]. Moreover, integrative lactylome analysis revealed various lactylated substrates and specific lactylation sites in different tumors, suggesting that Kla modifications have a wide range of substrates and functions beyond epigenetic regulation through Kla in histone proteins, which provides renewed perspectives for tumor metabolism, therapies and prognosis[24–26].

As we all know, cancer cells tend to exhibit enhanced glycolysis and excessive accumulation of cellular lactate, termed as the Warburg effect[27]. Moreover, hypoxia greatly facilitates glycolysis and lactate production, as a result of rapid proliferation and immense oxygen demand of tumor tissues, which becomes a key characteristic of solid tumors including OSCC[28, 29]. Our previous findings revealed that hypoxia promoted glycolysis activity through upregulating HKII, which in turn contributed to the invasion and migration of tongue squamous cell carcinoma. In addition, lactate dehydrogenase A (LDHA), as the key enzyme in lactogenesis, has been reported to promote the malignant progression of OSCC[30]. Therefore, it is legitimate to speculate that lactylation plays a crucial part in tumorigenesis and progression of OSCC. However, the patterns and biological functions of lactylation in OSCC still remain to be explored. Hence, in this study, we performed quantitative lactylome and proteome analyses in OSCC cells under normoxic and hypoxic conditions, to map the comprehensive patterns of lactylation in OSCC. We identified 1011 Kla sites across 532 proteins and 1197 Kla sites across 608 proteins in normoxia and hypoxia, respectively. Those lactylated proteins were mainly enriched in spliceosome, ribosome and glycolysis/gluconeogenesis pathway in both normoxic and hypoxic cultures.

2 Materials and methods

2.1 OSCC samples and Cell culture
Three OSCC tissues and matched adjacent noncancerous normal tissues were collected from OSCC patients without preoperative chemotherapy or radiotherapy at the Hospital of Stomatology, Sun Yat-sen University during January 2022 to December 2022. Two normal gingival epitheliums were derived from two healthy volunteers. All participants provided informed consent before enrollment, and the study was approved by the Institutional Research Ethics Committee of the Hospital of Stomatology, Sun Yat-Sen University (KQEC-2020-16-03). Human OSCC cell lines SCC25, HSC3, CAL33 and HN6 was purchased from the American Type Culture Collection. Normal oral keratinocyte (NOK) was provided by J. Silvio Gutkind (NIH, Bethesda, MD, USA). SCC25 and HN6 cells were cultured in DMEM/Ham’s F12 medium (DMEM/F12, Gibco, USA) supplemented with 10% FBS. HSC3 and CAL33 were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS. NOK cells were grown in keratinocyte SFM medium (Gibco, USA) supplemented with growth factor. For normoxia environment, cells were grown at 37°C in a humidified incubator containing 21% O₂, 5% CO₂ and 74% N₂. For hypoxia exposure, cells were cultured in a hypoxia humidified incubator (ThermoFisher, USA) with 1% O₂, 5% CO₂ and 94% N₂ for the indicated time. After 24 h of normoxia and hypoxia treatment, cells were harvested for subsequent proteome and lactylome analysis.

2.2 Protein extraction

The sample was first sonicated three times on ice using a high-intensity ultrasonic processor (Scientz) in lysis buffer consisting of 8 M urea, 1% protease inhibitor cocktail, 3 µM TSA, and 50 mM NAM. Then the samples were centrifuged at 12,000 g at 4°C for 10 min to separate residual debris. Finally, the supernatant was collected and the protein concentration was quantified using bicinchoninic acid (BCA) kit according to the instructions provided by the manufacturer.

2.3 Trypsin digestion

To precipitate the protein sample, a final concentration of 20% trichloroacetic acid (TCA) was slowly added, followed by vortexing and incubation for 2 h at 4°C. The precipitate was collected by centrifugation for 5 min at 4500 g and then washed 2–3 times with pre-chilled acetone. After drying, the precipitate was redissolved in 200 mM triethylammonium bicarbonate (TEAB) and dispersed ultrasonically. To facilitate digestion, trypsin was added at a ratio of 1:50 (trypsin:protein, m/m) and allowed to digest overnight. The protein sample was reduced with 5 mM dithiothreitol (DTT) for 30 min at 56°C and alkylated with 11 mM iodoacetamide (IAM) for 15 min at room temperature in darkness.

2.4 Affinity enrichment

NETN buffer (pH 8.0) containing 100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, and 0.5% NP-40 was used to dissolve tryptic peptides. Pre-washed antibody beads (Anti-L-Lactyl Lysine antibody, PTM-1404, PTM Bio) were next added to the mixture and incubated at 4°C for overnight period with gentle shaking. Then the beads were washed for four times with NETN buffer and twice with H₂O. Use of 0.1% trifluoroacetic acid allowed the bound peptides to be eluted from the beads. Finally, the resulting fractions were combined and vacuum-dried, and the peptides were desalted using C18 ZipTips (Millipore), according to the manufacturer’s LC-MS/MS analysis instructions.
2.5 LC-MS/MS analysis

For proteomic analysis, the peptides were dissolved in liquid chromatography mobile phase A (0.1% formic acid, 2% acetonitrile in water), and then separated using a NanoElute ultra-high performance liquid chromatography (UHPLC) system (Bruker Daltonics). The gradient settings of solvent B (0.1% formic acid in 80% acetonitrile) were: 0 ~ 70 min, 6%~24% solvent B; 70.0 ~ 84.0 min, 24%~35% solvent B; 84.0~87.0 min, 35%~80% solvent B; 87.0 ~ 90.0 min, 80% solvent B, all with a constant flow rate of 450 nl/min. The eluted peptides were ionized through a capillary source and then analyzed using the timsTOF Pro (Bruker Daltonics) mass spectrometry, with a voltage of 2.0 kV applied to the electrospray source. The precursors and fragments were detected and analyzed using a high-resolution TOF detector, with a mass spectrometry scan range of 100~1700 m/z. The parallel accumulation serial fragmentation (PASEF) mode of the timsTOF Pro was used. Ten PASEF MS-MS images were obtained per cycle after fragmenting precursors with charge states 2 to 5. The dynamic exclusion was set to 30 s to avoid repeated scanning of the precursors.

For lactylome analysis, the enriched Kla-peptides were dissolved in liquid chromatography mobile phase A (0.1% formic acid, 2% acetonitrile in water), and then separated using a NanoElute ultra-high performance liquid chromatography (UHPLC) system (Bruker Daltonics). The gradient settings of solvent B (0.1% formic acid in 100% acetonitrile) were: 0 ~ 42 min, 7%~24% solvent B; 42.0 ~ 54.0 min, 24%~32% solvent B; 54.0~57.0 min, 32%~80% solvent B; 57.0 ~ 60.0 min, 80% solvent B, all with a constant flow rate of 450 nl/min. The eluted peptides were ionized through a capillary source and then analyzed using the timsTOF Pro (Bruker Daltonics) mass spectrometry, with a voltage of 1.6 kV applied to the electrospray source. The precursors and fragments were detected and analyzed using a high-resolution TOF detector, with a mass spectrometry scan range of 100~1700 m/z. The parallel accumulation serial fragmentation (PASEF) mode of the timsTOF Pro was used. Ten PASEF MS-MS images were obtained per cycle after fragmenting precursors with charge states 0 to 5. The dynamic exclusion was set to 24s to avoid repeated scanning of the precursors.

2.6 Database search

To process the MS/MS data generated, MaxQuant (v.1.6.15.0) was employed as the search engine. Tandem mass spectra were compared against the reverse decoy database and the human SwissProt database (20376 entries). Trypsin/P was specified as cleavage enzyme with the following restrictions: two missing cleavages in the proteome and four in the lactylome. The initial search employed a mass tolerance of 20 ppm for precursor ions, whereas the main search utilized 5 ppm. The fragment ions' mass tolerance was set to 0.02 Da. A fixed modification was defined as carbamidomethylation on Cys, while acetylation at the protein's N-terminus, oxidation at Met, and lactylation at lysine were variable modifications. The false discovery rates (FDRs) of modified peptides, proteins and sites were set to 1%. Kla sites with localization probability below 0.75 and MaxQuant scores below 40 were removed.

2.7 Quantification of proteome and lactylome
The protein levels in all samples were quantified using the MaxLFQ algorithm in the MaxQuant software. The levels of Kla-peptides were calculated based on the raw spectral intensity. In order to remove the level variations of Kla-peptides resulting from protein level changes, the abundance of each detected Kla-peptide was normalized with its corresponding protein abundance. Levene's test in conjunction with the log2 transformed abundance were applied to determine the homogeneity of variance across groups.

2.8 Bioinformatics analysis

To analyze the motif characteristics of modification sites, MoMo analysis based on Motif-x algorithm was performed. The WoLF PSORT software was used to predict the subcellular location of each protein. Protein functions were annotated from three categories (biological processes, cellular components, and molecular functions) using Gene Ontology analysis (www.ebi.ac.uk/GOA/). Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) was used to explore the associated signaling pathways of the Kla-proteins. The secondary structures and surface accessibilities of peptides were analyzed using Pfam database (http://pfam.xfam.org/). Lastly, the STRING database version 11.5 was searched for protein-protein interactions (PPI) of all differentially expressed protein database accession or sequence.

2.9 Western blotting

Cells were lysed in RIPA lysis buffer (CW2333S, CWBIO, China) with the addition of protease inhibitor cocktail set I (539131, Millipore, USA) and then centrifuged at 14,000g at 4°C for 20 min. To extract histone proteins, the EpiQuik™ Total Histone Extraction Kit (OP-0006, Epigentek, USA) was applied following the manufacturer's protocol. The protein concentration was quantified using BCA kit according to the manufacturer's instructions. Next, protein samples were subjected to 10% SDS-PAEG (P0014B, CWBIO) followed by transfer to PVDF membranes (ISEQ00010, Millipore, USA). After blocking with 5% skim milk (232100, Corning, USA) at room temperature for 1 h, the membranes were incubated overnight at 4°C with the following primary antibodies: Anti-L-Lactyl Lysine Rabbit mAb(1:2000, PTM-1401RM, PTM Bio); Anti-Acetyllysine Rabbit mAb(1:2000, PTM-105RM, PTM Bio); PFKP Rabbit mAb (1:1000, D4B2, CST); beta-actin Rabbit mAb (1:12000, AF7018, Anity); histone H3 Rabbit mAb (1:2000, D1H2, CST); SF3A1 Rabbit pAb (1:2000, HA500235, Huabio), hnRNPA1 Rabbit mAb (1:2000, ET1704-52, Huabio). The membranes were incubated with HRP Goat Anti-Rabbit IgG secondary antibody (1:2000, 7074S, CST) at room temperature for 1 h. Finally, the protein bands were visualized with a chemiluminescent substrate (WBKLS0500, Millipore, USA). For Coomassie Brilliant Blue staining, the gels were incubated with staining solution (0.01% Coomassie Brilliant Blue, 50% distilled water, 40% methanol, and 10% acetic acid) for 2–4 h and then destained with destaining solution (50% distilled water, 40% methanol, and 10% acetic acid) for 4–8 h until protein bands appeared.

2.10 Intracellular lactate assay

Cells were collected and then added in Lactate Assay Buffer after exposing to different concentrates of oxamate for 24 h or cultured in hypoxia for the indicated times (0 h, 12 h, 24 h, 36 h). Samples were sonicated on ice for 5 min (power 200 W, sonication 3 s, interval 7 s, repeat 30 times) using a high
intensity ultrasonic processor (Scientz). To separate the supernatant, the samples were centrifuged at 12,000 g at 4°C for 5 min. Finally, the lactate concentration in the supernatant was quantified using CheKine™ Lactate Assay Kit (KTB1100, Abbkine, China), following the manufacturer’s instructions.

### 2.11 Immunoprecipitation (IP)

Cells were lysed in PIERCE IP lysis buffer (87787, ThermoFisher, USA) containing protease inhibitor cocktail set I (539131, Millipore, USA) and then centrifuged at 14,000 g at 4°C for 20 min. After Protein A/G Magnetic Beads pre-incubating with primary antibodies for 2 h at 4°C, the soluble supernatant was then used for immunoprecipitation with Magbeads-Ab complex for 2 h at 4°C (HY-K0202, MCE, USA). Dilute antibody (Ab) to the final concentration of with binding/wash buffer: anti-IgG (1:200, 2729S, CST), anti-L-Lactyl Lysine antibody (1:100, PTM-1401RM, PTM Bio). These Immunocomplexes were washed with washing buffer (0.5% PBST) three times before denaturing elution. Finally, the prepared samples were subjected to western blotting with corresponding antibodies as previously described to incubate overnight at 4°C: PFKP Rabbit mAb (1:1000, D4B2, CST), SF3A1 Rabbit pAb (1:2000, HA500235, Huabio), hnRNPA1 Rabbit mAb (1:2000, ET1704-52, Huabio). The membranes were incubated with VeriBlot for IP secondary antibody (HRP) (1:2000, ab131366, Abcam) at room temperature for 1 h. Finally, the protein bands were visualized with a chemiluminescent substrate (WBKLS0500, Millipore, USA).

### 2.12 Immunofluorescence staining

SCC25 cells (1*10^4) were inoculated in confocal dishes and cultured in the hypoxic environment for the indicated times (0 h, 12 h, 24 h, 36 h). Then, cells were fixed with 4% paraformaldehyde for 20 min, washed three times with PBS for 5 min, and permeabilized with 0.1% Triton x-100 for 10 min. Following this, the cells were blocked with 5% bovine serum albumin (BSA) at room temperature for 1.5 hours and then washed three times with PBS for 5 minutes. Primary antibody (anti-L-Lactyl Lysine antibody, 1:200, PTM-1401RM, PTM Bio) were added and incubated overnight at 4°C. Subsequently, the secondary antibodies were added for 1 h at room temperature followed by nuclear staining with DAPI. Finally, the cells were examined and captured using the confocal microscope (Olympus FV-3000).

### 2.13 Statistical analysis

Statistical analyses were conducted using GraphPad Prism 9. Two-tailed unpaired or paired Student’s t-test was used to determine statistical differences between two groups, while ANOVA with Tukey's multiple comparisons was performed for multiple groups. For the estimation of significant enrichment in signaling pathways and functional annotations, the two-tailed Fisher’s exact Test was utilized. To analyze the relationship between a categorical variable and a quantitative variable, the Wilcoxon rank-sum test was performed. P-values lower than 0.05 were deemed statistically significant.

### 3 Results

#### 3.1 Identification of lysine lactylation in OSCC
To determine whether Kla modification could occur in OSCC, we detected the overall protein lactylation level in NOK and four OSCC cell lines by western blotting. OSCC cell lines demonstrated significantly higher level of Kla compared with NOK (Fig. 1B), and the multiple protein bands indicated the presence of non-histones Kla in OSCC cell lines. In addition, we isolated proteins from 3 paired OSCC tissues and adjacent noncancerous normal tissues to perform western blotting, which revealed that the Kla level of tumors were higher than adjacent normal tissues (Fig. 1C). As SCC25 cells exhibited abundant Kla level, SCC25 was employed for subsequent LC-MS/MS analysis and experimental validation. The Kla levels were confirmed to be elevated in a dose-dependent manner in response to exogenous addition of L-lactate and sodium lactate (Nala) (Fig. 1D-E), which were in line with the dose-dependent increasing pattern of histone Kla in human breast cancer cell lines[16]. Oxamate was used to inhibit the activities of lactate dehydrogenase (LDH)[30], which significantly decreased the intercellular lactate level of SCC25 cells (Fig. 1F). Moreover, SCC25 cells exhibited a decrease of global Kla level with the increasing concentrations of oxamate (Fig. 1G). Collectively, these observations indicated that lysine-lactylation was prevalent in both histones and non-histones in OSCC.

3.2 Systematic analysis of lysine lactylome of SCC25 in normoxia

We carried out the proteome and lactylome LC-MS/MS for SCC25 to accurately elucidate the lactylation landscape of OSCC under normoxia (Supplementary Table 1–2). The lactylome LC-MS/MS included five vital steps: protein extraction, trypsin digestion, affinity enrichment, LC-MS/MS and bioinformatics analysis (Fig. 2A). The sample preparation complied with the quality control requirements, according to the lactylated peptides' length distribution and mass error analysis. (Fig. 2B-C). In total, we identified 1011 lactyllysine sites within 532 proteins (Supplementary Table 1), with more than half of the Kla proteins weighted below 100 kDa (Fig. 2D). Among these identified proteins, 65.04% contained only a single modification site, whereas 16.35% contained more than 2 Kla sites (Fig. 2E). As shown in Fig. 2F, 54.51% of all Kla proteins were located in the nucleus, 32.71% in the cytoplasm, 4.14% in the mitochondria and 2.26% in the extracellular. These results further confirmed that lysine-lactylated modification occurred in both histones and non-histones in OSCC.

Based on MoMo analysis tool in motif-x algorithm, we compared the amino acids surrounding lysine-lactylated sites from the − 10 to + 10 positions to identify six conserved sequences in SCC25: x(2)Kx(7)Kx(10) (138 peptides), x(9)S_K_x(10) (117 peptides), x(10)_K_x(7)Kx(2) (102 peptides), x(7)Kx(2)_K_x(10) (82 peptides), x(9)A_K_x(10) (77 peptides) and x(5)KGx(3)_K_x(10) (25 peptides) (Supplementary Fig. 1A-B). Notably, x(2)Kx(7)K_x(10) and x(10)_K_x(7)Kx(2) were consistent with those found in gastric cancer[25]. To further explore the Kla motifs of SCC25, we conducted the analysis of amino acids surrounding Kla sites against the human proteome, which prominently demonstrated that lysine(K) (from +5 to +10 and −2 to -10), alanine(A) (from −8 to +6) and proline(P) (from +2 to +4 and from −2 to -5) were overexpressed, while cysteine(C), leucine(L) and glutamic acid(E) were largely depleted in many positions (Supplementary Fig. 1C). Additionally, statistics of secondary structure between modified and un-modified proteins clearly clarified that 28.47% of those lactylated sites were
3.3 Enrichment analyses of Kla modified proteins of SCC25 in normoxia

To further comprehend the biological regulatory functions of these lactylated proteome in OSCC, we then conducted KEGG and GO enrichment analyses. The result of KEGG demonstrated that proteins involved in spliceosome and ribosome were more likely to undergo lactylation, inferring that Kla may affect RNA splicing and protein synthesis (Fig. 4A). The biology process (BP) analysis further indicated that the majority of the lactylated proteins were enriched in RNA splicing, RNA processing and RNA metabolism (Fig. 4B). As shown in Fig. 4C for cellular component (CC), proteins located in the nucleus were more prone to be lactylated. Then, the observation of molecular function (MF) analysis manifested that lactylated proteins were more involved in RNA binding and DNA binding (Fig. 4D). A total of 367 lactylated proteins were included in PPI network to depict three greatly interconnected clusters: spliceosome, ribosome and viral carcinogenesis (Fig. 4E). Finally, proteins that underwent Kla modification in the spliceosome pathway were depicted in Fig. 4F.

3.4 The characteristics of lactylation of SCC25 in hypoxia

To better investigate the lactylome of OSCC, we used hypoxic cultured SCC25 cells to simulate the internal hypoxia environment of OSCC. Hypoxia induced intracellular lactate production and increased the global Kla levels in SCC25 (Fig. 5A-C). After 24h hypoxic culture, lactylome and proteome LC-MS/MS analyses were performed (Supplementary Table 4–5). In order to confirm the effectiveness of hypoxia treatment, a proteome comparison between hypoxia and normoxia was performed, which captured 266 up-regulated proteins and 556 down-regulated proteins (Supplementary Table 6, Supplementary Fig. 2A-C). KEGG enrichment analysis suggested that ribosome, HIF-1a signaling and glycolysis pathway were
up-regulated under hypoxia (Supplementary Fig. 2D). Meanwhile, BP enrichment analysis revealed several up-regulated pathways associated with energy metabolism (Supplementary Fig. 2E). MF analysis revealed significant enrichment in ribosome subunit, and CC analysis showed the significant enrichment in structural constituent of ribosome, oxygen binding and ubiquitin protein ligase activity (Supplementary Fig. 2F-2G). In a variety of solid tumors, hypoxia has been confirmed to promote ribosomal proteins expression to facilitate adaptation to hypoxic environment[33, 34]. Collectively, the proteome of SCC25 in hypoxia versus normoxia evidently demonstrated that OSCC had undergone adaptive changes in response to hypoxia.

According to lactylome of SCC25 in hypoxia, 1197 Kla sites were identified for 608 proteins, and the weight of those proteins were still predominantly less than 100 kDa (Supplementary Table 4-5, Fig. 5D). What’s more, the proportion of proteins with more than one Kla site was increased to 38.1%, compared with 34.96% in normoxia (Fig. 5E). All Kla proteins remained mainly located in nucleus, accounting for 57.96% (Fig. 5C, 5F). Furthermore, 13 motif sequences were identified under hypoxia, with 4 of these sequences in line with those observed in gastric cancer [25] (Supplementary Fig. 3A-3B). The top four motif sequences were x(2)Kx(7)_K_x(10), x(9)S_K_x(10), x(10)_K_x(7)Kx(2) and x(7)Kx(2)_K_x, which were detected both in hypoxia and normoxia. The variation of amino acids surrounding lysine-lactylated sites from the − 10 to + 10 positions and the distribution of secondary structure were similar to normoxia (Supplementary Fig. 3C-3D). Overall, a total of lactylome of SCC25 combined normoxia and hypoxia captured 692 proteins with 1414 Kla sites, which mainly functioned in nucleus.

Unexpectedly, despite histones Kla levels were upregulated under hypoxic condition, the number of histones Kla sites did not exhibit a substantial increase compared with normoxia (Supplementary Fig. 4A-4C, Supplementary Table 7). As seen in Supplementary Fig. 4D, differential Kla modification level of histones were mainly reflected in H2 with a significant upregulation trend. In hypoxic environment, the histones Kla levels were up-regulated with the exogenous addition of L-lactate and Nala, while the histones Kla levels showed an opposite performance in response to oxamate addition (Supplementary Fig. 4E-4G). Together, hypoxia regulated global Kla level in OSCC.

### 3.5 Enrichment analyses of Kla modified proteins of SCC25 in hypoxia

Next, we performed enrichment analyses to provide a more comprehensive and detailed lactylome for SCC25 in hypoxia. The lactylproteins exhibited a notable enrichment of spliceosome and ribosome (Fig. 6A). Combined with the KEGG analysis of normoxia, neutrophil extracellular trap formation, cell cycle and glycolysis/gluconeogenesis pathway also should be of interest. The top enriched categories of BP, CC and MF analyses were similar to the results of normoxic condition (Fig. 6B-D). Then, PPI was carried out to verify the pathway enrichment results (Fig. 6E). Finally, we performed IP to confirm the lactylation of hnRNPA1 and SF3A1, which were the key proteins of spliceosome (Fig. 6F-G).

The Warburg effect is a prominent feature of many tumors including OSCC, with elevated glycolysis and lactate production. However, whether Kla can in turn bind to glycolytic enzymes of OSCC remains to be
determined. According to LC-MS/MS analyses (Supplementary Table 1, Supplementary Table 4) and KEGG analysis (Fig. 4A, 6A), we summarized the lactylated key enzymes of glycolysis (Supplementary Fig. 5A). ENO1, ALDOA and GADPH were considered more susceptible to undergo Kla modification based on the identification of more than 4 sites in normoxic and hypoxic environment, whereas PFKP had only one Kla site. Our previous research revealed that PFKP promoted the progression of OSCC by regulating starvation-mediated autophagy, glycolysis and EMT[35]. The MS/MS spectra of PFKP was shown in Supplementary Fig. 5B, and the three-dimensional structure of PFKP-K688la was illustrated in Supplementary Fig. 5C. Indispensably, we conducted IP to verify the Kla of PFKP under both culture conditions. Meanwhile, the Kla level of PFKP was enhanced after adding exogenous L-lactate in hypoxia for 24 hours, while oxamate inhibited the Kla level of PFKP. (Supplementary Fig. 5D-E).

3.6 Differentially analysis of lactylated proteins between hypoxia and normoxia

The heatmaps were drawn the overall differences in the proteins and sites modified by Kla between normoxia and hypoxia of SCC25 (Fig. 7A). Next, we set a ratio $\geq 1.3$ as up-regulation and $\leq 0.77$ as down-regulation thresholds to explore the differences. Based on proteome, 231 differentially lactylated proteins with 334 differentially lactylated sites were identified, which were primarily located in nucleus (Supplementary Table 8, Fig. 7B-D). KEGG enrichment analysis suggested that differentially lactylated proteins were not only enriched in glycolysis but also in MAPK signaling pathway and homologous recombination, compared with normoxia. (Fig. 7E). Meanwhile, BP analysis revealed enrichment in multiple pathways of glucose metabolism process (Fig. 7F). Furthermore, these differentially lactylated proteins were closely associated with the structure of ATP, chromosome and DNA, suggesting their potential role in regulating energy production and gene expression (Fig. 7G-H).

To deeply explore the relationship between the lactylome and proteome of OSCC under hypoxia and normoxia, we integrated the two omics data and observed a negative correlation between the lactylome and proteome ($r = -0.28, P < 0.0001$) (Supplementary Fig. 6A). Next, we divided all Kla sites into nine groups according to the corresponding protein’s expression (Supplementary Fig. 6B). Apparently, the number of Kla sites in the first region were more than others, and BP analysis of these lactylated proteins indicated the enrichment of multiple pathways associated to immunity (Supplementary Fig. 6C, Supplementary Table 9). Lactate is known to be an immunosuppression molecule by regulating metabolism that mediates the activation and proliferation of immune cells[36, 37]. In summary, proteomic and lactylomic correlation analyses suggested that Kla modification might regulate immunity response of OSCC in hypoxic environment.

4 Discussion

To date, over 450 unique protein modifications have been identified[12]. To some extent, protein modifications control a reversible “on” or “off” state in regulating protein stability, activity, degradation or localization due to the PTMs reversibility feature[38, 39]. Researchers have explored the relationship
between PTMs and the malignant progression of OSCC[40–44]. Furthermore, an acetylome analysis was demonstrated 282 up-regulated Kac sites in 234 proteins and 235 down-regulated Kac sites in 162 proteins from nine OSCC tissues compared to paired adjacent normal tissues, and these differential acetylated proteins were highly enriched in ribosome[45]. Next, Zhang et al. quantified and identified the differentially modified 617 2-hydroxyisobutylated (Khib) proteins with 938 Khib sites between cancerous and paracancerous tissue from nine OSCC patients, which were enriched in actin cytoskeleton regulatory pathway[46].

The discovery of lactylation has created a new perspective for lactic acid, the predominant end product of glycolysis[16]. Aside from some literatures focusing on the histones lactylation in regulating transcription, little is known about non-histone substrates, especially in cancers[22, 31, 32]. In 2020, Gao et al. reported the first lactylomic research in Botrytis cinerea with 273 Kla sites in 166 proteins highlighting the Kla enrichment in structural components of ribosome and proteins participating in fungal pathogenicity[47]. Meng et al. carried out a comprehensive lactylome profile to confirm 638 Kla sites within 342 proteins in rice grains, which were mainly associated with central carbon metabolism and protein biosynthesis[48]. In gastric cancer AGS cells, 2375 Kla sites in 1014 proteins were identified and the prognostic value of Kla was further investigated[25]. Subsequently, Hong et al. reported a survey of lactylome in HCC, normal liver tissues and HCC with lung metastasis samples and verified the Kla level of two tumor-related proteins[26]. Hence, we initiated the first lactylome for OSCC to map the systematic Kla map of SCC25 with 1011 sites in 532 proteins and 1197 sites in 608 proteins in normoxia and hypoxia, respectively.

Functional enrichment analyses of lactylome under hypoxia and normoxia uncovered that Kla proteins were significantly enriched in spliceosome. As a fundamental eukaryotic process, RNA splicing regulates gene expression and protein diversity, the dysregulation of which can promote neoplastic transformation, malignant progression and chemoresistance[49]. It has been reported that RNA splicing could exert a profound influence on lactate metabolism [50, 51]. Consistent with our study, Yang et al. revealed that lactylated proteins in AGS cells were mainly enriched in spliceosome functions, identifying 193 Kla sites in 70 spliceosome-related proteins[25]. Moreover, they confirmed that lactate treatment has altered RNA splicing events in AGS cells through RNA-seq analysis. In our study, Kla modification in spliceosome was further validated in splicing factors hnRNPA1 and SF3A1. Several PTMs have been reported to regulate the function of spliceosome, including lysine phosphorylation and arginine methylation catalyzed by type I and II protein arginine methyltransferase (PRMTs)[52, 53]. Since splicing factors are the most abundant substrates for arginine methylation, PRMTs emerges as promising therapeutic targets for various neoplasms, with PRMT5 inhibitors under clinical trials in advanced solid tumors and hematological malignancies[54, 55]. Therefore, it is plausible to speculate that lactylation modification, as a newly identified PTM in splicing factors, profoundly influences splicing events and may serves as novel therapeutic target for OSCC.

Hypoxia is the result of rapid and uncontrolled tumor proliferation, which plays an essential part in promoting tumor progression, metastasis and desensitizing chemotherapy or radiotherapy in OSCC[29]. In response to the severe hypoxic environment, tumor cells change their behavior patterns by metabolic
reprogramming and enhancing angiogenesis to sustain survival and proliferation, with increased glycolysis and massive lactate accumulation in tumor microenvironment[28, 56, 57]. Hence, we used hypoxic cultured OSCC cells to simulate the internal hypoxia environment of OSCC, to explore the potential role of lactylation in cellular adaptation to hypoxia. We identified 1197 sites in 670 proteins for SCC25 in hypoxia and confirmed the elevated Kla level of SCC25 with the prolonged hypoxia treatment. Moreover, differential enrichment analysis revealed that the differentially lactylated proteins under hypoxia were significantly associated with energy metabolism and gene expression. In line with our study, Zhang et al. revealed that hypoxia induced intracellular lactate production and promoted histone lactylation in breast cancer cells[16]. Li et al. reported that hypoxia facilitated histone lactylation and upregulated the expression of fibrosis-related genes in the placentas of patients with preeclampsia[58]. Considering the high-lactate production under hypoxia in solid tumors, we have reasons to believe that lactylation may be a promising mechanism mediating the hypoxia adaptation in OSCC.

As the core metabolite in tumor microenvironment, lactate is extensively involved in tumor progression, cancer cell immune escape and chemoresistance[59–61]. The novel discovery of lactylation opens a wide perspective into the multitude mechanisms of lactate, serving as a bridge linking lactate, tumor metabolism and tumor progression[62]. A number of studies have revealed that lactylation plays an influential role in regulating cellular metabolism including glycolysis, the pathway that generates lactate. Yang et al. conducted a global lactylome profiling in a hepatocellular carcinoma cohort and demonstrated that Kla modulates enzymes engaged in metabolic pathways preferentially, including the tricarboxylic acid cycle, glycolysis, amino acid and fatty acid metabolism[63]. Furthermore, they verified that lactylation at K28 impairs adenylate kinase 2(AK2) activity, thus promoting the proliferation and metastasis of HCC cells[24]. In pro-inflammatory macrophages, the lactylation of pyruvate kinase M2(PKM2) at K62 site regulated inflammatory metabolic adaptation, which enhanced its kinase activity and inhibited the Warburg effect[19]. In our lactylome profiling, a multitude of glycolytic enzymes were detected to be lactylated at multiple lysine sites, which was more notable under hypoxia. Moreover, we verified Kla modification of key enzyme PFKP with the addition of Kla substrates and inhibitors. Therefore, we propose a conceivable and significant metabolic feedback loop between glycolytic enzymes, lactate and lactylation, which may contribute to the malignant progression of OSCC. The crosstalk between lactylation and tumor metabolism deserves further investigation.

5 Conclusion

In this study, LC-MS/MS was used for the first time to conduct the quantitative lactylome analyses of OSCC under normoxia and hypoxia. We analyzed the lactylated substrates both in normoxia and hypoxia and identified the main biological pathways regulated by Kla, including spliceosome, ribosome and glycolysis/gluconeogenesis pathway. Moreover, there may be a hypoxia-glycolysis-Kla modification positive feedback in OSCC. And Kla modification may have an immunosuppressive effect on OSCC. In summary, our study presented a systemic profiling of Kla modification to provide a novel strategy for targeted therapy for OSCC.
Declarations

Acknowledgements We thank for J. Silvio Gutkind providing NOK cell lines.

Authors’ contributions FS, CH and J-SH conceived the project and designed experiment. Z-YW, Y-QJ, D-QQ collected clinical information and performed bioinformatics analyses. FS, CH, H-SC and J-FL conducted the experiments. FS, CH and H-SC analyzed data. FS and HC wrote the manuscript and made the figures. G-LT, H-SC, J-FL, Z-YW, Y-QJ, D-QQ and J-SH supervised this study and edited the manuscript. All authors read and approved the final manuscript.

Funding This research was funded by the National Natural Science Foundation of China (grant no. 81874128, 82072994), Sun Yat-sen University Clinical Research 5010 Program (grant no. 2015018).

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate This study involved human participants and was approved by the Institutional Research Ethics Committee of the Hospital of Stomatology, Sun Yat-Sen University (KQEC-2020-16-03)

Competing interest The authors declare no competing interests.

References


Figures
Figure 1

Identification of lysine-lactylation in OSCC. (A) Illustration of Kla structure. (B) The Kla levels of NOK and four OSCC cell lines by WB (left). Coomassie blue staining (right). (C) The Kla levels of three paired OSCC and adjacent tissues by WB (left). Coomassie blue staining (right). (D, E) Exogenous L-lactate and Nala boosted Kla levels. Coomassie blue staining (right). (F) Intracellular lactate levels were measured in SCC25 cells in response to different oxamate concentrations for 24 hours under normoxia. n=4 biological
replicates; statistical significance was determined using one-way ANOVA followed by Sidak’s multiple comparisons test. **$P<0.01$, ***$P<0.001$. (G) Oxamate decreased Kla levels of SCC25(left). Coomassie blue staining (right).
Systematic analysis of lysine lactylome of SCC25 cells in normoxia. (A) A schematic flow chart for identification of Kla-containing protein substrates in SCC25 cells. (B) Mass error distribution of the Kla peptides. (C) Distribution of Kla peptides based on their length. (D) Molecular weight distribution of the identified proteins. (E) Distribution of the number of Kla sites per protein. (F) The subcellular localization of Kla proteins.

Figure 3
The lactylated histones of SCC25 in normoxia. (A) Number of detected Kla sites on different histones. (B) MS/MS spectra of a representative histone Kla peptides from H2BC15. (C) Histones Kla levels in normal gingival epithelium of healthy person and four OSCC cell lines. (D-F) Histone Kla levels of SCC25 cells cultured in L-lactate (D), Nala (E) and oxamate (F) with different concentrations for 24 hours.

Figure 4
Enrichment analyses of Kla modified proteins of SCC25 in normoxia. (A) KEGG pathway enrichment analysis of Kla proteins. (B-D) GO enrichment analyses of Kla proteins according to (B) biological processes, (C) cellular components and (D) molecular functions. (E) PPI network of the lactylated proteins in SCC25. (F) Wiring diagrams showing the Kla proteins enriched in the spliceosomal pathway.

Figure 5
The characteristics of lactylation for SCC25 in hypoxia. (A) Intracellular lactate levels were measured in SCC25 cells in response to hypoxia. N=5 biological replicates; statistical significance was determined using one-way ANOVA followed by Sidak's multiple comparisons test. **P<0.01. (B) The Kla levels of SCC25 cells under hypoxia (1% oxygen) for indicated time points by western blotting (left). Coomassie blue staining (right). (C) The Kla modification levels was visualized by immunofluorescence staining at the indicated time points under hypoxia. Magnification: 100x (scale bars: 50 µm) (D) Molecular weight distribution of the identified proteins. (E) Distribution of the number of Kla sites per protein. (F) The subcellular localization of Kla proteins.
Figure 6

Enrichment analyses of Kla modified proteins of SCC25 in hypoxia. (A) Enriched KEGG pathway analysis for lactylated proteins (B) Function classification by GO of lactylated proteins according to (B) biological processes, (C) cellular components and (D) molecular functions. (E) PPI network analysis of Kla proteins in SCC25. (F-G) Immunoprecipitation analysis of Kla for two spliceosome proteins. (F) hnRNPA1 (G) SF3A1
Figure 7

Pathway alterations and differential expression of lactylome and proteome in hypoxia versus normoxia.

(A) The heatmap showing the overall lactylome expression. H: hypoxia; N: normoxia. (B) The number of differentially lactylated proteins and differentially lactylated sites between hypoxic and normoxic environment. H: hypoxia; N: normoxia. (C) Volcano map of differentially lactylated sites in hypoxia versus normoxia. H: hypoxia; N: normoxia. (D) The subcellular localization of differentially lactylated proteins.
(E) KEGG functional enrichment analysis for differentially lactylated proteins. (F-H) Barplots depicting the GO enriched pathways for differentially lactylated proteins involving (F) biological processes, (G) cellular components and (H) molecular functions in hypoxia versus normoxia.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.pdf
- SupplementaryFigure2.pdf
- SupplementaryFigure3.pdf
- SupplementaryFigure4.pdf
- SupplementaryFigure5.pdf
- SupplementaryFigure6.pdf
- SupplementaryTables.xlsx