Lipid but not glucose metabolism is associated with 18FDG-PET/CT false negative lymph nodes in head and neck cancer

Xiaoyan Meng  
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Jingjing Sun  
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Feng Xu  
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Zhonglong Liu  
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine

yue he (✉ xymeng@sjtu.edu.cn)  
Shanghai 9th Peoples Hospital Affiliated to Shanghai Jiaotong University School of Medicine  
https://orcid.org/0000-0003-2105-5249

Research Article

Keywords: 18FDG-PET/CT, head and neck cancer, lymph node metastasis, false negativity, CD36, GLS, metabolism

Posted Date: July 21st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1855027/v1

License: © ① This work is licensed under a Creative Commons Attribution 4.0 International License.  Read Full License
Abstract

Background: Lymph node metastasis frequently occurs in head and neck squamous cancer (HNSCC) patients, and $^{18}$F fluorodeoxyglucose (FDG) positron emission tomography (PET) with computed tomography (CT) ($^{18}$FDG-PET/CT) examination for lymph node metastasis could result in false negativity and delays following treatment. However, the mechanism and resolution for $^{18}$FDG-PET/CT false negatives remain unclear.

Methods: In our study, ninety-two patients diagnosed with HNSCC who underwent preoperative $^{18}$FDG-PET/CT and subsequent surgery in our institution between 2018 and 2021 were reviewed. Immunohistochemistry (IHC) examinations of glucose metabolism (GLUT1 and GLUT5), amino acid metabolism (GLS and SLC1A5), and lipid metabolism (CPT1A and CD36) markers were conducted on their primary lesion as well as lymph node sections.

Results: We identified metabolic rewiring in lymph node metastasis and specific metabolic activity patterns of the false negative group. More significantly, we found that CD36 and GLS in primary lesions could be promising biomarkers for distinguishing false negative nodes as CD36 IHC score of primary lesions was significantly higher in false negative group than true positive group while GLS showed the opposite association.

Conclusion: IHC examination of CD36 and GLS expression in primary lesions could be used in distinguishing lymph nodes false negatives in $^{18}$FDG-PET/CT.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [1]. A large proportion of HNSCC patients suffer from lymph node metastasis whose first-line therapy is dissection surgery [2]. Therefore, preoperative evaluation of lymph nodes is important. However, some clinical N0 patients showed lymph node metastasis according to the final pathological report, which referred to false negativity. The false negativity of lymph nodes may delay the patient’s treatment plan.

With the development of examination methods, positron emission tomography (PET) with computed tomography (CT) using $^{18}$F fluorodeoxyglucose (FDG) is widely performed for the detection of lymph node metastases in HNSCC. However, although it is considered to be more sensitive than CT and ultrasound, $^{18}$FDG-PET/CT still exhibits a false negative rate of 10.5%-37.0% [3–7]. The mechanisms for false negativity remain unclear, and the resolution to improve false negativity has not yet been developed.

Since $^{18}$FDG-PET/CT was performed using $^{18}$FDG, an analog of glucose that is abnormally taken up by cancer cells [8], we hypothesized that the glucose metabolic status as well as other metabolic activities of the examined region could be causative factors for false negativity. Therefore, we chose six metabolism-related molecules (GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36) to represent the three main metabolic activities and examined their expression levels by immunohistochemistry (IHC). GLUT1 is the main glucose transporter on tumor cells [9]. GLUT5 is the isoform of GLUT1 and mediates fructose uptake [10]. GLS is the key enzyme for glutamine metabolism [11], and SLC1A5 controls glutamine uptake in various tumor types [12]. CD36 controls the uptake of fatty acids [13], and CPT1A mainly mediates fat oxidation in tumor cells [14].

By comparing these metabolic markers expression between primary lesions and metastatic nodes, we concluded specific metabolic activity patterns of the false negative group. More significantly, we found that CD36 and GLS in primary lesions could be promising biomarkers for distinguishing false negative nodes, which means that by primary lesion biopsy and IHC examinations, we are able to screen out patients with metastasis nodes but not detected in $^{18}$FDG-PET/CT examinations. This would be of great importance for improving outcomes of HNSCC patients.
2. Materials And Methods

Patient cohort

We conducted a retrospective cohort study to investigate the expression of 6 metabolic markers (GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36), \(^{18}\)FDG-PET/CT diagnosis and pathological diagnosis in a sample of HNSCC patients consecutively admitted to our hospital. Our analysis included patients between 1 January 2018 and 31 December 2021. They underwent \(^{18}\)FDG-PET/CT examination and then surgery in our hospital.

The inclusion criteria were 18–90 years of age, primary lesion in head and neck region, squamous cell carcinoma for final pathological diagnosis, and \(^{18}\)FDG-PET/CT examination before surgery. The exclusion criteria were as follows: no squamous cell carcinoma for final pathological diagnosis, preoperative adjuvant treatment, a history of radiation in the head and neck area, a history of chemotherapy or immunotherapy before surgery, and a delay of more than 6 weeks between \(^{18}\)FDG-PET/CT and surgery. The final study population for statistical analysis was 92 patients (Fig. 1).

The study data were obtained by reviewing patient records, \(^{18}\)FDG-PET/CT reports, pathological reports, and immunohistochemistry staining. Briefly, true negative patients were those who had no pathologically metastatic node as well as no suspicious nodes noted in \(^{18}\)FDG-PET/CT reports. False positive patients were those who had no pathologically metastatic node while had suspicious nodes in neck dissection side noted in \(^{18}\)FDG-PET/CT reports (interpreted by two independent radiologists). False negative patients were those who had pathologically metastatic nodes while had no indication in neck dissection side in \(^{18}\)FDG-PET/CT reports True positive patients were those who had metastatic nodes pathologically as well as indications radiologically.

The expression of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36 in the primary lesion and lymph nodes was compared between the false negative, true positive, true negative and false positive groups. Clinicopathological variables such as primary lesion size, clinical stages, and standard uptake value max (SUV\(_{\text{max}}\)) of the primary lesion were also compared between the four groups.

Immunohistochemical examinations and evaluations

Immunohistochemical staining was performed according to the staining protocol. In brief, tissue specimens were fixed in 4% formaldehyde and embedded in paraffin. Then, the tissue sections were deparaffinized, rehydrated, and incubated overnight at 4°C with primary antibodies against GLUT1 (ab115730, 1:250, Abcam), GLUT5 (ab76316, 1:250, Abcam), GLS (ab156876, 1:100, Abcam), SLC1A5 (ab237704, 1:1000, Abcam), CPT1A (ab234111, 1:1000, Abcam), and CD36 (ab252922, 1:250, Abcam). After washing, the bound antibody was detected with horseradish peroxidase (HRP)-conjugated secondary antibody at 37°C for 30 min and then visualized using a DAB kit. IHC staining is shown in Fig. 2.

The staining evaluation was conducted by pathologists. The staining intensity was scored as follows: 0 (colorless), 1 (light yellow), 2 (brownish yellow), or 3 (brown). The IHC score equals the sum of the intensity score plus the percentage of cells of corresponding intensity [15].

Public data retrieval and preprocessing

Publicly available HNSCC datasets were obtained from the TCGA-HNSC. Data on RNA-seq were FPKM or TPM transformed. Genes with low expression were eliminated. After PCA, any outlier samples were removed.

Functional characterization of differential expression analysis (DEGs)

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database and Gene Ontology (GO) category databases were used for functional annotation of DEGs. Enrichment analysis of GO categories was performed by the R clusterProfiler (v3.14.3) package, and enrichment analysis of pathways was tested upon hypergeometric distribution by the R 'phyper' function. Those
GO categories with an FDR < 0.05 were considered significantly enriched. Pathways with a p value < 0.05 were regarded as enriched. Only those GO categories or pathways containing ≥ 5 DEGs were kept for further analysis. For the RNA-seq data, the edgeR and DEseq2 R packages were used. Genes with an FDR < 0.05 were considered differentially expressed.

**Enrichment analysis of specific gene sets**

Single-sample gene set enrichment analysis (ssGSEA) was performed to calculate the enrichment score (ES) of each sample using the R package ‘GSVA’ [16] and identify up- or downregulated genes or pathways of interest in different subtypes within each tumor type by limma [17]. The KEGG and biological process signature and hallmark gene sets were obtained from the Molecular Signatures Database (MSigDB, V7.2) [18]. The immunologic signature was downloaded from the Immport database [19].

**Transcriptome deconvolution of the TIME**

The abundance of infiltrating immune cell populations was estimated by several deconvolution methods, such as MCP [20], CIBERSORT [21], and TIMER XCELL [22]. All these methods were integrated in R (immunedeconv).

**Cell culture and transient transfection**

A total of two cell lines including human HNSCC SCC9 and SCC25 (the American Type Culture Collection) were used in current research. They were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA). The culture was maintained in a humidified incubator with 37°C, 5% CO2. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used to transfet Negative Control (NC) and CD36 siRNAs (GenePharma, Shanghai, China) into HNSCC cells according to the manufacturer's instruction. Sequences for CD36 siRNAs were sense (5’-3’): GCAGCAACAUUCAAGUUATT and antisense (5’-3’): UUAACUUGAAUGUUGCUGCTT.

**Transwell assay**

The migration and invasive abilities of HNSCC cells were determined by Transwell assays (8.0 µm pores Transwell, Corning, USA) after transfection with siRNAs. Cells (1.0 × 10^5 for migration and 2.0 × 10^5 for invasion) were cultured in serum-free H-DMEM in the upper chambers. H-DMEM containing 10% FBS was added to the lower chambers. After cells were cultured for 24 h, cells that had migrated to the opposite side of the Transwell filter were fixed with 4% paraformaldehyde and stained with crystal violet staining solution (Beyotime, Shanghai, China). In Transwell-invasion assay, the top chamber was coated with Matrigel (1:10 in H-DMEM dilution, Corning, USA), other procedure was the same as Transwell-migration assay. Five fields were randomly selected under 100× microscope for photo recording.

**Quantitative real time PCR**

Total RNA was isolated from frozen tissues and cell lines using Trizol reagent (Thermo Fisher Scientific, USA). The RNA was used to synthesize cDNA with RT Master Mix kit (TaKara, China). The qRT-PCR experiment was performed using a TB Green Premix Ex Taq Kit (TaKaRa, China) in the Applied Biosystems Viia TM 7 Real-time PCR system (Life Technologies, CA). We used GAPDH as an internal control for normalization. The following primers were used:

- **CD36**, forward: 5’-CTGTTATGGGCTATAGGGATC-3’; reverse: 5’- ACTCCATCTGCAGTTGTTGT-3’;
- **Snai2**, forward: 5’-CTGTGACAAGGAATATGTGAGC-3’; reverse: 5’- CTAATGTGTCCTTGAAGCAACC-3’;
- **N-cadherin**, forward: 5’- AGGAGTCAGTGAAGGAGTCAGCAG-3’; reverse: 5’- TTCTGGCAAGTTGATTGGAGGGATG-3’;
- **Vimentin**, forward: 5’- CCTTCGTGAATACCAAGACCTGCTC-3’; reverse: 5’- AATCCTGCTCTCCTCGCCTTCC-3’;
- **S100A8**, forward: 5’-CTAATGTGTCCCTGAAAGCAACC-3’; reverse: 5’- TCTGCACCCTTTTTCCTGATAT-3’
S100A9, forward: 5'- CCTTCCACCAATACTCTGTGAA-3'; reverse: 5'- GGTCCCTCCATGATGTGTTAT-3'

Statistical analysis

Continuous variables were compared using Student’s t test, and categorical variables were compared with the χ² test. The Spearman correlation coefficient was used to assess the correlation between two IHC scores. P values of < 0.05 were considered to indicate statistical significance. All of the data were analyzed using SPSS 22.0 (IBM).

3. Results

3.1. Description of the study population

The patient cohort consisted of 92 patients (female n = 28, male n = 64, age 62 (mean) ± 12 (SD) years) with primary diagnosed HNSCC. The median SUV\textsubscript{max} of the primary lesions and lymph nodes were 12.3 and 3.9, respectively.

Then, we investigated the association of these clinical factors and lymph node metastasis. We found a significant association of advanced pT stage (T3-T4 vs. T1-T2, p = 0.007), advanced clinical stage (III-IV stage vs. I-II stage, p < 0.001), and larger depth of invasion (DOI) (>10 mm vs. ≤10 mm, p = 0.038).

Additionally, we also looked into radiological factors. Since there was no criteria of SUV\textsubscript{max} for diagnosis yet, we used median SUV\textsubscript{max} as cut-off value to compared lymph node metastasis between high-SUV\textsubscript{max} group and low-SUV\textsubscript{max} group. We found larger SUV\textsubscript{max} of primary lesions (≥12.3 vs. <12.3, p = 0.031) and lymph nodes (≥3.9 vs. <3.9, p < 0.001) were significantly associated with lymph node metastasis.

The complete demographic, clinical and pathological data are listed in Table 1.
## 3.2 Association between clinical factors and 18FDG-PET/CT false negativity of lymph nodes

To determine which clinical factors may be associated with 18FDG-PET/CT false negative diagnosis, we reviewed the clinical characteristics of the false negative, true negative, and true positive groups.

When comparing the false-negative group with the true-negative group (Table 2), we found that false-negative lymph nodes were significantly associated with a larger DOI (> 10 mm vs. ≤10 mm, p = 0.002) and advanced pT stage (T3-T4 vs. T1-T2, p
< 0.001) of the primary lesion. However, diagnosis had no association with the SUV\textsubscript{max} of the primary lesion, which suggested that the SUV\textsubscript{max} of the primary lesion was unable to distinguish false negative and true negative nodes.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Clinical characteristics of false negative group compared to true negative group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Primary lesion site</td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>21 (56.76%)</td>
</tr>
<tr>
<td>Others</td>
<td>16 (43.24%)</td>
</tr>
<tr>
<td>SUV\textsubscript{max} of primary lesion</td>
<td></td>
</tr>
<tr>
<td>&lt; 9.7</td>
<td>18 (50.00%)</td>
</tr>
<tr>
<td>≥ 9.7</td>
<td>18 (50.00%)</td>
</tr>
<tr>
<td>SUV\textsubscript{max} of lymph node</td>
<td></td>
</tr>
<tr>
<td>&lt; 2.7</td>
<td>15 (51.72%)</td>
</tr>
<tr>
<td>≥ 2.7</td>
<td>14 (48.28%)</td>
</tr>
<tr>
<td>DOI</td>
<td></td>
</tr>
<tr>
<td>≤ 10 mm</td>
<td>16 (43.24%)</td>
</tr>
<tr>
<td>&gt; 10 mm</td>
<td>21 (56.76%)</td>
</tr>
<tr>
<td>pT</td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>16 (43.24%)</td>
</tr>
<tr>
<td>T3-T4</td>
<td>21 (56.76%)</td>
</tr>
<tr>
<td>Neuron/vessel invasion</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12 (32.43%)</td>
</tr>
<tr>
<td>Negative</td>
<td>25 (67.57%)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Moderate-low</td>
<td>28 (75.68%)</td>
</tr>
<tr>
<td>High-moderate</td>
<td>9 (24.32%)</td>
</tr>
</tbody>
</table>

p values were calculated with χ\textsuperscript{2} test.

* p value < 0.05, ** p value < 0.01, *** p value < 0.001

Then, we compared the false negative group with the true positive group (Table 3). We found that false negative lymph nodes were significantly associated with a larger DOI (> 10 mm vs. ≤ 10 mm, p = 0.008) and advanced pT stage (T3-T4 vs. T1-T2, p = 0.002) of the primary lesion. Moreover, false negativity was significantly associated with a smaller SUV\textsubscript{max} of lymph nodes (≤ 7.4 vs. > 7.4, p = 0.001), which indicated that estimation based on the SUV\textsubscript{max} of lymph nodes may lead to incorrect diagnoses. Therefore, there is an urgency to find a more solid marker to distinguish false negative and true negative lymph nodes.
| Clinical characteristics of false negative group compared to true positive group |
|-------------------------------------------------|-----------------|-----------------|-----------|
| n                                              | False negative | True positive   | P value   |
| Primary lesion site                            |                 |                 |           |
| Tongue                                         | 30(58.82%)      | 9               | 21        | 0.518    |
| Others                                         | 21(39.22%)      | 4               | 17        |          |
| \(\text{SUV}_{\text{max}}\) of lymph node      |                 |                 |           |
| \(\leq 14.4\)                                  | 23(50.00%)      | 9               | 14        | 0.190    |
| \(\geq 14.4\)                                  | 23(50.00%)      | 4               | 19        |          |
| DOI                                             |                 |                 |           |
| \(\leq 10\text{mm}\)                          | 20(39.22%)      | 1               | 19        | 0.008**  |
| \(> 10\text{mm}\)                             | 31(60.78%)      | 12              | 19        |          |
| pT                                             |                 |                 |           |
| T1-T2                                          | 18(35.29%)      | 0               | 18        | 0.002**  |
| T3-T4                                          | 33(64.71%)      | 13              | 20        |          |
| pN                                             |                 |                 |           |
| N1                                             | 11(21.57%)      | 4               | 7         | 0.439    |
| N2-N3                                          | 40(78.43%)      | 9               | 31        |          |
| Neuron/vessel invasion                         |                 |                 |           |
| Positive                                       | 17(33.33%)      | 7               | 10        | 0.069    |
| Negative                                       | 34(66.67%)      | 6               | 28        |          |
| Differentiation                                |                 |                 |           |
| Moderate-low                                   | 34(66.67%)      | 11              | 23        | 0.175    |
| High-moderate                                  | 17(33.33%)      | 2               | 15        |          |
| Extranodal extension (ENE)                     |                 |                 |           |
| Positive                                       | 20(39.22%)      | 5               | 15        | 0.949    |
| Negative                                       | 31(60.78%)      | 8               | 23        |          |

\(p\) values were calculated with \(\chi^2\) test.

* \(p\) value < 0.05, ** \(p\) value < 0.01, *** \(p\) value < 0.001

3.3 CD36 and GLS expression in primary lesions is related to false negative \(^{18}\)FDG-PET/CT results in lymph nodes
The $^{18}$FDG-PET/CT examination makes use of the abnormal glucose uptake of tumor cells; therefore, we hypothesized that tumor samples in the false negative group exhibited different metabolic patterns from other groups. Thus, we examined the expression of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36 in primary lesions in the four groups. As shown in Fig. 3 and Supplementary Table 2, the CD36 IHC score of the primary lesion was significantly higher in the pathologically positive group than the pathologically negative group (FN vs. TN $p = 0.028$, FN vs. FP $p = 0.031$, TP vs. TN $p = 0.016$, TP vs. FP $p = 0.018$) while the GLS IHC score of the primary lesion was significantly lower in the false negative and true positive group than the false-positive group (FN vs. FP $p = 0.025$, TP vs. FP $p = 0.045$). Representative case pictures are shown in Fig. 4. These results indicated that by preoperative biopsy of the primary lesion and IHC examination of CD36 and GLS, we could exclude $^{18}$FDG-PET/CT false negative diagnosis of lymph nodes.

The association of the IHC scores of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36 in primary lesions with clinical factors is shown in Supplementary Table 1 and Fig. S1.

### 3.4 Metabolic rewiring of metastasis lymph node

Next, we investigated the expression of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36 in lymph nodes and compared them with those in primary lesions (representative pictures are shown in Fig. S2). As shown in Fig. 5A-C, for the true positive group, the GLUT1 IHC score in the lymph nodes was equal to that in the primary lesion, while the GLS IHC score ($p = 0.046$) increased and the CD36 IHC score ($p = 0.022$) decreased in the lymph nodes. Meanwhile, for the false negative group, GLUT1 ($p = 0.034$) and CD36 ($p = 0.044$) IHC scores both decreased in lymph nodes, while GLS IHC scores showed no significant change. These results indicated that tumor cells underwent a metabolic rewiring for metastasizing to lymph nodes and that the metabolic transition pattern of the false negative group was different from that of the true positive group: the overall metabolic activity of the false negative group was weakened in the lymph nodes. Moreover, we found that GLS and CD36 IHC scores in false negative nodes were significantly lower than those in true positive nodes (Fig. 5D-E, $p = 0.025$ and 0.036, respectively).

We investigated the metabolic rewiring pattern further by calculating the correlation of the IHC scores of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36. In primary lesions, the GLUT1 IHC score was positively correlated with GLS ($R = 0.51$, $p = 0.01$), and CD36 was negatively correlated with GLS ($R = -0.43$, $p = 0.04$) (Fig. 5F-G), which represented the canonical characteristics of tumor cell metabolism in primary lesions. When looking into lymph nodes, metabolic markers still kept correlation in true positive group as shown in Fig. 5H-I (GLS with GLUT1: $R = 0.91$, $p = 0.01$, GLS with CD36: $R = 0.90$, $p = 0.01$), but lost their correlation in false negative group as shown in Fig. 5J-K, which suggested that tumor cells in lymph nodes in false negative groups may have noncanonical metabolic pattern.

In conclusion, these results suggested that tumor cells underwent metabolic reprogramming in metastatic lymph node. Malignant cells in false negative nodes had lower metabolic activity than those in true positive nodes and that amino acid and lipid metabolism were promising targets for developing new clinical examinations of lymph node metastasis.

### 3.5 CD36 is related to tumor invasive characteristic

As CD36 was demonstrated to promote tumor metastasis, we turned to a public database to determine biological activities related to CD36. By analyzing the TCGA-HNSC database (Fig. 6A-C), we found that CD36 was related to extracellular matrix structural constituents, metalloendopeptidase activity, apical junctions, and epithelial mesenchymal transition, all of which focused on metastasis-promoting functions.

We validated the metastasis promoting characteristics of CD36 in vitro in two oral cancer cell lines: SCC9 and SCC25, both of which exhibit relatively high endogenous CD36 levels (Fig.S3A). We knocked down CD36 in SCC9 and SCC25 cells (Fig.S3B) and observed CD36 silencing significantly inhibited aggressive behaviors of oral cancer cells by transwell migration and invasion assay (Fig. 6D-E). The PCR results showed that expression levels of EMT markers and genes related to metastasis such as S100A8 and S100A9 were significantly decreased by knocking down CD36 in oral cancer cells (Fig. 6F).
Moreover, when analyzing immune infiltration status, we found that the CD36 expression level was negatively related to CD8+ T cells, which indicated that CD36+ tumor cells could promote tumor progression by exhibiting immune infiltration (Fig. 6G).

In summary, we concluded metastasis-promoting characteristics of CD36 by investigating public databases and validated that by experiments in vitro.

4. Discussion

Lymph node metastasis frequently occurs in HNSCC, influences the clinical therapy plan and poses a limiting factor for patient outcomes. Although 18FDG-PET/CT is considered a good examination method to identify lymph node metastasis, the false negative problem is also unavoidable. Previous studies on this topic mainly focused on comparing different diagnostic methods [3, 5] or evaluating the influences of diagnosis results on clinical decisions [4, 6]. Instead, few, if any, researchers analyzed primary lesions for a biomarker of false negative patients.

In our study, we performed a histopathological examination of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36 expression in primary lesions as well as lymph nodes from HNSCC patients. CD36 in the primary lesion showed higher expression, while GLS showed lower expression in the false negative group than in the true negative group.

In our study, we performed a histopathological examination of GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36 expression in primary lesions as well as lymph nodes from HNSCC patients. CD36 in the primary lesion showed higher expression, while GLS showed lower expression in the false negative group than in the true negative group.

GLS is the enzyme critical for glutamine utilization. Glutamine addiction is common in cancer cells, and GLS was reported to promote cancer cell proliferation and invasion [23, 24]. We found that after metastasizing into lymph nodes, the GLS expression level was higher than that in primary lesions, which was consistent with previous research [24]. However, the correlation of GLS with lymph node metastasis is still unclear, and the results in existing articles are inconsistent [23, 25]. In our study, we found that in patients with lymph node metastasis, GLS expression in their primary lesion was significantly lower than that in patients with no metastasis. The mechanism behind this phenomenon requires further exploration.

Regarding CD36, a series of studies have demonstrated its metastasis-promoting functions [26–29]. However, CD36 has not been applied with 18FDG-PET/CT for distinguishing false negatives. We validated that CD36 expression in primary lesions was higher in false-negative patients than in true-negative patients. This meant that by preoperative biopsy and histopathological examination of CD36, we could distinguish patients with lymph node metastasis, although their 18FDG-PET/CT showed N0 results.

Since we only had retrospective paraffin samples of the 18FDG-PET/CT cohort, we tried to further explore metabolic characteristics of primary lesions and lymph nodes between groups by analyzing metabolic marker expression based on IHC. We found that GLUT1, GLS, and CD36 expression levels were significantly changed between them, and false negative nodes showed an overall lower metabolic activity than true positive nodes. This may be one of the causes for false negatives in 18FDG-PET/CT examinations. These results inspired us to use IHC for confirming lymph node metastasis status beyond 18FDG-PET/CT examinations.

Compared to GLS, there are more studies focused on CD36 and tumor metastasis, so we validated this marker in oral cancer cell lines in vitro. Transwell migration and invasion assays showed that when CD36 was knockdown in cancer cells, their migration and invasion abilities were significantly impaired. Epithelial-mesenchymal transition is a hallmark of cancer metastasis, whose marker genes includes snai2, N-cadherin and vimentin [30]. The expression of these three genes decreased after CD36 knockdown. Also, S100A8 and S100A9 were down-regulated after CD36 knockdown, which are classical ligands related to cancer aggressiveness [31, 32].

The following shortcomings of the present study need to be discussed.

First, the significance of our study lies in clinical translation so we did not pay close attention to the molecular mechanism. Second, since the nuclear medicine department in our hospital was not established for a long time and the cases included in our study were limited. We can only acquire retrospective paraffin samples, which means that novel sequencing methods and
multiomics technology are not allowed to use. We will design a prospective study to consolidate the significance of CD36 and GLS in distinguishing false negatives in subsequent studies with the aid of high-throughput sequencing and multiomics technologies.

5. Conclusion

CD36 expression was higher in the $^{18}$FDG-PET/CT false negative group than in the pathologically negative group, while GLS showed the opposite association. Tumor cells in false negative nodes had lower metabolic activity than those in true positive nodes. Therefore, CD36 and GLS could be promising biomarkers for primary lesion biopsy to detect false negative lymph nodes in $^{18}$FDG-PET/CT.

Declarations

Acknowledgements: The research activities led by ZLL and YH are made possible by the funder who had no role in writing this manuscript: National Natural Science Foundation of China (No. 81900969 and No. 82173451), Project of Biobank (No. YBKB202105) from Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine.

Statement of author contributions: YH and ZLL conceived and carried out experiments; XYM, JJS, and FX conceived experiments and analysed data; XYM carried out experiments. All authors were involved in writing the paper and had final approval of the submitted and published versions.

Data availability: The datasets presented in this study can be found in online repositories. The datasets TCGA-HNSC can be found in the TCGA database.

Informed consent: Informed consent was obtained from all subjects involved in the study.

Competing interests: The authors declare no conflict of interest.

References


Figures

**Figure 1**

Flowchart of the study population, inclusion and exclusion criteria.
Figure 2

Tissue sections stained for GLUT1, GLUT5, GLS, SLC1A5, CPT1A, and CD36. GLUT1, GLUT5, SLC1A5, and CD36 were stained mainly in the membrane, while GLS and CPT1A showed cytoplasmic positivity. Representation of low expression (upper line) and high expression (below line). Scale bar: 25um.

Figure 3

Expression of CD36 (A-E) and GLS (F-J) in primary lesions. (A, F) Expression of CD36 (A) and GLS (F) in primary lesions in the false negative (FN), true positive (TP), true negative (TN), and false positive (FP) groups. (B-E, G-J) Representative images of each group are shown. Scale bar in the upper line: 100um, lower line: 25um. *p < 0.05, **p < 0.01, ***p < 0.001
Figure 4

Representative cases of false negative (A, E, I), true positive (B, F, J), true negative (C, G, K) and false-positive (D, H, L) groups. (A-D) $^{18}$FDG-PET/CT pictures of the primary lesion and lymph node. (E-H) GLS IHC images of the primary lesion. (I-L) CD36 IHC images of the primary lesion. Scale bar: 25um
Figure 5

Expression patterns of GLUT1, GLS, and CD36 in primary lesions and lymph nodes. (A-C) GLUT1 (A), GLS (B), and CD36 (C) expression in primary lesions and lymph nodes in false negative and true positive groups. (D-E) Comparison of GLS (D) and CD36 (E) IHC scores between false negative and true positive nodes. (F-K) Correlation of GLS and GLUT1 (F, H, J) and GLS and CD36 (G, I, K) IHC scores in primary lesions (F-G), true positive nodes (H-I) and false negative nodes (J-K). *p < 0.05, **p < 0.01, ***p < 0.001

Figure 6

CD36 is associated with aggressive characteristics of tumor cells. (A-C) GO (A), GSEA (B) and KEGG (C) analysis of CD36 in the TCGA-HNSC database. (D-E) Images of Transwell assays for migration and invasion (D) in negative control and CD36 knockdown group. The quantitative analysis of migration and invasion ability is shown in (E). (F) Differences in expression of
EMT-related genes between negative control and CD36 knockdown group. (G) Association of CD36 expression and immune infiltration. *p < 0.05, **p < 0.01, ***p < 0.001

Supplementary Files

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

- figuresupplementarylegend.pdf
- supplementarytablefinal.docx