

Increased PD1 and Glycolysis in CD4+ T Cell Promotes Lymph Node Metastasis in Oral Squamous Cell Carcinoma Patients

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Research article

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Abstract

Background: The presence of cervical lymph node metastasis is one of the poorest prognostic factors in oral squamous cell carcinoma (OSCC) with 50% reduction in survival of patients with lymph node positive involvement compared to those without. However, it is unknown whether immune checkpoint contributed to metastatic lymph nodes in OSCC patients.

Methods: Flow cytometry and immunofluorescence staining were used to analyze the differences of CD4⁺ PD1⁺ T cells between metastatic and negative lymph nodes. RT-PCR was performed to clarify the expression of immune checkpoints and glycolysis related enzymes in metastatic and negative lymph nodes. Kruskal-Wallis tests, Mann-Whitney tests or nonparametric paired test (Wilcoxon matched paired test) were used to analyze the non-parametric distribution of samples.

Results: We found that frequency of CD4⁺ T cells decreased in metastatic lymph nodes ($p = 0.0019$). In following experiments, immune checkpoints (PD1, PDL1 and CTLA4) of CD4⁺ T cells were detected in metastatic lymph node (LN+) and paired negative lymph node (LN-) of OSCC patients. The PD1 expression of LN+ was increased markedly compared to LN- ($p = 0.0205$). Similarly, the PD1 of CD4⁺ T cells in LN+ was increased significantly compared to LN-. We also found that glycolysis related enzymes levels in CD4⁺ T cells from LN+ were elevated dramatically compared to LN-. Moreover, PD1 and Hk2 expression of CD4⁺ T cells was increased in metastatic lymph nodes of OSCC patients with prior surgical treatment compared to those without.

Conclusions: These findings suggested that increased PD1 and glycolysis in CD4⁺ T cell may serve as a pivotal regulator of OSCC metastatic lymph nodes via elevating glycolysis related enzymes level, especially in Hk2.

Background

Oral squamous cell carcinoma (OSCC) is a major devastating oral cancer subtype, accounting for over 90% of malignant tumor in the oral cavity[1]. The presence of cervical lymph node metastasis is one of the poorest prognostic factors in OSCC with 50% reduction in survival of patients with lymph node positive involvement compared to those without[2]. Hence, the underlying molecular mechanism of cervical lymph node metastasis was fully illuminated to decrease the mortality of patients with OSCC.

The host immune tolerance and activation depended on the balance of positive and negative signals which were determined by immune checkpoints[3]. Malignant tumor cells evade antitumor immune responses by facilitating negative signals such as PD1/PDL1[4]. More specifically, upregulation of PD1 inhibited effector functions of T cells and expansion in the tumor microenvironment, thus enabling tumor cells to escape immune surveillance[5]. Moreover, immune checkpoint receptors in T cells could determine their activation, expansion, and effector functions[6] via regulating metabolic activity[7, 8]. T cell has highly dynamic metabolism and specific metabolic pathways to support specific cell functions, such as

effector, memory, regulatory and alloreactive T cells[9]. Therefore, activation of T cells causes a large increase needs in glucose metabolism and aerobic glycolysis fuel[10].

Lymph nodes are pivotal peripheral immune organs that respond to disseminated tumor cells through presenting tumor cells antigens and the subsequent priming of effector cells such as antigen-specific T cells[11, 12]. However, how immune checkpoint contributed to metastatic lymph nodes in OSCC patients are still unclear. Furthermore, the correlations between glycolysis and immune checkpoint expression in CD4⁺ T cells have not been elucidated.

In present study, we aimed to investigate the effect of immune checkpoints in metastatic lymph nodes. Furthermore, experiments were performed to identify the glycolysis in CD4⁺ T cells from metastatic lymph nodes. This study revealed that increased PD1 and glycolysis in CD4⁺ T cells promotes lymph node metastasis in OSCC patients.

Methods

Ethics

The Ethics Committee of Second Xiangya Hospital ratified this study. Written informed consents were obtained from all participants prior to enrollment. All experimental methods abided by the Helsinki Declaration.

Patients and Specimens

All OSCC samples were collected from Department of Oral and Maxillofacial Surgery, Second Xiangya Hospital of Central South University. More specifically, lymph nodes were obtained from the OSCC patients who underwent surgery treatment between June 2018 and July 2019. Half of each lymph node was stored for experiments, and other half was sent for pathological diagnosis. The clinical parameters were obtained from medical records.

RNA extraction and real-time PCR (RT-PCR) analysis

Total RNA of CD4⁺ T cells were isolated by TRIzol reagent (Takara, Japan) and cDNA was synthesized with a PrimeScript RT Reagent Kit (Takara, Japan). Real-time PCR was applied using SYBR Premix Ex Taq Reagent Kit (Takara, Japan) by the StepOne Real-Time PCR System (Life Technologies, USA) according to manufacturer's instructions. In tissue lysates, the mRNA levels were normalized against β -actin levels. The primer sequences used in the present study are listed in Supplementary Table 1.

Immunofluorescence analysis

Briefly, Paraffin-embedded sections were deparaffinized, rehydrated and submerged into EDTA buffer for heat-induced antigen retrieval. Then, the sections immersed into 0.3% hydrogen peroxide, blocked with 10% goat serum, incubated with specific primary antibodies at 4°C overnight, and incubated the Alexa

Fluor 488-cojugated secondary antibody (Invitrogen, USA) or Alexa Fluor 549-cojugated secondary antibody (Invitrogen, USA) in the dark at room temperature. Sections stained with DAPI (Sangon Biotech, China) to detect nuclei. Sections were imaged using a TCS SP2 laser-scanning confocal microscope (Leica Microsystems, Germany) and Gen5 software (Bio Tek, USA).

Flow cytometry

The cell surface markers were analyzed by flow cytometry (FCM). The living cells were stained with antibodies in PBS containing 0.1% (weight/volume) BSA and 0.1% NaN_3 in 50 μL FACs buffer for 30min on ice. The following antibodies-fluorochrome combinations were used: anti-CD4 BB515 (RPA-T4), anti-CD8a BB700 (RPA-T8), anti-CD19 Percp (HIB19), anti-CD20 FITC (2H7), anti-CD11c PE (3.9), anti-MHCII Percp (G46-6), anti-CD68 FITC (Y1/82A), anti-CD86 Percp (FUN-1), anti-CD274 FITC (MIH1), anti-CD279 APC (EH12.2H7), anti-CD152 APC (BNI3). The antibodies were obtained from BioLegend or BD Pharmingen.

T cell isolation

CD4⁺ T cells were sorted from a single-cell suspension drawn from lymph node with the CD4⁺ T cell Isolation Kit (Biolegend), and purity levels were greater than 95%, as determined by using the BD FACSCalibur. CD4⁺ T cells were used for Real-time PCR analysis.

Immunohistochemical analysis

Briefly, the OSCC tissue were fixed, paraffin-embedded, sectioned, dehydrated and, then the sections were incubated with anti-KRT14 (1:100, Abcam) at 4 °C overnight, followed by DAKO ChemMate envision kit/HRP (Dako-Cytomation, USA) according the manufacturer's instructions. Pictures were taken with light microscopy (Olympus, Tokyo, Japan) and Gen5 software (Bio Tek, USA).

Statistical Analysis

Kruskal-Wallis tests, Mann-Whitney tests or nonparametric paired test (Wilcoxon matched paired test) were used to analyze the non-parametric distribution of samples. All statistical analyses were calculated using SPSS 17.0 (SPSS, Chicago, IL, USA). All values were two sided, and $p < 0.05$ was considered to be significant.

Results

Metastatic lymph nodes in OSCC patient

The lymph node metastasis is one of the poorest prognostic factors in OSCC patients[2]. The fresh sample of metastatic lymph nodes (LN+) and negative lymph node (LN-) was showed in Figure 1A. The keratinocyte was found in HE stained of LN+ (Fig. 1B).

Decreased frequency of CD4⁺ T cells in metastatic lymph nodes

The lymph node is a secondary lymphoid organ[13], representing a pivotal meeting point of various immune cell types for adaptive immune responses[14]. To identify the immune cell types in metastatic lymph nodes, LN+ and paired LN- collected from each OSCC patient were analyzed by flow cytometry. The clinical parameter of 3 OSCC patients were in shown Table 1. The percent of T cells are significantly decreased in LN+ compared with LN- ($p = 0.0394$) (Fig. 2A). However, there was no significant alternation between the percent of B cell ($p = 0.2203$), Dendritic cell ($p = 0.5909$) or Macrophage ($p = 0.5462$) in LN+ and paired LN- (Fig. 2A). In further analysis, we found that CD4⁺ T cell dramatically decreased in LN+ compared with LN- ($p = 0.0019$) (Fig. 2B), while the CD8⁺ T cells were not changed in LN+ (Fig. 2B). This result indicated that the decreased frequency of CD4⁺ T cells could contribute to promotion of OSCC metastatic lymph nodes.

Increased frequency of PD1 of CD4⁺ T cells in metastatic lymph nodes

Immune checkpoint receptors on T cells can negatively determine their expansion, activation, and effector functions via inhibitory signals generated by binding with their receptors[6]. Interacting of PDL1 to its cognate ligand PD1 on activated T cells inhibits anti-tumor immunity by counteracting T cell-activating signals[15]. To clarify the expression of immune checkpoint receptors PD1, PDL1 and CTLA4 in metastatic lymph nodes, the immune checkpoint receptors transcriptional levels were detected by RT-PCR. The LN+ and paired LN- collected from each OSCC patient were collected and the clinical parameter of 8 OSCC patients were in shown Table 2. Only the PD1 expression level of CD4⁺ T cells was notably upregulated in LN+ compared to LN- ($p = 0.0205$) (Fig. 3A). To further determine changes of immune checkpoints in metastatic lymph nodes, PD1, PDL1 and CTLA4 protein level were detected by flow cytometry. As we expected, the PD1 protein level of CD4⁺ T cells was significantly upregulated in LN+ compared to LN- (Fig. 3B). But there was no significant alternation in PDL1 and CTLA4 between LN+ and LN- (Fig. 3B). In immunofluorescence analysis, the PD1 was predominantly expressed in CD4⁺ T cells, and markedly upregulated in LN+ compared to LN- (Fig. 3C). Our findings revealed that the increased PD1 of CD4⁺ T cells in LN+ could facilitate the lymph node metastasis progression.

Elevated glycolysis related enzymes levels in CD4⁺ T cells from metastatic lymph nodes

T cells depend on dramatic increases in glucose metabolism as fuel to support the growth, function, survival, and differentiation of an activated T cells[9, 16]. To determine whether the glycolysis related enzymes contribute to CD4⁺ T cells in metastatic lymph nodes, the mRNA expression levels of Glut1, Hk2, Hk3, Tpi1, Gpi1, Eno1, PKM, LDHa and MCT4 in CD4⁺ T cells were detected by RT-PCR. The mRNA expression levels of Glut1, Hk2, Tpi1, Gpi1, Eno1 and LDHa in CD4⁺ T cells were dramatically increased in LN+ compared to LN- (Fig. 4). Even though there was no statistical difference between Hk3, PKM and MCT4 expression level in LN+ and LN-, the average value of Hk3, PKM and MCT4 expression level in LN+

is higher than in LN- (Fig. 4). The results suggested that increased PD1 of CD4⁺ T cells was linked to glucose metabolism and aerobic glycolysis fuel.

Increased PD1 and Hk2 expression of CD4⁺ T cells in metastatic lymph nodes of OSCC patients with prior surgical treatment compared to those without

According to prior surgical treatment history (underwent neck lymph node dissection) of OSCC patients in Table 2, LN+ in OSCC patients with prior surgical treatment history were defined as P-LN+, and LN+ in OSCC patients without prior surgical treatment history were defined as N-LN+. The PD1 expression level of CD4⁺ T cells were markedly upregulated in P-LN+ compared to N-LN+ ($p = 0.0286$), while there is no statistical difference in PDL1 and CTLA4 expression between P-LN+ and N-LN+ (Fig. 5A and 5B). To determine whether the glycolysis related enzymes contribute to upregulation PD1 of CD4⁺ T cells in P-LN+, the mRNA expression levels of Glut1, Hk2, Hk3, Tpi1, Gpi1, Eno1, PKM, LDHa and MCT4 in CD4⁺ T cells were analyzed according to prior surgical treatment history. Only the Hk2 expression levels of CD4⁺ T cells were dramatically increased in P-LN+ compared to N-LN+ ($p = 0.0302$) (Fig. 5C). These data suggested that increased PD1 of CD4⁺ T cells in P-LN+ was associated with elevated Hk2.

Discussion

In present study, we demonstrated that the percentage of CD4⁺ T cells decreased in LN+ compared to LN-. We also found that expression of PD1 and glycolysis related enzymes elevated in CD4⁺ T cells from metastatic lymph nodes. These results indicated that increased PD1 of CD4⁺ T cells in LN+ facilitates the lymph node metastasis progression via glucose metabolism and aerobic glycolysis fuel. In following experiments, the findings revealed that PD1 and Hk2 of CD4⁺ T cells were upregulated in P-LN+ compared with N-LN+.

T cells inhibit tumor cells in various ways, either directly by killing tumor cells via cytolytic mechanisms or indirectly by modulating the tumor microenvironment[17]. Emerging evidences revealed that CD4⁺ T cells is necessary to initiate and maintain anticancer immune responses[18, 19]. Furthermore, CD4⁺ T cells could increase the quality and magnitude of B cell and CD8⁺ cytotoxic T lymphocytes responses in lymph nodes[17]. Our findings suggested that the decreased percentage of CD4⁺ T cells in LN+ inhibit anti-tumor immunity, which contribute to progression of OSCC metastatic lymph nodes. The previous study showed that the OSCC patients with elevated CD4⁺ T cells had a poorer prognosis[20]. This difference may attribute to different cell types between lymph node and primary tumor. Lymph nodes include various immune cells and primary tumor composed mostly of tumor cell. Metastatic lymph nodes contain tumor cells and immune cells, while the percent of immune cells decreased correspondingly.

A previous study in cervical carcinomas reported that PD1 was expressed by a vast number of infiltrating CD8⁺ T cells, suggesting that PD1 could serve as a potential therapeutic target[21]. In additionally, recent study suggested that CD4⁺ T cells as pivotal regulators of PDL1 levels, determined the immune

responsiveness to PD1-based immune checkpoint therapy in OSCC patients[22]. The present study found that the increased PD1 expression of CD4⁺ T cells in LN+ could promote lymph nodes metastasis, suggesting that blocking of PD1 could have therapeutic potential in OSCC patients. Chronic antigen exposure, such as occurs with cancer, can lead to upregulated PD1 expression levels, which induces a state of anergy or exhaustion among cognate antigen-specific T cells[23].

T cells do not have enough internal glycogen stores, making them highly dependent on uptake of extracellular glucose to meet increased metabolic needs during an immune response[7]. If T cells fail to meet increase glucose metabolic needs due to direct metabolic inhibition or inadequate nutrients, T cells proliferation and activation are suppressed[9]. Moreover, the immune checkpoint PD1 associated with T cell metabolism. The study of Bengsch have shown that PD1 regulates glycolysis, metabolism and mitochondrial function of virus-specific CD8⁺ T cells in chronic lymphocytic choriomeningitis virus infection[24]. Our results demonstrated that increased PD1 of CD4⁺ T cells in LN+ was associated with glycolysis related enzymes, indicating that increased PD1 of CD4⁺ T cells inhibit anti-tumor immunity via glucose metabolism and aerobic glycolysis fuel. Furthermore, we also found that PD1 and Hk2 of CD4⁺ T cells were increased in P-LN+ compared to N-LN+, suggesting that Hk2 is a key enzyme in glycolysis, contributing to progression of metastatic lymph nodes in OSCC.

Conclusions

In summary, our study suggests that increased PD1 and glycolysis in CD4⁺ T cell may serve as a pivotal regulator of OSCC metastatic lymph nodes via elevating glycolysis related enzymes level, especially in Hk2.

Abbreviations

OSCC: oral squamous cell carcinoma; LN+: metastatic lymph node; FCM: flow cytometry.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Second Xiangya Hospital (Approval No. 2020530, 2020/9/14) and was performed in accordance with the Helsinki declaration.

Consent for publication

Written informed consent was obtained from all participants prior to enrollment

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WK and HNN analyzed and interpreted the data. LY design this study. WK was a major contributor in writing the manuscript. ZS and LY edited the manuscript. ZS and WK collected the specimen. All authors read and approved the final manuscript

Acknowledgements

Not applicable.

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Tables

Table 1. The clinical parameter of OSCC patients were used for determining the immune cell types in lymph node

NO.	T classification	Lymph node status	Tumor site	Perineural invasion	Prior Radiotherapy/ Chemosensitivity
1	T2	pN positive	Tongue	No	No
2	T3	pN positive	Buccal mucosa	Yes	No
3	T3	pN positive	Tongue	Yes	No

pN: pathology lymph nodes status

Table 2. The clinical parameter of OSCC patients were used for screening immune check point and glycolysis related enzymes

Table 2. The clinical parameter of OSCC patients were used for screening immune check point and glycolysis related enzymes

NO.	T classification	Lymph node status	Tumor site	Perineural invasion	Prior Radiotherapy/Chemosensitivity	Prior surgical treatment
1	T3	pN positive	Tongue	Yes	No	Yes
2	T2	pN positive	Tongue	Yes	No	Yes
3	T2	pN positive	Tongue	Yes	No	No
4	T4	pN positive	Buccal mucosa	No	No	Yes
5	T3	pN positive	Oral floor	No	No	No
6	T2	pN positive	Tongue	No	No	No
7	T4	pN positive	Tongue	No	No	Yes
8	T3	pN positive	Gingiva	No	No	No

pN: pathology lymph nodes status

Figures

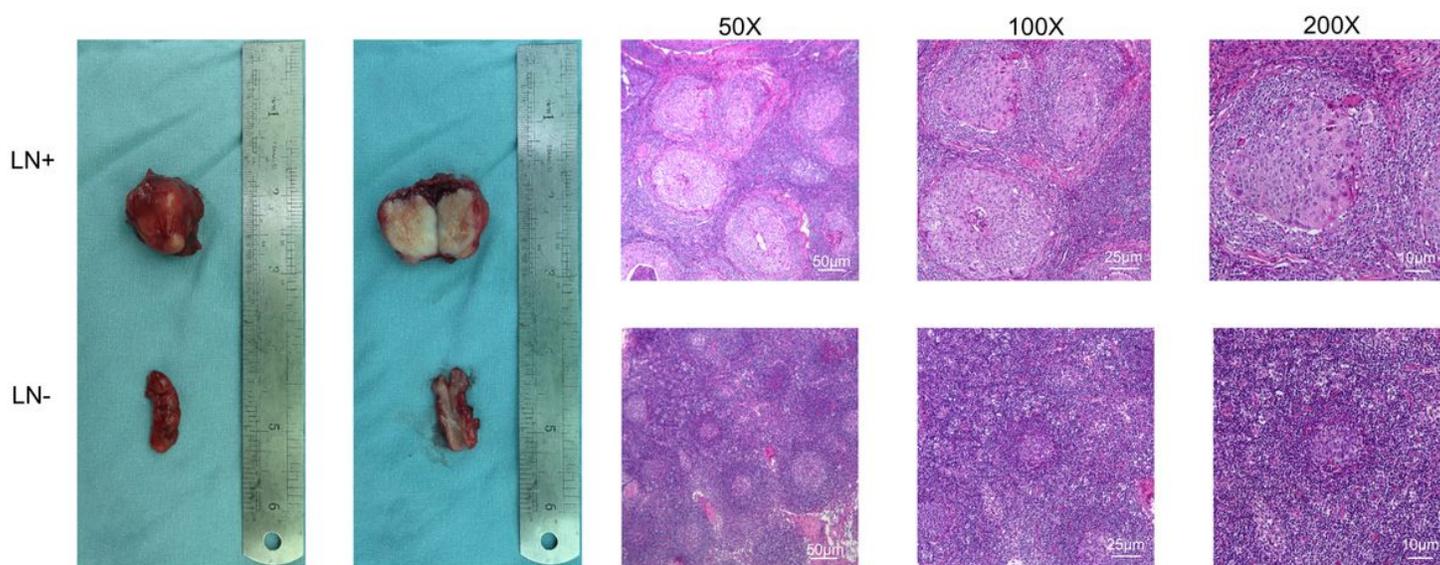


Figure 1

Metastatic lymph nodes (LN+) and negative lymph nodes (LN-) of OSCC patient (A) fresh samples (B) stained with HE (Resolution: 300 dpi)

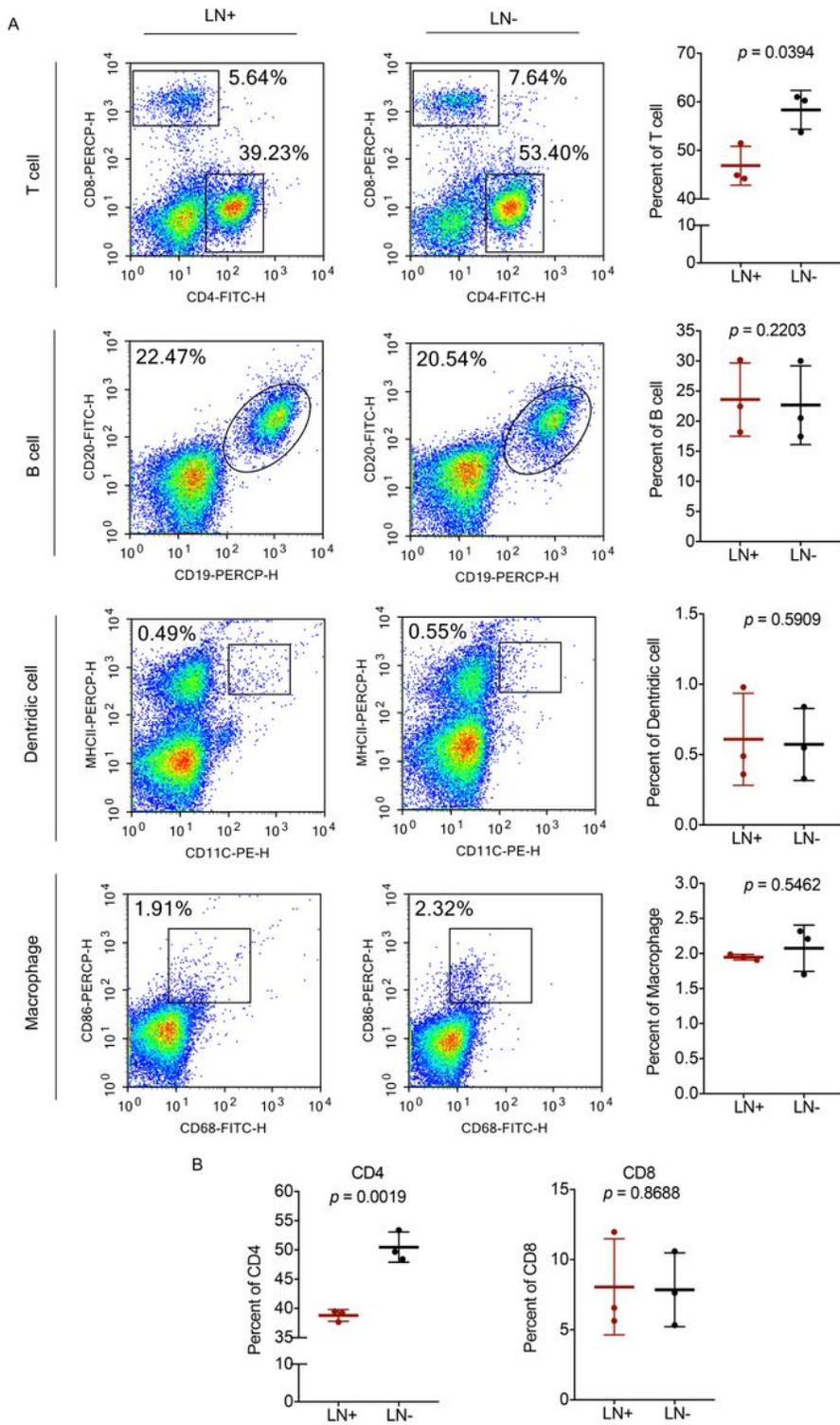


Figure 2

The immune cells types of LN+ and LN- from OSCC patients (A) Representative flow cytometry analysis of T cell, B cell, Dendritic cell and Macrophage isolated from 3 OSCC patients (B) The percentage of CD4 cell and CD8 cell were analyzed

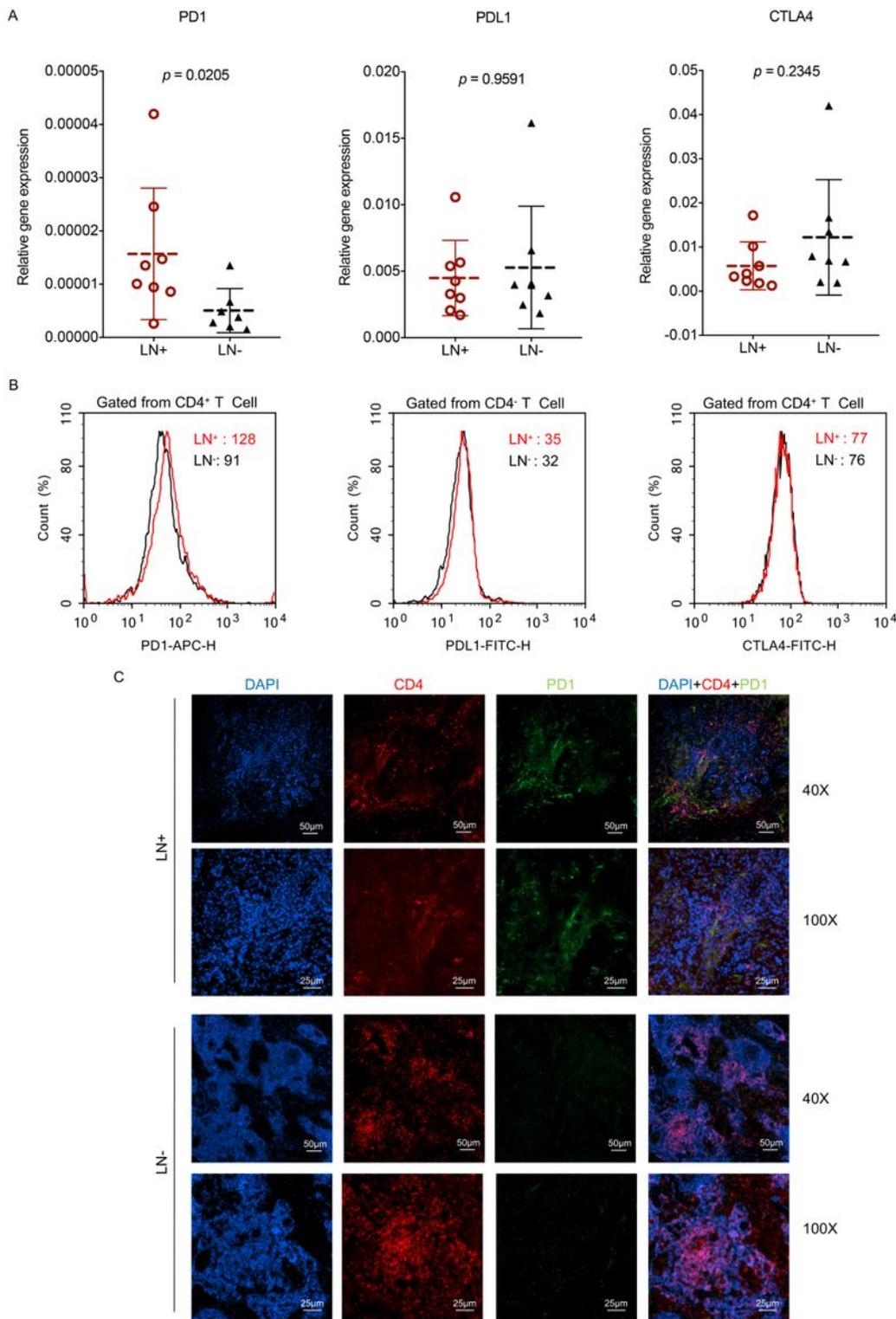


Figure 3

Immune checkpoint of LN⁺ and LN⁻ from OSCC patients (A) The mRNA expression of PD1, PDL1 and CTLA4 were performed by RT-PCR (B) PD1, PDL1 and CTLA4 of LN⁺ and LN⁻ were measured by flow cytometric analysis (C) PD1 and CD4 of LN⁺ and LN⁻ were detected by immunofluorescence analysis (Resolution: 300 dpi)

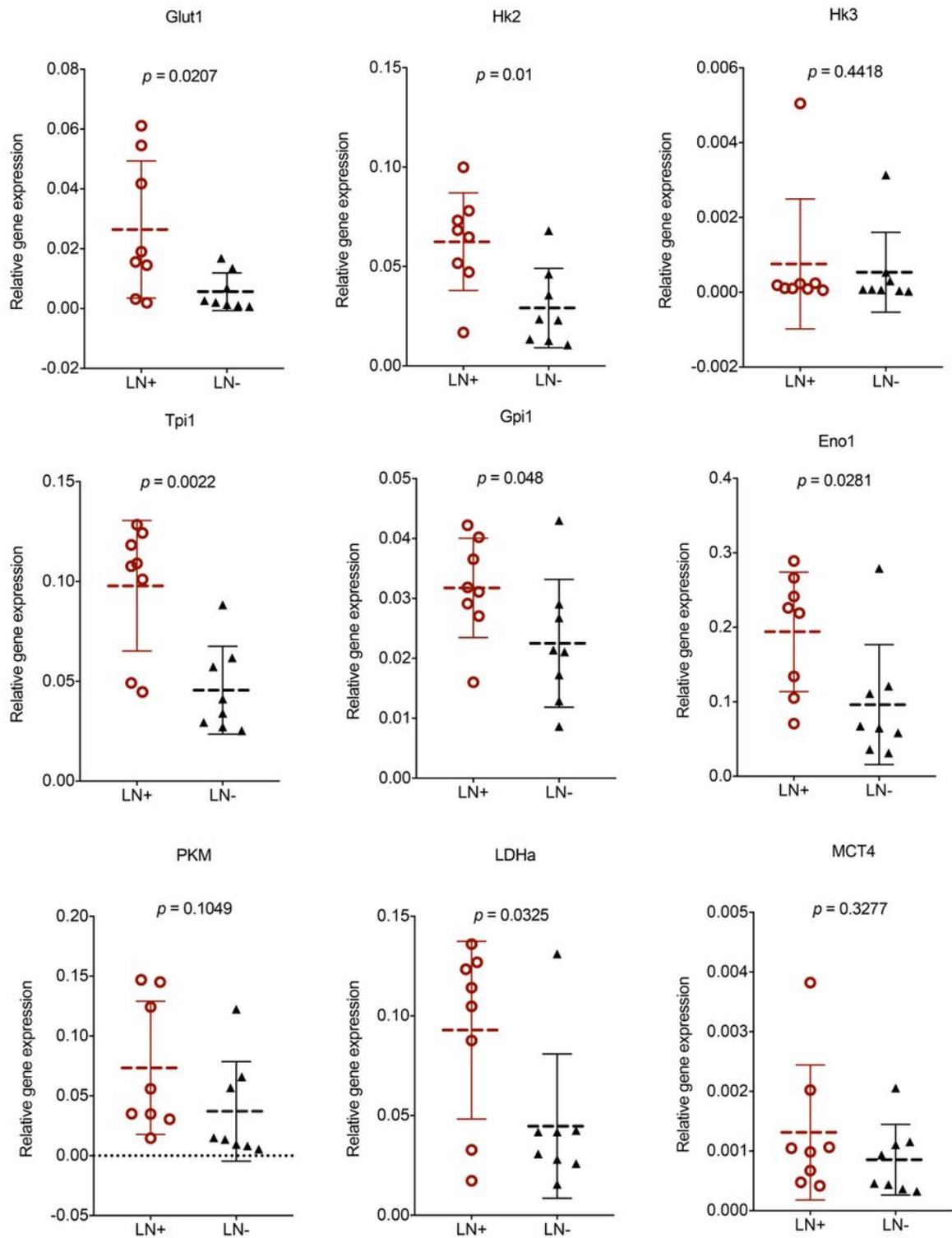


Figure 4

Glut1, Hk2, Hk3, Tpi1, Gpi1, Eno1, PKM, LDHa and MCT4 in CD4+ T cells were detected by RT-PCR.

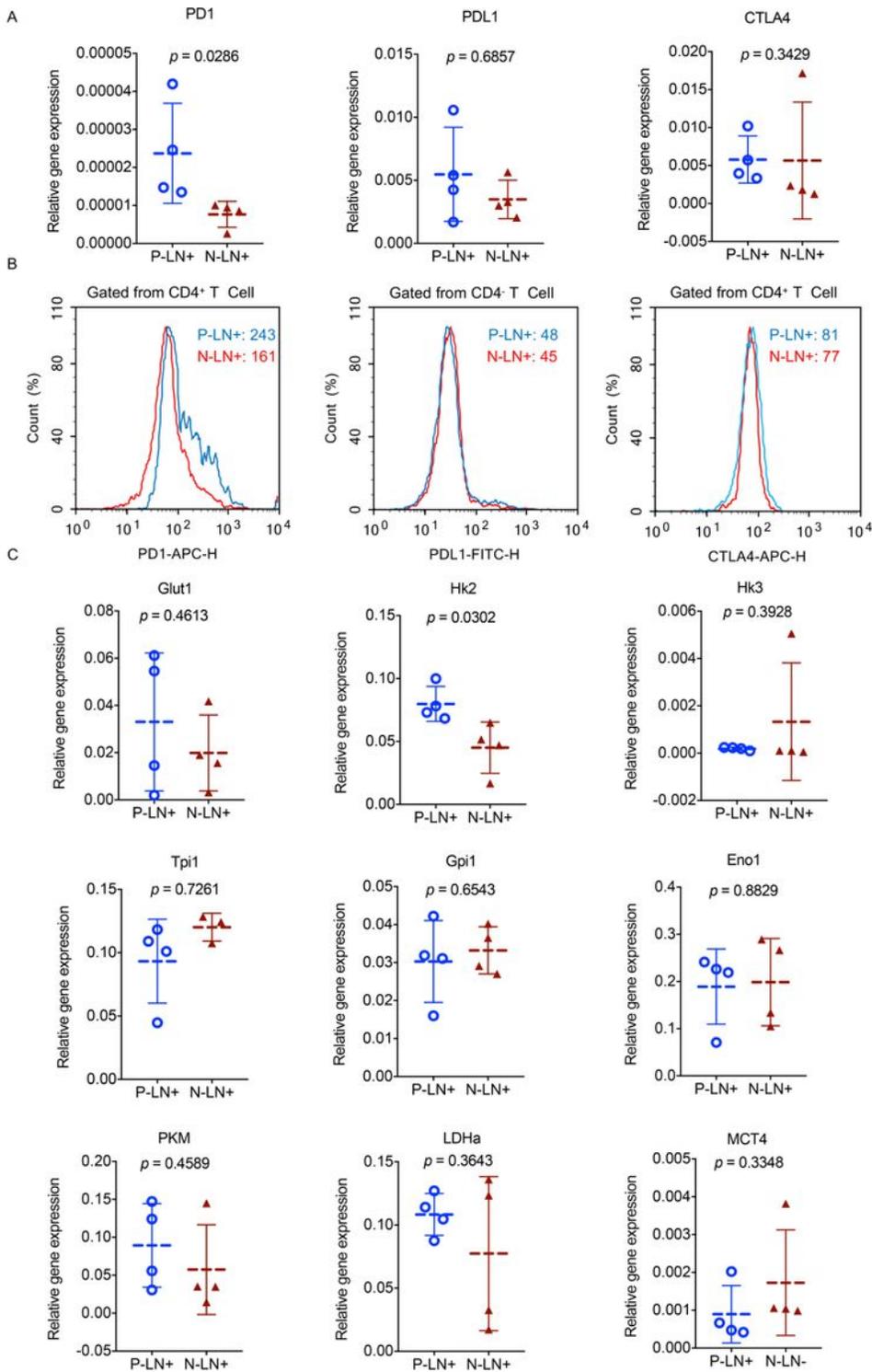


Figure 5

Immune checkpoint and glycolysis related enzymes were analyzed according to prior surgical treatment history. (A) The mRNA expression of PD1, PDL1 and CTLA4 were performed by RT-PCR according to prior surgical treatment history (B) PD1, PDL1 and CTLA4 of LN+ and LN- were measured by flow cytometric analysis according to prior surgical treatment history (C) Glut1, Hk2, Hk3, Tpi1, Gpi1, Eno1, PKM, LDHa and MCT4 in CD4⁺ T cells were detected by RT-PCR according to prior surgical treatment history

Supplementary Files

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