

Increased Levels of PD1 and Glycolysis in CD4⁺ T Cells Promote Lymph Node Metastasis in Oral Squamous Cell Carcinoma Patients

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Research Article

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Abstract

Background: Cervical lymph node metastasis is one of the poorest prognostic factors in oral squamous cell carcinoma (OSCC). Activated immune cells and cancer cells generally have metabolic similarities in tumor microenvironment. However, it is unknown whether abnormal glycolysis in T cells could facilitate metastatic lymph nodes in patients with OSCC.

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

Results: The frequency of CD4⁺ T cells decreased in the metastatic lymph nodes ($p = 0.0019$). Immune checkpoints (PD1, PDL1, and CTLA4) of CD4⁺ T cells were detected in metastatic (LN+) and paired negative lymph nodes (LN-) of OSCC patients. The PD1 expression of LN+ increased markedly compared to that of LN- ($p = 0.0205$). Similarly, the PD1 of CD4⁺ T cells in LN+ increased significantly compared to that of LN-. Glycolysis-related enzyme levels in CD4⁺ T cells from LN+ were dramatically higher than those in LN-. Moreover, PD1 and Hk2 expressions in CD4⁺ T cells increased in metastatic lymph nodes of OSCC patients with prior surgical treatment compared to those without.

Conclusions: These findings suggest that increased PD1 and glycolysis in CD4⁺ T cells may serve as pivotal regulators of OSCC metastatic lymph nodes, which are closely associated with elevated glycolysis.

Background

Oral squamous cell carcinoma (OSCC) is a major and devastating oral cancer subtype, accounting for over 90% of all malignant tumors in the oral cavity [1]. Cervical lymph node metastasis is one of the poorest prognostic factors in OSCC, with a 50% reduction in the survival of patients with a lymph node positive diagnosis compared to those without [2]. Accordingly, we have detailed the underlying molecular mechanism of cervical lymph node metastasis in an attempt to decrease the mortality of patients with OSCC.

The host immune tolerance and activation depend on the balance of positive and negative signals, which are 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 inhibits effector functions of T cells and expansion in the tumor microenvironment, thereby enabling tumor cells to escape immune surveillance [5]. Moreover, immune checkpoint receptors in T cells can determine their activation, expansion, and effector functions [6] through regulating metabolic activity [7; 8]. T cells have a highly dynamic metabolism and specific metabolic pathways that can support specific functions in

various cells, such as effector, memory, regulatory, and alloreactive T cells [9]. Therefore, activating T cells causes a large increase in glucose metabolism and aerobic glycolysis fuel [10].

Lymph nodes are pivotal peripheral immune organs that respond to disseminated tumor cells via presenting tumor cell antigens and the subsequent priming of effector cells, such as antigen-specific T cells [11; 12]. However, it remains unclear how immune checkpoints contribute to metastatic lymph nodes in patients with OSCC. Furthermore, the correlation between glycolysis and immune checkpoint expression in CD4⁺ T cells has not been explored.

In this study, we investigate the effects of immune checkpoints in metastatic lymph nodes. Furthermore, experiments are performed to identify glycolysis in CD4⁺ T cells from metastatic lymph nodes. This study reveals that increased PD1 and glycolysis in CD4⁺ T cells promote lymph node metastasis in patients with OSCC.

Methods

Ethics

The Ethics Committee of the Second Xiangya Hospital approved this study. Written informed consent was obtained from all participants prior to their enrollment. All experimental procedures were performed in accordance with the Helsinki Declaration.

Patients and Specimens

All OSCC samples were collected from the Department of Oral and Maxillofacial Surgery at Second Xiangya Hospital of Central South University. Specifically, lymph nodes were obtained from patients with OSCC who underwent surgery between June 2018 and July 2019. A single metastatic lymph node (LN+) and one paired negative lymph node (LN) were collected from 11 patients with OSCC. Half of each lymph node was stored for the experiments, and the other half was sent for pathological diagnosis. Clinical parameters were obtained from medical records.

T cell isolation

Lymph nodes preparation and digestion were using 1 mg/ml Collagenase IV and 40 µg/ml Dnase I as followed [13]. The cells processed from the lymph nodes were used for T cell isolation and flow cytometry analysis. CD4⁺ T cells were sorted from a single-cell suspension drawn from lymph nodes with the CD4⁺ T cell Isolation Kit (BioLegend), purity levels were greater than 95%, as determined by using the BD FACSCalibur. CD4⁺ T cells were used for real-time PCR analyse.

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

Total RNA from CD4⁺ T cells was isolated using the TRIzol reagent (Takara, Japan), and cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara, Japan). Real-time PCR was performed using a

SYBR Premix Ex Taq Reagent Kit (Takara, Japan) via the StepOne Real-Time PCR System (Life Technologies, USA) according to the manufacturer's instructions. In tissue lysates, mRNA levels were normalized to β -actin levels. The primer sequences used in this study are listed in Table 1.

Immunofluorescence analysis

Paraffin-embedded sections were deparaffinized, rehydrated, and submerged in an EDTA buffer for heat-induced antigen retrieval. The sections were then immersed in 0.3% hydrogen peroxide, blocked with 10% goat serum, incubated with specific primary antibodies at 4°C overnight, and incubated with an Alexa Fluor 488-cojugated secondary antibody (Invitrogen, USA) or Alexa Fluor 549-cojugated secondary antibody (Invitrogen, USA) in the dark at room temperature. Sections were stained with DAPI (Sangon Biotech, China) to detect the nuclei. Sections were imaged using a TCS SP2 laser-scanning confocal microscope (Leica Microsystems, Germany) and Gen5 software (Bio Tek, USA).

Flow cytometry

Cell surface markers were analyzed using flow cytometry (FCM). The living cells were stained with antibodies in PBS containing 0.1% (w/v) BSA and 0.1% NaN_3 in 50 μL FACS buffer for 30 min on ice. 7-Amino-Actinomycin D (7-AAD) was used for the exclusion of nonviable cells in flow cytometric assays. The following antibody-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), and anti-CD152 APC (BNI3). The antibodies were obtained from BioLegend or BD Pharmingen).

Statistical Analysis

Kruskal-Wallis, Mann-Whitney, or nonparametric paired tests (i.e., the Wilcoxon matched paired test) were used to analyze the non-parametric distribution of the samples. All statistical analyses were performed using SPSS (version 17.0; SPSS, Chicago, IL, USA). All values were two-sided, and statistical significance was set at $p < 0.05$.

Results

Metastatic lymph nodes in OSCC patients

Lymph node metastasis is one of the poorest prognostic factors in patients with OSCC [2]. A fresh sample of metastatic lymph nodes (LN+) and negative lymph nodes (LN-) were collected and keratinizing cells were observed in LNs + (Fig. 1A-1B).

Decreased frequency of CD4⁺ T cells in metastatic lymph nodes

The lymph node is a secondary lymphoid organ [14] that represents a pivotal meeting point of various immune cell types for adaptive immune responses [15]. To identify the immune cell types in metastatic lymph nodes, LN+ and paired LN- collected from each OSCC patient were analyzed via flow cytometry. The clinical parameters of the 11 OSCC patients are shown in Table 1. The percentage of T cells was significantly lower in LN+ than in LN- ($p = 0.0028$) (Fig. 2A). However, there was no significant difference between the percentage of B cells ($p = 0.9825$), dendritic cells ($p = 0.7674$), or macrophages ($p = 0.1625$) in LN+ and paired LN- (Fig. 2A). In a further analysis, CD4⁺ T cells dramatically decreased in LN+ compared with LN- ($p < 0.0001$) (Fig. 2B), whereas CD8⁺ T cells did not change in LN+ (Fig. 2B). This result indicated that the decreased frequency of CD4⁺ T cells was closely associated with metastatic lymph nodes in OSCC.

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

Immune checkpoint receptors on T cells can negatively determine their expansion, activation, and effector functions via inhibitory signals generated through binding to the receptors [6]. The interaction of PDL1 with its cognate ligand PD1 on activated T cells inhibits anti-tumor immunity by counteracting T cell-activating signals [16]. To detail the expression of immune checkpoint receptors PD1, PDL1, and CTLA4 in metastatic lymph nodes, immune checkpoint receptor transcriptional levels were detected using RT-PCR. The LN+ and paired LN- from each OSCC patient were collected, and the clinical parameters of 11 OSCC patients are shown in Table 1. Only the PD1 expression level of CD4⁺ T cells was notably upregulated in LN+ compared to that in LN- ($p = 0.0158$) (Fig. 3A). To further determine changes in immune checkpoints in metastatic lymph nodes, PD1, PDL1, and CTLA4 protein levels were detected using flow cytometry. As expected, the PD1 protein level of CD4⁺ T cells was significantly upregulated in LN+ compared to LN- ($p < 0.0001$) (Fig. 3B and Fig. 3C, respectively). However, there was no significant difference in PDL1 and CTLA4 between LN+ and LN- (Fig. 3B and Fig. 3C, respectively).

Immunofluorescence analysis showed that PD1 was predominantly expressed in CD4⁺ T cells and markedly upregulated in LN+ compared to LN- cells (Fig. 3D). Our findings revealed that the increased PD1 of CD4⁺ T cells in LN+ was related to 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 activated T cells [9; 17]. To determine whether 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 via RT-PCR. The mRNA expression levels of Glut1, Hk2, Tpi1, Gpi1, Eno1, and LDHa in CD4⁺ T cells dramatically increased in LN+ compared to LN- (Fig. 4). Although there was no statistical difference between Hk3, PKM, and MCT4 expression levels in LN+ and LN-, the average values of Hk3, PKM, and MCT4 expression levels in LN+ were higher than those in LN- (Fig. 4). These results suggest that an increase in PD1 of CD4⁺ T cells is linked to glucose metabolism and aerobic glycolysis.

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

LN+ in OSCC patients with a surgical treatment history (i.e., underwent neck lymph node dissection) was defined as P-LN+ (n=7), and LN+ in OSCC patients without a prior surgical treatment history was defined as N-LN+ (n=4). The PD1 expression level of CD4⁺ T cells was markedly upregulated in P-LN+ compared to N-LN+ ($p = 0.0286$), whereas there was no statistical difference in the PDL1 and CTLA4 expressions between P-LN+ and N-LN+ (Fig. 5A and 5B, respectively). To determine whether glycolysis-related enzymes contributed to the upregulation PD1 of in 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 the patients' surgical treatment history. Only the Hk2 expression levels of CD4⁺ T cells dramatically increased in P-LN+ compared to N-LN+ ($p = 0.0061$) (Fig. 5D). These data suggest that the increase in PD1 of CD4⁺ T cells in P-LN+ was associated with elevated Hk2.

Discussion

We have demonstrated in this study that the percentage of CD4⁺ T cells decreased in LN+ compared to LN-. Additionally, the expressions of PD1 and glycolysis-related enzymes were elevated in CD4⁺ T cells from metastatic lymph nodes. These results indicate that increases in PD1 of CD4⁺ T cells in LN+ facilitates lymph node metastasis progression and is closely related to glucose metabolism and aerobic glycolysis. In the following experiments, the findings revealed that PD1 and Hk2 of CD4⁺ T cells were upregulated in P-LN+ compared to N-LN+.

T cells are regarded as the principal weapons of immunity against cancer [18]. T cells inhibit tumor cells in various ways, either directly by killing tumor cells via cytolytic mechanisms, or indirectly by modulating the tumor microenvironment [19]. Emerging evidence has revealed that CD4⁺ T cells are necessary to initiate and maintain anticancer immune responses [20; 21]. Furthermore, CD4⁺ T cells may increase the quality and magnitude of B cells and CD8⁺ cytotoxic T lymphocyte responses in lymph nodes [19]. Changes in the number of CD4⁺ T cells are vital in creating robust hosts against tumors, especially for lymph node metastasis [22]. Our findings suggest that a decrease in the percentage of CD4⁺ T cells in LN+ inhibits anti-tumor immunity, which leads to the progression of OSCC metastatic lymph nodes. Tumor-draining lymph nodes are the major sites for priming tumor-reactive T cells and tumor metastasis. Metastatic lymph nodes contain tumor cells and immune cells, and the percentage of immune cells decreases correspondingly [23]. It is uncertain why the immune cells in the lymph nodes cannot recognize and clear the invading tumor cells, as well as tend towards tumor metastasis.

The PD-1 checkpoint blockade has revolutionized the field of cancer immunotherapy. As immune checkpoint blockade therapies fail to induce responses in the majority of cancer patients, increasing the objective response rate has therefore become an urgent challenge [24]. A previous study on cervical carcinomas reported that PD1 was expressed by a vast number of infiltrating CD8⁺ T cells, thus

suggesting that PD1 could serve as a potential therapeutic target [25]. In addition, a 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 [26]. The expression of the inhibitory receptor PD1 through lymph node and tumor-infiltrating regulatory T cells have been shown to be correlated with lymph node metastasis in pancreatic ductal adenocarcinoma [27]. The presence of metastatic neck nodes and tumor recurrence is associated with poor prognoses [28; 29]. A recent study showed that the PD1 protein expression was significantly related to the PDL1 expression, a higher tumor infiltrating lymphocyte abundance, and distant metastasis [30]. Nonetheless, the relationship between lymph node metastasis of tumor cells and immune checkpoints remains unclear. Our study demonstrates that increasing the PD1 expression of CD4⁺ T cells in LN+ may promote lymph node metastasis, thereby suggesting that blocking PD1 may have therapeutic potential in these patients.

Activated immune cells and cancer cells often share metabolic similarities in the tumor microenvironment. This demonstrated that a striking increase in glycolysis was the main feature of T cell activation [31]. Glucose can be used by T cells to support effector functions [32]. Accordingly, T cells do not have extensive internal glycogen stores, making them highly dependent on the uptake of extracellular glucose to meet increased metabolic needs during an immune response [7]. A study by Bengsch showed that PD1 regulates glycolysis and the mitochondrial function of virus-specific CD8⁺ T cells in chronic lymphocytic choriomeningitis virus infection [33]. Melanoma patients with a high expression of glycolysis-related genes also showed worse progression-free survival rates following anti-PD1 treatment [34]. Our study has demonstrated that increasing PD1 of CD4⁺ T cells in LN+ is associated with glycolysis-related enzymes, thereby indicating that increased PD1 of CD4⁺ T cells inhibits anti-tumor immunity and is associated with glucose metabolism and aerobic glycolysis fuel. Furthermore, PD1 and Hk2 of CD4⁺ T cells also increased in P-LN+ compared to N-LN+. This suggests that Hk2 may be a key enzyme in glycolysis, thereby contributing to the progression of metastatic lymph nodes in OSCC.

Conclusions

In summary, our study suggests that lymph node metastasis and recurrence in OSCC are closely associated with increases of PD1 and glycolysis in CD4⁺ T cells; this response may serve as a potential regulator in OSCC progression.

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 analyzed during the current study are available from the corresponding author upon 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 designed this study. WK was a major contributor in writing the manuscript. ZS and LY edited the manuscript. ZS and WK collected the specimens. All authors have read and approved the final manuscript.

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Tables

Table 1. The clinical parameter of OSCC patients were used.

NO.	Age	Gender	T classification	Lymph node status	Tumor site	Perineural invasion	Prior Radiotherapy/ Chemosensitivity	Prior surgical treatment
1	78	Female	T2	pN positive	Tongue	No	No	Yes
2	49	Female	T3	pN positive	Buccal mucosa	Yes	No	Yes
3	51	Male	T3	pN positive	Tongue	Yes	No	Yes
4	59	Male	T3	pN positive	Tongue	Yes	No	Yes
5	71	Male	T2	pN positive	Tongue	Yes	No	Yes
6	68	Male	T2	pN positive	Tongue	Yes	No	No
7	85	Male	T4	pN positive	Buccal mucosa	No	No	Yes
8	45	Female	T3	pN positive	Oral floor	No	No	No
9	52	Male	T2	pN positive	Tongue	No	No	No
10	66	Male	T4	pN positive	Tongue	No	No	Yes
11	58	Female	T3	pN positive	Gingiva	No	No	No

pN: pathology lymph nodes status

Figures

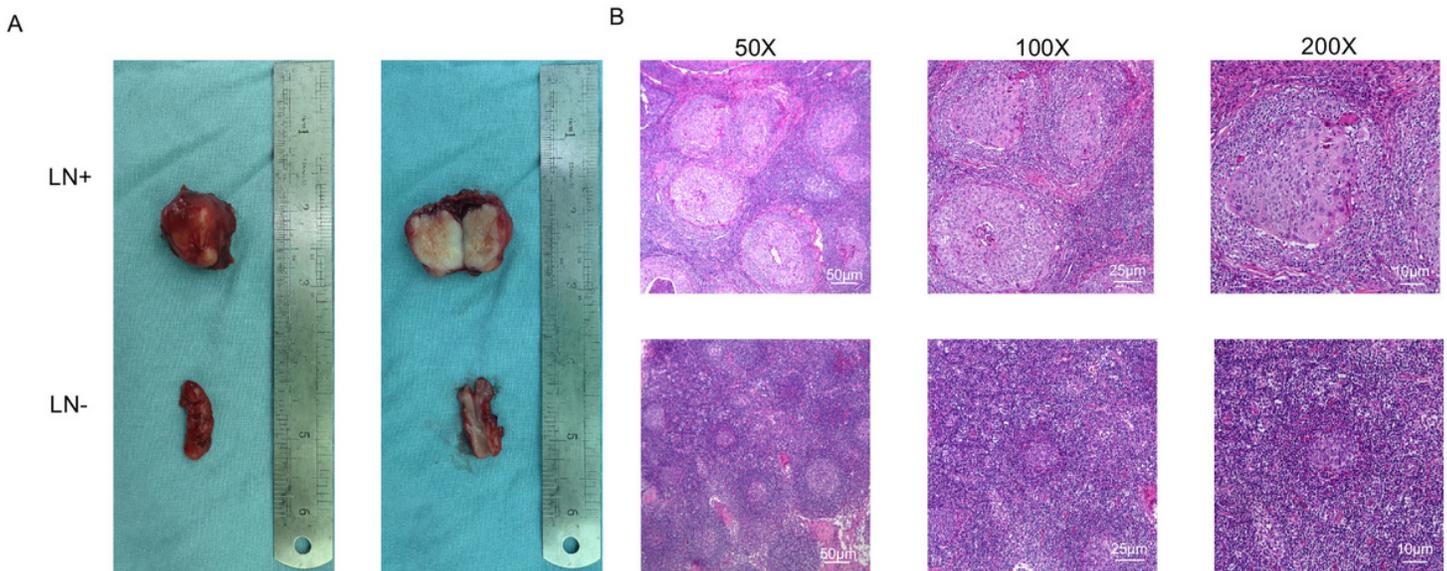


Figure 1

Metastatic lymph nodes (LN+) and negative lymph nodes (LN-) of OSCC patients.

(A) Macroscopic view images of LN+ and LN- fresh tissue samples from OSCC patients. (B) Representative hematoxylin & eosin staining (H&E) of LN+ and LN- samples. n=11 (Resolution: 300 dpi).

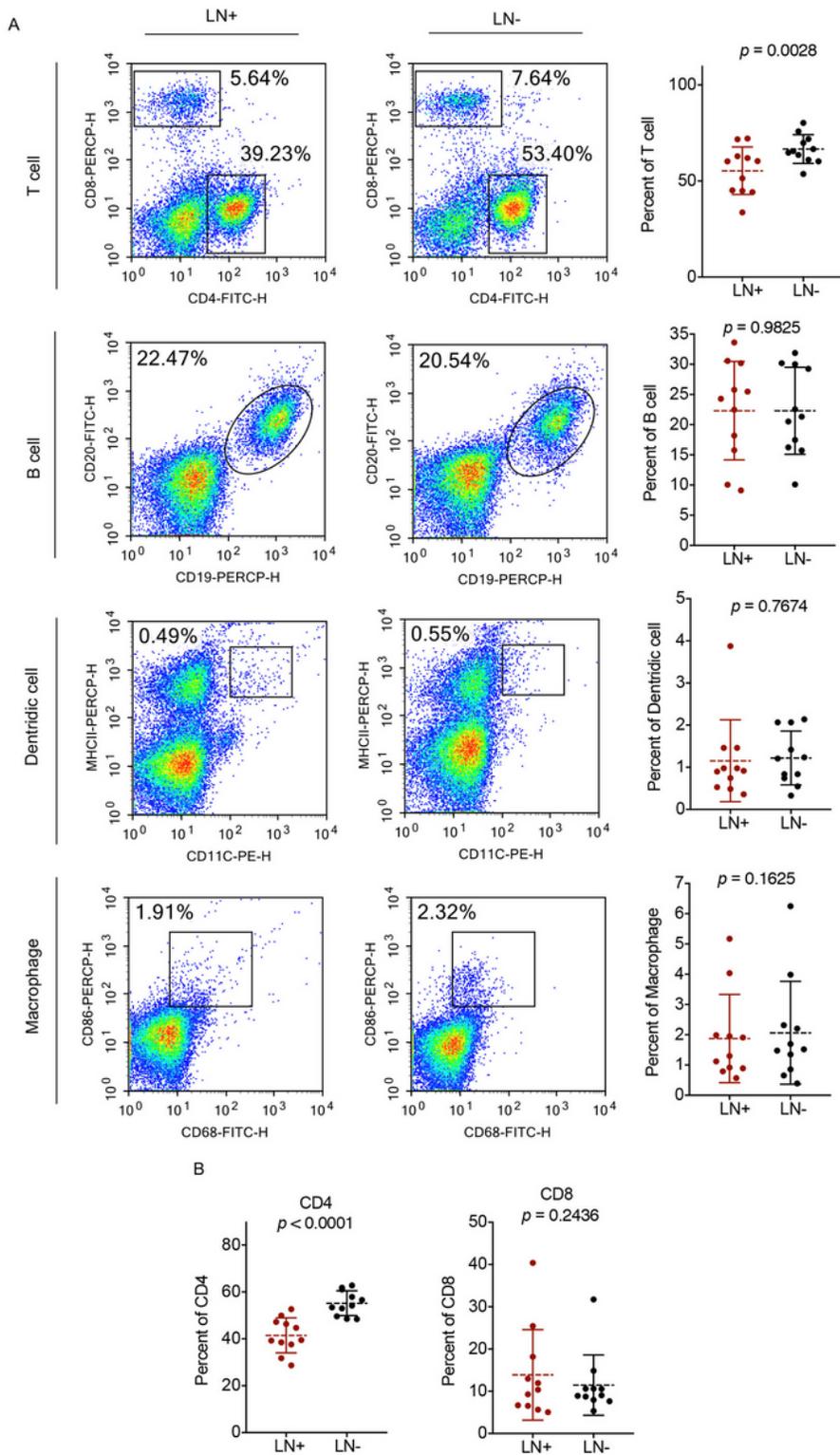


Figure 2

Immune cell types of LN+ and LN- from OSCC patients

(A) Cells of LN+ and LN- from OSCC patients stained with 7AAD, anti-CD45 mAb, anti-CD4 mAb, anti-CD8 mAb, anti-CD19 mAb, anti-CD20 mAb, anti-CD11c mAb, anti-MHCII mAb, anti-CD68 mAb and anti-CD86 for flow cytometry analysis. Representative flow cytometry analysis of T cells, B cells, Dendritic cells, and macrophages isolated from LN+ and LN- in OSCC patients.

(B) Percentages of CD4 and CD8 cells analyzed in LN+ and LN- samples. n=11.

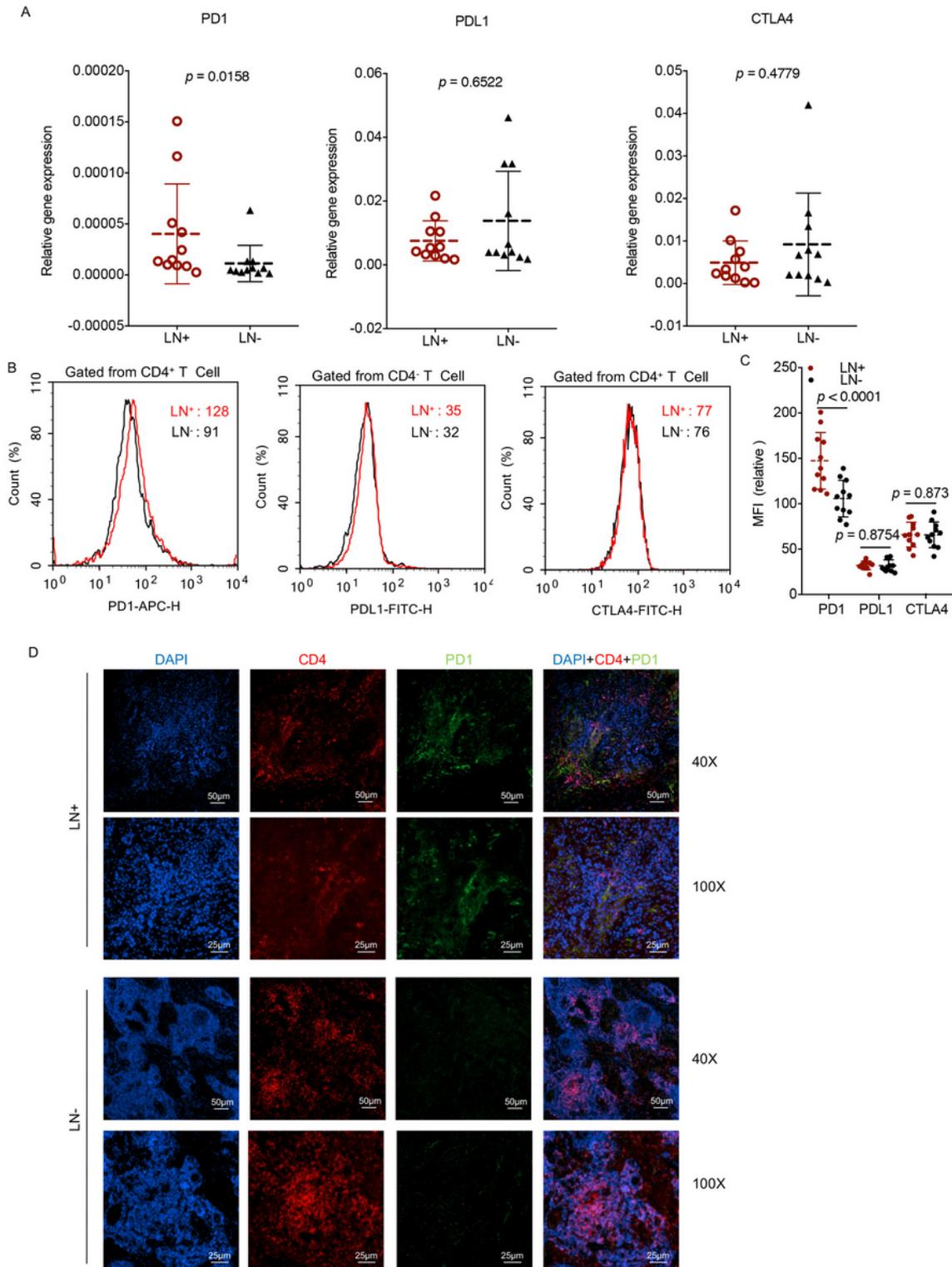


Figure 3

Immune checkpoint expression of LN+ and LN- from OSCC patients.

(A) mRNA expression of PD1, PDL1 and CTLA4 as performed by RT-PCR

(B) PD1, PDL1 and CTLA4 of LN+ and LN- measured through flow cytometric analysis.

(C) PD1 and CD4 of LN+ and LN- detected using immunofluorescence analysis. n=11, (Resolution: 300 dpi).

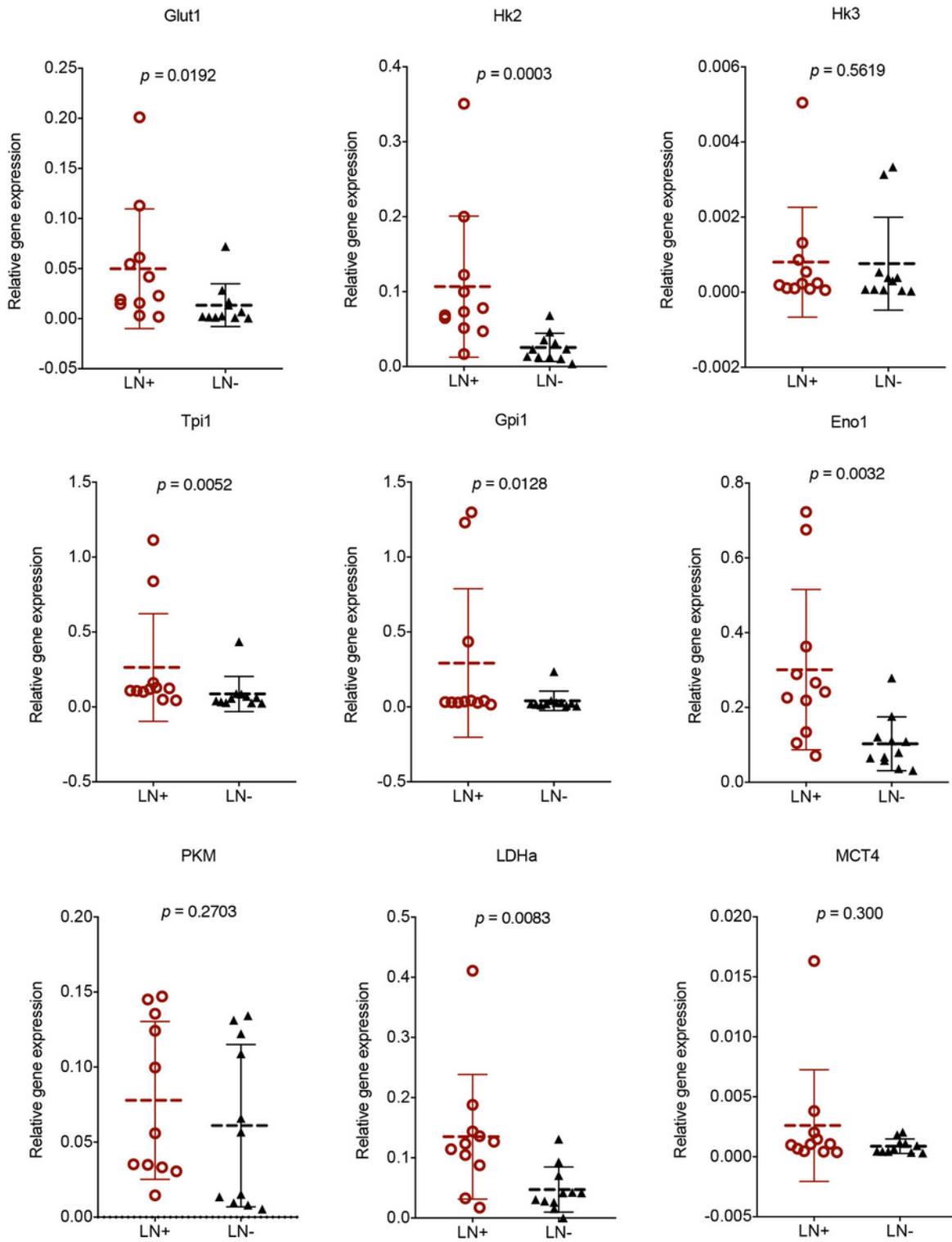


Figure 4

The mRNA expression of Glut1, Hk2, Hk3, Tpi1, Gpi1, Eno1, PKM, LDHa and MCT4 in CD4⁺ T cells as detected by RT-PCR. n=11.

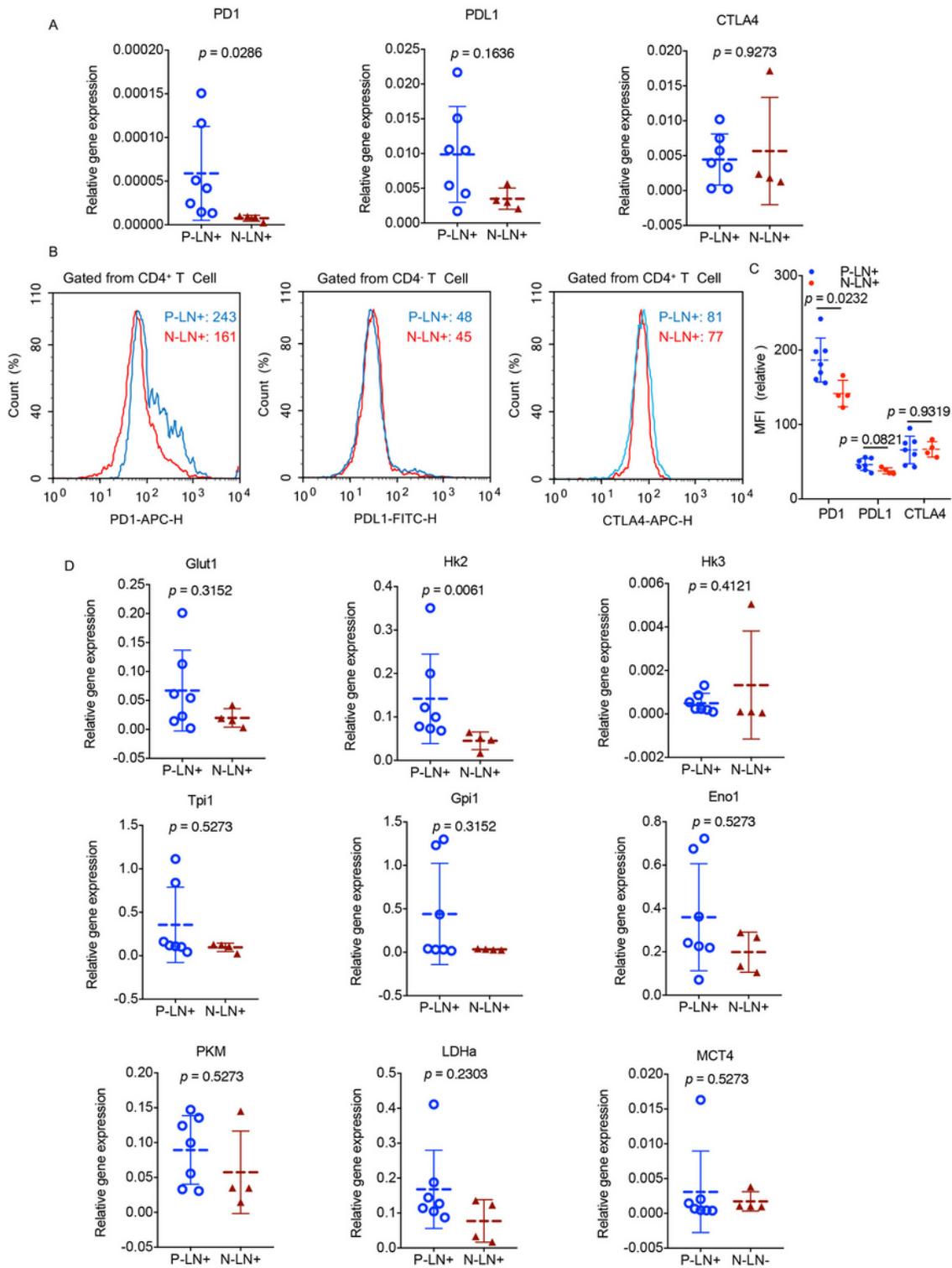


Figure 5

Immune checkpoint and glycolysis related enzymes analyzed according to prior surgical treatment history.

(A) mRNA expressions of PD1, PDL1 and CTLA4 in LN+ and LN- as performed by RT-PCR according to the surgical treatment history. n=11.

(B) PD1, PDL1 and CTLA4 of LN+ and LN- as measured via flow cytometric analysis according to the surgical treatment history. n=11.

(C) Glut1, Hk2, Hk3, Tpi1, Gpi1, Eno1, PKM, LDHa and MCT4 of CD4⁺ T cells in LN+ and LN- as detected by RT-PCR according to the surgical treatment history. n=11.

Supplementary Files

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- [Supplementarytable1.docx](#)