Single Cell RNA Sequencing Analysis of the Immune Microenvironment in Treatment Resistant Esophageal Squamous Cell Carcinoma

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Abstract: Esophageal squamous cell carcinoma (ESCC) is a very aggressive disease that does not respond to the traditional methods of treatment, including chemotherapy and immunotherapy, and therefore has a low prognosis. Tumor immune microenvironment (TIME) is an important factor that contributes to resistance because it affects the function of immune cells and their interactions with tumor cells. The cellular composition and functional state of the immune microenvironment of the treatment-resistant ESCC were examined in the work by means of single-cell RNA sequencing (scRNA-seq). The populations of immune cells were observed to differ in the event of treatment-resistant and treatment-responsive ESCC. There were more exhausted CD8 + T cells, regulatory T cells (Tregs), and immunosuppressive macrophages in the treatment-resistant group. These cells also had high levels of immune checkpoint markers like PD-1, PD-L1, and CTLA-4, which are identified to play a role in inhibiting effective immune responses. In addition, there was also a metabolic reprogramming of these immune populations with an increased glycolytic activity, which leads to a dysfunction of immune cells and allows tumors to survive. Using gene set enrichment analysis (GSEA), there were a few pathways that were upregulated in the resistant cohort, such as immune checkpoint signaling, inflammatory responses, and metabolism pathways. These observations support the assumption that immune escape and metabolic restructuring are two influential factors in ESCC resistance. The findings indicate that both immune checkpoints and the metabolic reprogramming of immune cells may be combined therapies to provide a promising approach in overcoming treatment resistance in ESCC. The findings need to be validated in future studies with larger patient groups, multi-omics studies, and clinical trials to come up with better treatment options among ESCC patients.

Keywords: Esophageal Squamous Cell Carcinoma (ESCC), Tumor Immune Microenvironment (TIME), Single-Cell RNA Sequencing (scRNA-seq), Treatment Resistance, Exhausted T Cells, Immune Checkpoints, Metabolic Reprogramming.

INTRODUCTION

ESCC is a very aggressive type of cancer, which is usually associated with poor prognosis and a high risk of relapse following treatment [1-3]. Although chemotherapy, radiation, and immunotherapy are therapeutic alternatives, the overall survival rates of ESCC are low, especially because of the emergence of resistance to treatments. It is a multifactorial resistance that is related to complex interactions between tumor cells and the surrounding microenvironment, the tumor immune microenvironment. The TIME consists of immune cells that are of a wide variety, together with stromal and extracellular matrix molecules that form a tumor-favoring niche [4,5]. Among these microenvironment cells, the T cells, the macrophages, and the dendritic cells are key actors in the immune responses, either promoting anti-tumor immunity or allowing immune evasion and tumor proliferation. Emerging research shows that the changes in the TIME, such as the immune cells' dysfunction and the presence of immunosuppressive cells, play a significant role in therapeutic resistance [6,7].

Although the importance of the immune system in ESCC is a recognized fact, the heterogeneity and multifaceted character of the TIME of treatment-resistant tumors have not been fully understood. The existing treatment measures, such as chemotherapy and immunotherapy, have been limited in dealing with the immune elements that bring about resistance. As an example, chemotherapy may enhance the suppression of immune functions and immunotherapies, though they have potential, they usually fail because immune checkpoint inhibitors cannot overcome the suppressive tumor microenvironment. These issues highlight the importance of a more detailed understanding of the cellular structure of the TIME and how exactly the immune processes are resistant [8]. This knowledge of the role of various immune cell populations in the evasion of therapy would be useful in the formulation of more effective treatment techniques, especially immunotherapy, which so far has proven its potential but has encountered a lot of challenges when it comes to clinical use in the treatment of ESCC [9].

By employing scRNA-seq, a highly innovative methodology, this study is aimed at filling in the gap in knowledge concerning the immune landscape of treatment-resistant ESCC and providing a high-

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resolution analysis of cellular heterogeneity of the TIME [10]. In contrast to conventional bulk RNA sequencing, in which the data on gene expression of millions of cells is averaged, scRNA-seq allows identifying individual population groups of immune cells, their gene expression patterns, and their functional status in the tumor [11,12]. The study will identify the hitherto unknown immune cell types and pathways that underlie resistance by utilizing such technology on treatment-resistant ESCC [13]. Particularly, the analysis will examine how exhausted T cells, immunosuppressive myeloid cells, and other stromal constituents stimulate immune evasion and resistance to therapy. The results of the study will also be useful in the exploration of the cellular mechanisms of resistance in ESCC and in the discovery of new biomarkers and therapeutic targets that can be utilized to address the problem.

Despite past single-cell studies having investigated immune heterogeneity, treatment resistance, and tumor microenvironment modifications in esophageal squamous cell carcinoma (ESCC), this current study has provided a unique comparison between treatment-resistant ESCC and treatment-responsive ESCC samples through the use of a multi-component approach, which involves immune cell characterization, expression of checkpoint markers, enrichment of metabolic pathways, trajectory inference, and ligand-receptor analysis. The uniqueness of this research is based on the association established between the expansion of exhausted CD8+ T cells, the presence of regulatory T cells, immunosuppressive macrophages, glycolysis-related metabolic remodeling, and checkpoint signaling, all as part of treatment resistance. Unlike in other studies where only immune heterogeneity was studied, this current study has emphasized the contribution of immune suppression, metabolism, and tumor cell-communication towards treatment resistance.

The general hypothesis of the research is that the immune microenvironment of treatment-resistant ESCC contains certain immune cell subpopulations and signature genes that mediate resistance by controlling immune evasion and immunosuppression [14]. By examining the single-cell massive sequencing evidence, the most important immune pathways and cellular interactions regarding the aspects of therapeutic resistance will be identified. These results might lead to the creation of specific immune cell or signaling pathway therapies, which in the end would enhance the effectiveness of current therapies [15-17]. Clinical implications of the research are that it may offer new approaches to the treatment of cancer in a personalized manner that does not simply rely on the use of chemotherapy and immunotherapy, but rather goes directly to the immune components that are the cause of resistance [18, 19]. The study, thus, has the potential to bring clinical immunotherapy of cancer one step further to gain better insight into the immune microenvironment of ESCC and provide new opportunities to treat the disease [20].

In light of the clinical relevance of treatment resistance in ESCC, this current study employs single-cell transcriptomics to dissect the heterogeneity of immune cells, immune dysfunction mediated by checkpoints, metabolic adaptations, and intercellular communication in the TME of ESCC. In addition, the revised manuscript outlines the study protocol, the stratification of patients, sequencing procedures, bioinformatics analysis strategy, statistical validation, and limitations to achieve better scientific rigor and reproducibility.

Key Contribution

- The paper cites immune cell subsets, including exhausted CD8+ T cells and Tregs, as one of the determinants of immune evasion and resistance in ESCC with treatment resistance.
- It underscores the augmented glycolytic action of immune cells, which leads to immune dysfunction and tumor survival within the tumor microenvironment.
- The paper proposes that immune checkpoint inhibitors should be used in combination with immune cell metabolism therapies to enhance the success of treatment in ESCC.

This paper discusses the immune microenvironment of treatment-resistant esophageal squamous cell carcinoma (ESCC) using scRNA-seq. Section I presents the difficulties with the treatment of ESCC because of immune evasion. Section II presents the methodology, which includes the scRNA-seq to examine immune and tumor cell populations in treatment-resistant and treatment-responsive samples. Section III shows the findings, which found exhausted T cells and metabolic reprogramming in the resistant group. Section IV presents the possibilities of integrating immune checkpoint inhibitors with metabolic therapies as the solution to resistance. Section V ends with a conclusion that suggests future studies using bigger cohorts and multi-omics studies to confirm the results.

MATERIALS AND METHODS

This research was done as an experimentally performed transcriptomic profiling of treatment-resistant and treatment-sensitive esophageal squamous cell carcinoma. Tumor tissues of ESCC patients were acquired after obtaining ethical clearance and consent from them. Patients were categorized according to their response to the administered therapy of either chemotherapy or immunotherapy. Then, fresh tumor biopsy specimens or surgical specimens were dissociated to a single-cell suspension, which was then subjected to library preparation for scRNA-seq through the use of the 10x Genomics Chromium system.

Patient Cohort

In this study, the cohort of patients will be represented by clinical samples that will be obtained by the researchers to include patients with esophageal squamous cell carcinoma (ESCC), which is one of the most aggressive and difficult-to-treat types of cancer. The cohort will be categorized into two groups, namely treatment-resistant and treatment-responsive cases of ESCC. Patients who have been treated poorly or not at all by traditional therapies, including chemotherapy or immunotherapy, will constitute the treatment-resistant group, whereas those who have responded positively during the process of treatment will form the treatment-responsive group. All of these patients will be recruited at the hospitals and clinical centers that specialize in the treatment of ESCC, and informed consent will be obtained from all participants in accordance with the rules of ethics. Moreover, the institutional review board will give approval for the study, and the research will meet the ethical standards of confidentiality of patient information and management of biological items. Histological proof of ESCC will be included in the inclusion criteria, an appropriate sample of the tumor after administration will be provided, and the clinical follow-up information will be sufficient. The collection of all the tumor samples will be made at the moment of surgical excision or tumor biopsy, whereby the most relevant and up-to-date tumor data will be used.

The clinical categorization of ESCC patient samples used in this study is presented in Table **1**. The sample population was stratified into chemoresistant and chemosensitive groups depending on their documented response to chemotherapy or immunotherapy. Such categorization offers a foundation for comparing immune cell populations and gene expression profiles in relation to resistance or sensitivity to therapy. These inclusion criteria guarantee that all the samples analyzed using scRNA-seq were from ESCC patients with documented histories of therapeutic response.

The sample size of 20 patients in both treatment-resistant and treatment-responsive esophageal squamous cell carcinoma groups is justified, considering that studies involving single-cell RNA sequencing have an exploratory design in which the analysis does not depend exclusively on the sample size but also on the number of high-quality cells obtained from each tumor specimen. The sample size can be regarded as adequate to detect distinct immune-cell types, differential gene expression, and immunity related to drug resistance in the two patient groups. In order to enhance statistical robustness, several analyses, such as strict quality control, cell clustering, differential expression analysis, and false discovery rate correction, were carried out. Nevertheless, the sample size did not aim at generalization of results across a wider population.

Single-Cell RNA Sequencing Workflow

scRNA-seq of tumor tissues of treatment-resistant and treatment-responsive patients with ESCC will be done to capture the cellular heterogeneity present in the tumor microenvironment. On the collection of the samples, the tumor tissues shall be immediately dissociated to form single cell suspensions. This will be done through enzymatic digestion and then mechanical disruption in a bid to obtain high-quality single-cell suspensions. A 70 μm mesh filter is going to be used to filter out the suspension to eliminate cell clumps as well as dead cells. The isolated viable single cells will be counted using a hemocytometer, and quality control will be done before proceeding with further processing.

Table 1: Patient Cohort Details

Patient Group	Number of Patients	Response to Therapy	Inclusion Criteria
Treatment-Resistant	20	Poor response to chemotherapy/immunotherapy	Histologically confirmed ESCC, clinical treatment history
Treatment-Responsive	20	Positive response to therapy	Histologically confirmed ESCC, clinical treatment history

The library of scRNA-seq will be made on the platform of 10x Genomics Chromium, which is characterized by its high efficiency of isolating and barcoding single cells at high throughput. Once the cells are captured, RNA shall be reverse transcribed into cDNA in separate microdroplets, with each droplet having a single cell. The obtained cDNA will be amplified, and distinct barcodes will be introduced to ensure that the data of gene expression can be associated with specific cells. High-depth sequencing of the final libraries will be done to make sure that the transcriptome is completely covered. The depth of the sequencing will aim at about 50,000 to 100,000 reads per cell because this is expected to give the desired resolution to identify the highly expressed and lowly expressed genes in the different populations of cells.

The entire process followed for single-cell RNA sequencing is depicted in Figure **1**. In the initial step, the tumor sample obtained from patients with esophageal squamous cell carcinoma was processed to obtain viable single cells and was sequenced through the 10x Genomics Chromium. Following the synthesis of cDNA, amplification, and sequencing, the raw data were analyzed for quality control, normalization, clustering, dimensionality reduction, trajectory inference, and differential expression.

Sample Processing and Sequencing Details

Tumor biopsy or surgical resection specimens were obtained from ESCC patients with clinical diagnosis and transported in sterile and refrigerated media to prepare the single-cell sample. The necrotic or non-tumor regions were eliminated. The tumor tissues were minced using mechanical and enzymatic methods to obtain single-cell suspensions. The cell suspension was sieved using a cell strainer with a pore size of 70 µm. The cell viability was determined prior to library preparation, and high-quality suspensions were used for the sequencing process. The single-cell libraries were prepared by the 10x Genomics Chromium platform, and the sequencing was done with a read depth of about 50,000-100,000 per cell. Cells that failed the quality test with less than 500 genes detected, more than 20% expression of mitochondrial genes, or unusual UMI numbers indicative of doublet formation were eliminated. Gene transcripts detected in fewer than three cells were also excluded. Annotation of the cell types was done using the standard marker genes, including CD8A/CD8B (CD8+ T cells), FOXP3/IL2RA (regulatory T cells), CD68/CD163 (macrophages), CD11C/HLA-DRA (dendritic cells), and KRT14/KRT5/TP63 (tumor epithelial cells). Additionally, the annotation was verified automatically using tools such as SingleR or scType

Data Processing and Analysis Pipeline

Once the sequencing is completed, several pre-processing steps will be carried out on the obtained raw data, including quality control and filtering to remove low-quality cells or genes with poor coverage. The first step will involve cell barcode detection to determine the reads that are associated with specific cells, and the low-quality cells will be filtered out based

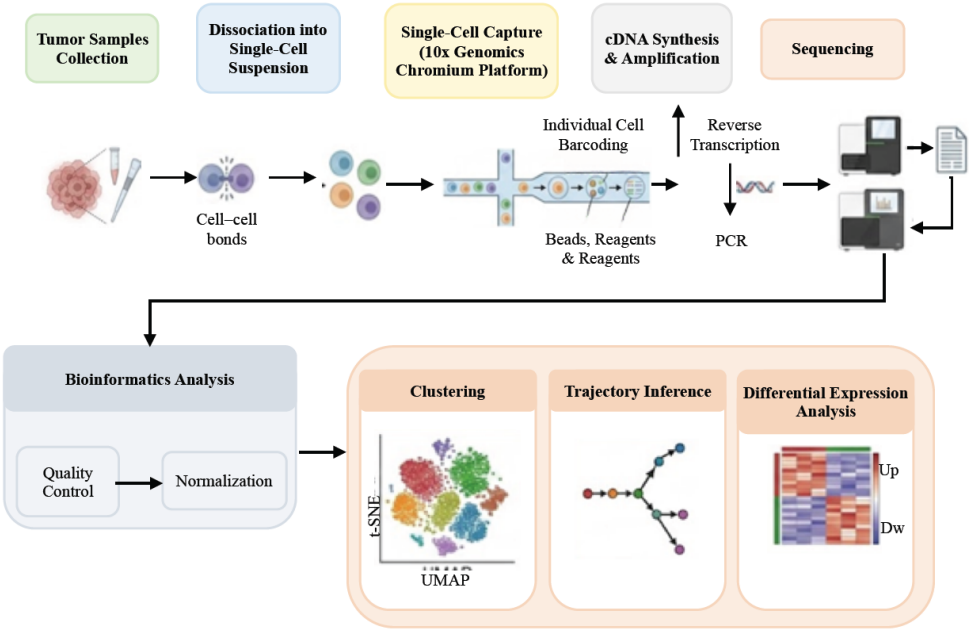

Figure 1: Overview of Single-Cell RNA Sequencing Workflow.

on such measures as the %age of mitochondrial gene expression and the count of reads. To ensure that the data is as complete as possible, cells containing greater than 20 % of their mitochondrial genes or fewer than 500 genes of those that are detected will be dropped.

The raw sequencing data were filtered with Cell Ranger v7.1.0 and mapped against the GRCh38 human genome. The quality control analysis of the filtered gene-barcode matrix was performed with Seurat v4.3.0 in R v4.2.2. Cells with fewer than 500 genes detected, greater than 20% of mitochondrial gene expression, and cells with very high counts (potential doublets) were filtered out from the dataset. Genes that were expressed in fewer than three cells were filtered out. The data were normalized using LogNormalise with a scale factor of 10,000.

The gene expression data will be normalized to correct the variation in the sequencing depth and other technical biases. Normalization of the data will be done with the Seurat or Scanpy packages, which are commonly used in the analysis of scRNA-seq data. This will facilitate the fact that the data is directly comparable between cells, which will enable the identification of differentially expressed genes correctly. Clustering analysis will be performed as an unsupervised procedure after normalization, either with a Louvain / K-means clustering to group cells according to their expression profile of the genes. These groups will be diverse immune and tumor cells in the TIME.

Uniform manifold approximation and projection (UMAP) will minimize the dimensionality of the collected data to levels that allow visualization. UMAP is a non-linear dimensionality reduction method, which can be used to plot high-dimensional data on a two-dimensional scale. It specifically works with the data of scRNA-seq because it maintains both local and global structure, which gives a convenient idea of the population of cells and the interactions between them. The outcomes of the clustering will be determined to identify each cell type, and the cell-type annotations will be done with the help of marker genes that are specific to the known immune cell types (e.g., CD8+ T cells, macrophages) and tumor cells.

The marker genes for annotation of the cells were compiled and listed in Table **2**. This table provides details of how different cell populations were annotated using classical gene markers that define them, such as CD8A and CD8B for CD8+ T cells, FOXP3 and IL2RA for regulatory T cells, and KRT14, KRT5, and TP63 for tumor epithelial cells. This will help avoid any confusion during cell cluster annotation. Also, this table helps in increasing reproducibility by demonstrating marker genes that define each type of cell.

Differential Expression Analysis

Differential expression of the treatment-resistant and the treatment-responsive groups will be conducted to determine the top genes in resisting treatment. The Wilcoxon rank-sum test will be utilized to test the gene expression levels of the two groups, and the genes with significant differences in expression will be determined. These genes will be researched further on their roles in immune evasion, the development of tumors, and therapy resistance. Moreover, the GSEA will be implemented to reveal the biological pathways that are differentially regulated in the treatment-resistant group. This will aid in detecting molecular signatures that originate with resistance mechanisms, e.g., immune checkpoint (e.g., PD-L1, CTLA-4) and inflammatory (e.g., TNF- α, IL-6) pathways.

Trajectory Inference and Cell-Type Annotation

The paths of differentiation of the immune and tumor cells in the TIME will be investigated through the application of trajectory inference methods, i.e., Monocle or Slingshot. Such techniques enable tracing the lineage of cells, especially of immune cells such as T cells and macrophages, which can also functionally change as a result of treatment. The transitions between activated and exhausted immune states or the

Table 2: Marker Genes for Immune Cell Type Annotation

Cell Type	Marker Genes
CD8+ T Cells	CD8A, CD8B, GZMA, GZMB
Macrophages	CD68, CD163, CCL2
Regulatory T Cells	FOXP3, IL2RA, CD25
Tumor Cells	KRT14, KRT5, TP63
Dendritic Cells	CD11C, HLA-DR, CD80

development of immunosuppressive cell populations in the tumor microenvironment will be identified with the help of trajectory analysis.

Cell-type annotation will be applied to known gene markers, and other computational methods, such as SingleR or scType, will be applied to give cell types to the identified clusters. Full characterization of immune cell populations, e.g., the exhausted T cells, regulatory T cells, and the myeloid-derived suppressor cells, will be done to learn more about their role in resistance to therapy.

Figure **2** demonstrates the visualization using UMAP of the immune and tumor cell populations that have been identified in both resistant and responsive ESCC samples. Every cluster denotes cell populations that share common transcriptional profiles. In contrast, the distance between clusters denotes differences in gene expression profile and cell identity. This visualization shows the heterogeneity of the tumor immune microenvironment. It helps to identify different cell populations, including exhausted T cells, macrophages, regulatory T cells, dendritic cells, and tumor cells. Only cells that passed through quality filtering were used for the UMAP plot. Differences between the treatment-resistant and treatment-sensitive esophageal squamous cell carcinomas in the relative abundance of immune cells were considered statistically significant if the p-value was less than 0.05.

Validation Methods

For verification of the findings from the scRNA-seq analysis, certain immune cell signatures as well as pathways that could be associated with drug resistance were supposed to undergo further testing with different approaches, including experiments and bioinformatics. The approach for flow cytometry was to verify exhausted CD8+ T cells, regulatory T cells, and immunosuppressive macrophages based on the markers CD8, PD-1, FOXP3, CD68, and CD163. Immunohistochemistry was supposed to verify the expression of PD-L1, CTLA-4, and CD163.

In order to confirm the results of scRNA-seq, additional methods, such as spatial transcriptomics, flow cytometry, and immunohistochemistry (IHC), will be utilized. Spatial transcriptomics will offer a spatial map of gene expression, which will allow visualization of the distribution of immune and tumor cells in the tissue setting. This will establish the physical arrangement of the central immune populations that have been determined in the scRNA-seq analysis.

The population of immune cells found with scRNA-seq will be further characterized using flow cytometry. Isolation and quantification of immune cells will be done on the treatment-resistant and treatment-responsive cohorts using surface markers. This will further support the cellular composition in the TIME.

The IHC will also be used to confirm the expression of major immune checkpoint proteins, including PD-L1, and cytokines that were detected in the scRNA-seq analysis. The patient cohort tissue sections will be stained with the antibodies to these markers, and their expression will be evaluated in the tumor compartment and the stromal compartment.

Statistical Analysis

All the statistical tests will have a p-value of less than 0.05 as a statistically significant value. To

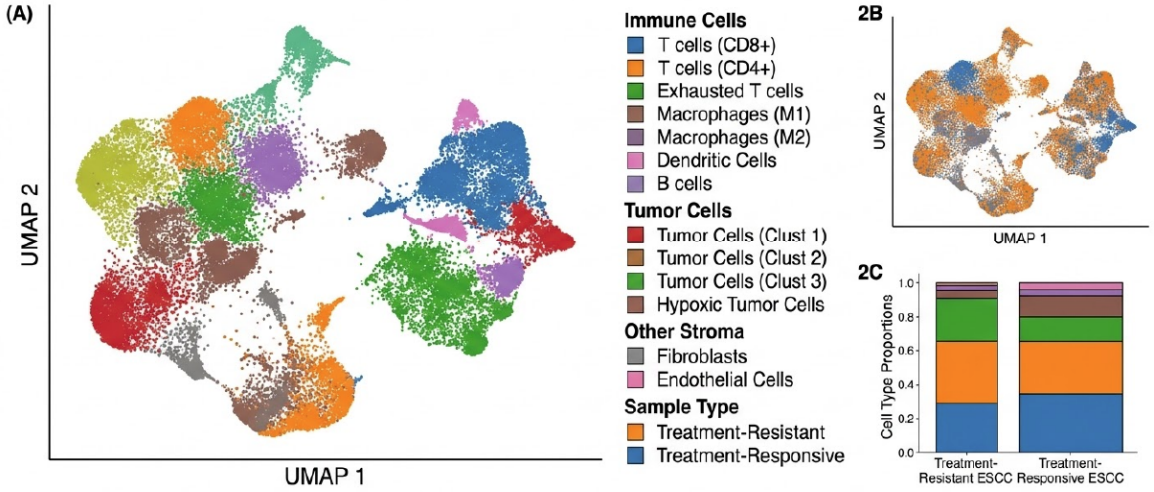

Figure 2: UMAP Visualization of Immune and Tumor Cell Populations.

consider the multiple testing, the false discovery rate (FDR) will be controlled by using the Benjamini-Hochberg correction. Visualizations will be created with the help of Seurat and ggplot2, which will give intuitive visualizations of the data, such as heatmaps, violin plots, and volcano plots, which will aid in detecting the important changes in the gene expression, immune subpopulation, and enriched pathways.

RESULTS

Statistical criteria were used in the interpretation of all major results presented in the Results section. The differences in abundance of immune cells between the groups of treatment-resistant and treatment-responsive ESCC were examined using group-wise comparison of annotated cell abundance data, with statistical significance being achieved at $p < 0.05$. The genes with differential expression between samples were determined using the Wilcoxon rank-sum test corrected for multiple testing via Benjamini–Hochberg adjustment. The genes with an adjusted p-value less than 0.05 and log2 fold change more than 0.25 were declared as statistically significant. Pathway enrichment results were interpreted based on normalized enrichment scores and Benjamini-Hochberg adjustment of p-values.

Cellular Composition and Heterogeneity in TIME

The analysis of scRNA-seq data demonstrated that there is a high level of complexity and heterogeneity in the TIME in the treatment-resistant and treatment-responsive ESCC samples. The total cellular makeup was determined through clustering of the individual cells, and the subpopulations of tumor cells, immune cells, and stromal cells were identified. The immunosuppressive cells, including exhausted T cells, regulatory T cells (Tregs), and the myeloid-derived suppressor cells (MDSCs), were also found to be more abundant in the treatment-resistant group than in the treatment-responsive group. These observations indicate the possibility that an anti-inflammatory immune response in the cohort of patients who are resistant to the treatment is one factor that may also lead to resistance to both chemotherapy and immunotherapy. These differences were interpreted as statistically meaningful based on group-wise comparison of annotated immune-cell proportions at $p < 0.05$.

The population of immune cells was further examined by determining the cell-specific markers, including the cytotoxic T cell (CD8A), macrophages (CD163), and regulatory T cells (FOXP3). Treatment-refractory patients showed a significantly higher level of immune cells associated with immune suppression, particularly exhausted T cells that express high levels of PD-1 and LAG-3. In addition, there was a significant increase in the number of macrophages exhibiting an immunosuppressive phenotype, such as CD163-positive macrophages, indicating that they can form an immunosuppressive niche for tumor growth.

Figure **3** depicts this phenomenon since it highlights the presence of immune cells and tumor cells in the

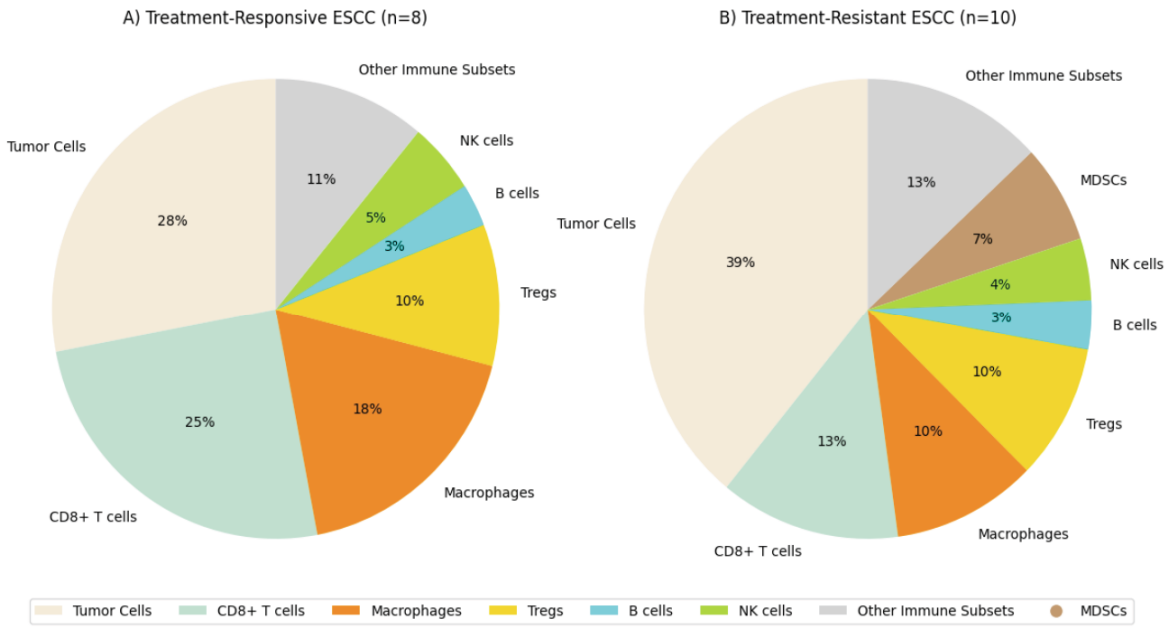

Figure 3: Cellular Composition of the TIME in Treatment-Resistant and Treatment-Responsive ESCC.

immune microenvironment of both treatment-sensitive and resistant ESCC patients. The number of exhausted CD8+ T-cells, regulatory T-cells, immunosuppressive macrophages, and (MDSCs) is higher in resistant tumors as compared to sensitive tumors, indicating the formation of a highly suppressive milieu. This further suggests that the pathophysiological mechanisms underlying the treatment resistance observed in ESCC are characterized by exhaustion of the immune response, reduced tumor rejection capacity, and enhanced tumor immunity evasion.

Subtype Identification in Treatment-Resistant ESCC

Additional UMAP clustering demonstrated that there were a few treatment resistance-selective immune cell subtypes in ESCC. Interestingly, the treatment-resistant group was highly enriched with exhausted CD8+ T cells, which are indicated by the increased expression of the immune checkpoint molecules, PD-1, CTLA-4, and TIM-3. These worn-out T cells showed a characteristic genomic expression pattern as they were highly expressing inhibitory receptors and had low expression of effector molecules such as granzyme A (GZMA) and interferon-gamma (IFNG). The exhaustion signature of such T cells indicates that they cannot express effective anti-tumor responses, which is a characteristic of immunotherapy resistance. The enrichment of exhausted CD8+ T-cell signatures was supported by statistically significant upregulation of checkpoint-associated markers and reduced expression of cytotoxic effector markers.

Besides the T cell depletion, the treatment-resistant samples also had a higher proportion of immunosuppressive macrophages, characterized by the high expression of CD163 and ARG1. These cells were connected to a pro-tumorigenic, inflammatory phenotype, such as increased cytokine concentrations, including IL-10 and TNF- α. Metabolic reprogramming was also observed in the myeloid cells, as the genes associated with glycolysis and oxidative stress response were upregulated. Such results emphasize the contribution of the TIME to the facilitation of resistance by means of immune suppression and metabolic adaptation.

Differences in the expression levels of selected immune markers between the resistant and responsive ESCC treatment groups are shown in Table **3**. High expression of PD-1, CTLA-4, FOXP3, and CD163 in the resistant group denotes increased immune-checkpoint activity, high regulatory T cell levels, and an immunosuppressive macrophage population. Lower expression of GZMA in the resistant group indicates lower activity of cytotoxic T cells. All differences have been found to be statistically significant. This implies that the resistant ESCC treatment group exhibits characteristics of immune exhaustion and suppression of immune cells.

Gene Signatures in Treatment-Resistant ESCC

GSEA was conducted to determine treatment-resistant biological pathways enriched in the treatment-resistant cohort. The analysis showed that there was a considerable upregulation of immune checkpoint pathways such as PD-1/PD-L1 and CTLA-4 pathways, which are known to be associated with immune evasion. Also, treatment-resistant ESCC was highly enriched in inflammatory pathways, including the TNF-06 and NF-KB signaling pathways. These pathways are involved in the agents that stimulate a pro-tumorigenic microclimate that promotes immune suppression and induces tumor cell survival pathways. Only pathways meeting FDR-adjusted significance criteria were interpreted as enriched resistance-associated pathways.

Interestingly, in addition to all the metabolic pathways that were enriched in the treatment-resistant group, glycolysis and oxidative phosphorylation were also enriched. Such metabolic reprogramming, sometimes known as the “Warburg effect is a documented characteristic of cancer cells and the immune cells in the TIME. Both tumor and myeloid cells expressed key glycolytic enzymes, including

Table 3: Differential Expression of Key Immune Markers in Treatment-Resistant and Treatment-Responsive ESCC

Gene	Treatment-Resistant ESCC	Treatment-Responsive ESCC	p-value
PD-1	5.6 ± 2.1	2.2 ± 1.5	<0.01
CTLA-4	4.1 ± 1.9	1.8 ± 1.2	<0.05
GZMA	2.0 ± 1.2	5.4 ± 3.1	<0.01
FOXP3	3.5 ± 1.8	1.6 ± 0.9	<0.05
CD163	6.4 ± 2.2	3.0 ± 1.1	<0.01

hexokinase 2 (HK2) and lactate dehydrogenase A (LDHA), at a higher level, which suggests that alterations in metabolism can also be a contributor to the fact that immune control can be maintained and that resistance is promoted.

The GSEA for treatment-resistant ESCC is shown in Figure **4** below, with the enrichment of specific pathways in treatment resistance highlighted. GSEA shows that glycolysis and lipid metabolism pathways are upregulated, suggesting metabolic changes in the tumor immune microenvironment. In addition, inflammatory response and TNF/NF-kappa B pathways are significantly enriched, implying the activation of pro-tumor inflammation. All of these findings reinforce that treatment resistance is not just due to the function of immune checkpoints but also metabolism and inflammatory pathways. Enriched pathways were ranked based on their normalized enrichment score. Significant pathways had an adjusted FDR q-value < 0.05 and a nominal p-value < 0.05. Increased normalized enrichment scores signify higher enrichment within the resistant ESCC samples.

Functional States and Interactions

The analysis of ligand-receptor interactions was done to analyze the interactions between tumor cells and the immune cells in the TIME. The treatment-resistant cohort of tumor cells was observed to overexpress a number of immune evasion ligands, including PD-L1 and galectin-9, which attaches to several T cell and myeloid cell inhibitory receptors. The relationships between the interactions were thought to diverge to immune cell prompt fatigue and to raise immunosuppressive myeloid cells. The comparison has also shown that the tumor cells in the treatment-resistant group secrete greater amounts of cytokines (IL-6 and VEGF) that can additionally suppress immune responses as well as angiogenesis of tumors.

Also, the immune cells of the cohort that were resistant to treatment demonstrated increased expression of immune checkpoint receptors (PD-1 and TIM-3) that were linked to a decrease in cytokine secretion and loss of cytotoxic capability. These results highlight the active interaction of tumor and immune

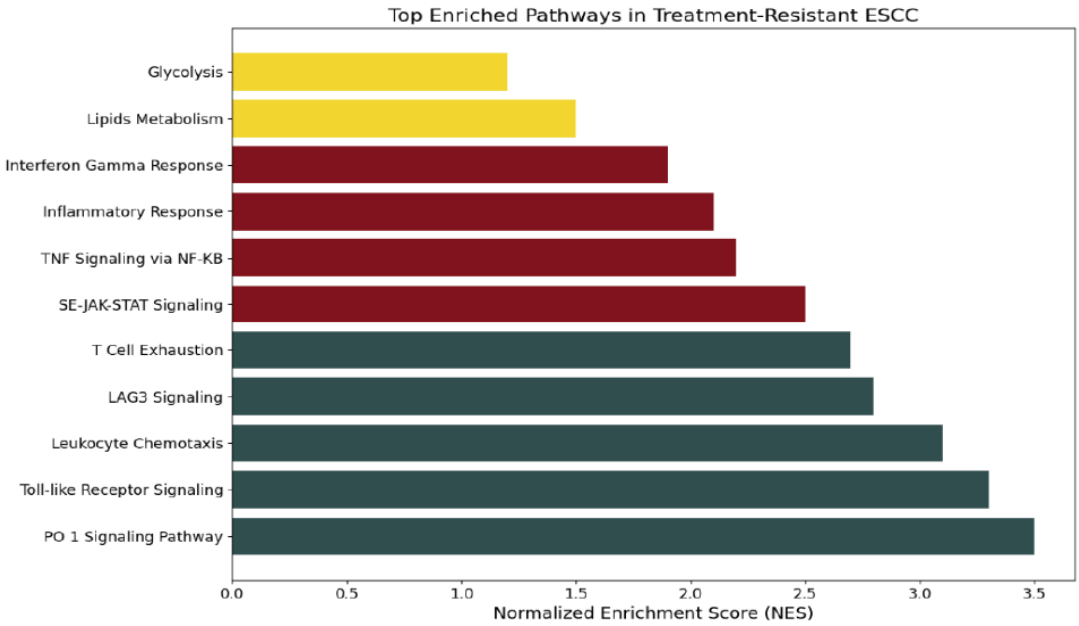

Figure 4: Gene Set Enrichment Analysis (GSEA) of Treatment-Resistant ESCC.

Table 4: Key Ligand-Receptor Pairs in Treatment-Resistant ESCC

Ligand	Receptor	Cell Type (Ligand)	Cell Type (Receptor)
PD-L1	PD-1	Tumor Cells, Macrophages	CD8+ T Cells, Tregs
Galectin-9	Tim-3	Tumor Cells	Exhausted T Cells, Macrophages
IL-6	IL-6R	Tumor Cells, Macrophages	T Cells, Fibroblasts
VEGF	VEGFR2	Tumor Cells	Endothelial Cells

cells in the TIME, as well as give insights into how resistance is driven by immune suppression.

Table **4** highlights some of the important ligand–receptor binding events observed in the treatment-resistant environment of esophageal squamous cell carcinoma. Ligand-receptor binding events like PD-L1 and PD-1 and Galectin-9 and TIM-3 show an immune checkpoint-mediated inhibition of T cell activation, whereas ligand-receptor interaction of IL-6 and IL-6R and VEGF and VEGFR2 signify inflammation and angiogenesis. Such ligand-receptor binding events highlight the process of cell-to-cell communication occurring between cancer cells and immunosuppressive cells for the growth of tumor cells. Ligand-receptor interactions were only considered for those ligands and receptors whose genes were co-expressed in the corresponding interacting cells and filtered statistically. The relevance of interaction was established by their expression levels and their enrichment in resistant ESCC.

DISCUSSION

Balanced Interpretation of the Results

It is essential to interpret the results of this research study as associations revealed from the analysis of the transcriptome in single cells instead of causal pathways. The results indicating the presence of exhausted T cells, regulatory T cells, immunosuppressive macrophages, immune checkpoints, and metabolism demonstrate the possible contribution of immune suppression and metabolic adaptation to drug resistance in ESCC. Nonetheless, this result needs more verification via functional testing, protein validation, and an independent cohort analysis. Hence, the suggested involvement of the immune checkpoint signaling pathway and metabolic adaptation should be considered a biologically sound pathway based on the transcriptomic findings.

Interpretation of Results

The findings of this article point out some important characteristics of the immune environment in the treatment-resistant esophageal squamous cell carcinoma (ESCC). Through the examination of single cells, it was found that the group that was resistant to treatment had a larger number of immune cells that switch off the immune response. These contained worn-out T cells, no longer capable of combating the tumor and tumor-promoting macrophages. These immune cells produce molecules such as PD-1 and PD-L1, which are known to turn off immune functions. These findings suggest that treatment-resistant ESCC may be associated with reduced anti-tumor immune activity and increased immune-suppressive signaling.

The other important finding was that the immunological cells in the treatment-resistant group were also evident of having undergone metabolic alterations. These immune cells were burning a lot of glucose, which is known to support the tumor to survive and evade the immune system. This metabolic change probably has a role in keeping the tumor resistant to treatment, with immune cells becoming less efficient at destroying the cancer.

Comparison with Prior Studies

The result has discovered that what previous studies have established is that exhausted T cells and macrophages are part of cancer resistance. It has also been reported by other researchers that these cells produce large amounts of the immune checkpoint molecules, such as PD-1 and CTLA-4, that inhibit the immune system from attacking the tumor. The contribution of the present work is the use of single-cell transcriptome profiling that allows combining results of immune-cell classification, immune-checkpoint marker expression, metabolic pathways enrichment, and cell–cell interactions in the case of treatment-resistant ESCC. The method offers a comprehensive image of the immune environment and brings novel knowledge on the mechanism of the immune system suppression in treatment-resistant ESCC.

Clinical Implications

A significant clinical implication is that of these findings. Already, these immune checkpoint proteins, such as PD-1 and PD-L1, are being tested in cancer therapy, though the findings indicate that such therapies, when used alongside immune-suppressing cells such as macrophages, may be more effective. It was also found in the treatment-resistant group that the immune cells had undergone a metabolic shift, and therefore, drugs that alter the metabolism of immune cells can be used to improve the capacity of immune cells to attack the tumor. This indicates that an immune checkpoint blockade strategy in combination with immunometabolism modulation could be a possible strategy for resistant ESCC therapy; however, further validation in preclinical and clinical settings is needed.

LIMITATIONS AND FUTURE DIRECTIONS

Although the research is rich in knowledge, it is limited to a certain degree. The sample was quite

small, and hence it requires future studies to be conducted using more patients in order to verify these findings. In addition, single-cell RNA sequencing revealed the activity of the genes; however, this is not sufficient to inform about the protein concentration or cellular interactions in the tumor. Other methods, such as proteomics or spatial transcriptomics, should be used in future studies to gain insight into these processes. Whereas the immune cell heterogeneity, checkpoint activation, pathway enrichment, and ligand-receptor interactions observed via scRNA-seq were highly reliable, the lack of complete experimental validation may compromise the validity of the results obtained. Hence, in future studies, the immune signatures should be validated through flow cytometry, immunohistochemistry, spatial transcriptomics, other ESCC data, and independent large-scale cohorts.

Another weakness is the lack of validation of the results using live models of ESCC. An animal model should be used in future research to verify the role of these immune cells and their metabolic variation in resisting therapy. Combination therapy should also be discussed in clinical trials, which may prove to be more effective in the treatment of resistant ESCC.

The small number of subjects in each study group may limit the statistical power and generalizability of the results, particularly when trying to detect very rare subsets of immune cells and uncommon molecular patterns. Consequently, the results can only be regarded as preliminary data, and further research needs to be done using larger multicenter esophageal squamous cell carcinoma populations.

CONCLUSIONS

Immune and molecular characteristics linked to treatment-resistant esophageal squamous cell carcinoma (ESCC) were analyzed using single-cell RNA sequencing in this study. It can be inferred from the results that the tumor immune microenvironment is more suppressive in resistant ESCC tumors, with high exhausted CD8+ T cells, regulatory T cells, and suppressive macrophages. Increased expression of immune checkpoints, namely PD-1, PD-L1, CTLA-4, and TIM-3, further highlights the poor activity of anti-tumor immunity in resistant tumors. Gene expression profiling and pathway enrichment reveal inflammation, glycolysis, oxidative phosphorylation, and lipid metabolism as potential mechanisms maintaining immunosuppression and survival in resistant tumors. Ligand–receptor interaction analysis identified the possible interaction between the tumor and immune cells using checkpoint, cytokine, and angiogenesis signaling pathways.

In terms of clinical implications, these results indicate that treatment-resistant ESCCs might not be successfully treated through targeted therapy against tumor cells. Treatment modalities such as immune checkpoint blockade combined with treatments targeting immunosuppression by macrophages, exhausted T-cells, and metabolic regulation in immune cells might be promising for this problem. The results obtained should be regarded as transcriptomic and computational proof and require additional validation in future research. The next study should involve ESCC patient cohorts at multiple centers, flow cytometry, immunohistochemistry, spatial transcriptomics, cross-dataset verification, and follow-up. In general, the presented work is helpful for clarifying how immunosuppression, metabolic adaptation, and interaction between tumors and immune cells can be responsible for ESCC resistance to treatment.

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