Inhibition of anti-tumour reactivity of immune cells in the salivary gland cancer: A proteomic approach

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ABSTRACT

Background: Adenoid cystic carcinoma (ACC), mucoepidermoid carcinoma (MEC), and oral squamous cell carcinoma (OSCC) respond differently to immunotherapy. Pembrolizumab, an immune checkpoint inhibitor, has been approved by the Food and Drug Administration for the treatment of squamous cell carcinomas of the head and neck region. While MEC has shown some response to pembrolizumab; however, ACC is the least responsive. At the molecular level, head and neck cancers produce immunosuppressive molecules, resulting in immune evasion. Therefore, we hypothesised that salivary gland cancer cells produce a higher number of immunosuppressive proteins that cause suppression of the immune system’s anti-tumour reactivity.

Method: To determine differential protein expressions in OSCC, MEC, and ACC, we constructed cancer-immune cell co-culture models using different oral and salivary gland cancer cells. We performed SWATH, proteome profilers, gene ontology biological function, functional annotation clustering and protein interaction network analysis of all cancer samples in the co-culture models.

Results: Analysis of the acquired data showed that the overexpressed proteins in the OSCC cells and participated more in metabolic process, while in the salivary gland cancer cells, overexpressed proteins participated more in immune processes, immune checkpoint pathway. Upon protein function analysis of salivary gland cells, the overexpressed proteins found negatively affecting immune process and checkpoint pathway proteins.

Conclusion: Overall, we conclude that salivary gland cancer is less responsive to immunotherapy, possibly because of the high presence of immunosuppressive proteins. However, further analysis is needed to verify the biological functions and interactive partners of each differentially expressed protein in ACC cells.

1. Background

Immunotherapy is the first-line treatment for head and neck cancers and can be broadly divided into adoptive T-cell therapy and immune checkpoint inhibitor therapy [1]. Pembrolizumab is a programmed cell death protein 1 (PD-1) inhibitor that has been approved by the Food and Drug Administration for the treatment of head and neck squamous cell carcinomas (SCCs) [1]. Recently, pembrolizumab has shown promise for the treatment of mucoepidermoid carcinoma (MEC) [2]; however, its efficacy in adenoid cystic carcinoma (ACC) is low [3]. The treatment outcomes of immunotherapy differ widely between oral and salivary gland cancers [1]. Numerous theories have evolved regarding the function of proteins involved in suppressing immune cells against cancer, including proteins involved in T-cell effector functions, immune regulation, cytokine regulation, and immune cell activation [4]. However, the most convincing of these is the checkpoint proteins PD-L1 and PD-1 present on tumour cells and T-cells, respectively, which inhibit or suppress the anti-tumour reactivity of immune cells [5]. Additionally, the release of immunosuppressive cytokines such as transforming growth factor-beta and interleukins IL6 and IL10 inhibit T-
cell proliferation and effector function [6]. T-cell proliferation is also impeded by the exhaustion of tryptophan by indole amine 2,3-dioxygenase produced by tumour cells [7]. Most of the previous research on protein changes has been done using highly heterogeneous patient samples or cancer cell lines not co-cultured with immune cells. In general, the understanding of protein-level changes during head and neck cancers is limited to chemokines and interleukins. Additionally, to the best of our knowledge, the reason for the different immune response to oral and salivary gland cancers remains elusive.

Therefore, we hypothesised that the protein expression of salivary gland cancer cells, particularly ACC, differs from that of oral cancer cells, affecting the anti-tumour response of immune cells. To test this hypothesis, we constructed various oral and salivary gland cancer-immune cell 3D co-culture models. To determine differential protein expression, we conducted a discovery phase proteomics assay, a proteome profiler assay, gene ontology, and a protein interaction prediction assessment.

2. Methods

2.1. Cancer immune cell co-culturing

3D-cancer-immune co-culture models were constructed using UM-HACC-2A (adenoid cystic carcinoma) (Cat T8326) (abm) cells, A-253 (mucoepidermoid carcinoma) (HTB-41, ATCC) cells, SCC4 (CRL-1624, ATCC), CAL27 (CRL-2095, ATCC), SCC9 (CRL-1629, ATCC), SCC25 (CRL-1628, ATCC) (OSCC) cells, and OKF6 (CVCL_1225) (normal oral) cells were used during the project. The cells were co-cultured in presence of NK-92 (natural killer) (CRL-2407, ATCC) cells and Jurkat, Clone E6-1 (Acute T cell leukemia) (TIB-152, ATCC) cells. Cell culture inserts (Transparent PET membrane) 0.4 μm × 25 mm (Thermo Fisher Scientific, Cat NUNJ40640), Falcon cell culture insert (Transparent PET membrane) 6 well 3.0 μm (in vitro technologies, Cat 353,091) and, Falcon cell culture insert (Transparent PET membrane) 6 well 0.4 μm (in vitro technologies, Cat 353,090) were used for co-culturing. All adherent cell lines were cultured following coating of the plates with extracellular matrix fibronectin human plasma [Sigma, Cat F0895-2 MG]. Details of the cell lines and medium used are provided in supplementary (Supplementary Table 1). After 16 h of cancer-immune cell interaction in the co-culture model, the cancer cells were collected for protein extraction.

2.2. Protein extraction

Proteome profiler samples were prepared from the acquired different cell types from the co-culture models. The cells were added to 100 μl of lysis solution, supplied in Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Cat RDSARY003C) and 10 μl of protease inhibitor (Protease Inhibitor Cocktail I, Tocris, Cat S500). The lysates were stored in –80 °C freezer for further assays.

2.3. Proteome profiler assay

Proteome Profiler Human Phospho-Kinase Array Kit, R&D Systems, Cat RDSARY003C was used to identify the phosphorylated proliferation mechanism proteins. All materials used during the project are provided in the supplementary.

2.4. Preparation of sample for LC-MS/MS

The acquired cell samples were lysed using sodium deoxycholate triethylammonium bicarbonate (1 % in 100 mM) (Sigma Aldrich). The lysates were sonicated for 5–10 s short cycle sonication and then de-natured at 95 °C for 5 min in heating block. The protein amount in each sample was determined using Pierce BCA protein assay kit (Thermo Fischer Scientific, Cat 23,225). Samples (each 100 μg) added to 1 % of 1 M dithiothreitol (DTT) (Sigma Aldrich, Cat D0632-5G) for 30 min at 60 °C. Then 4 % of 0.5 M iodoacetamide (IAA) (Sigma Aldrich, Cat 16,125-25 GM) added to the sample and incubated for 30 min at room temperature. Then sequencing grade trypsin (Sigma Aldrich, Cat T6567-20UG) added to the samples and incubated at room temperature overnight. Then centrifuged at 14000 g for 5 min and supernatant was collected and vacuum centrifuged. Finally, 1 μg processed protein sample was added to 0.1 % formic acid and mass spectrometry at IDA mode performed using Triple TOF 6600 mass spectrometer.

2.5. High pH and reversed phase HPLC

The ion library was constructed after pooling all the samples together. The cancer and immune samples were pooled separately for separate SWATH library. The pooled samples were fractionated using High pH reverse phase-High performance liquid chromatography. For, ion library generation through high pH (HpH) fractionation, 2 μg of each sample was pooled, and fractionated by High pH RP-HPLC. The pooled sample was first vacuum dried then resuspended in mobile phase buffer A (5 mM ammonium hydroxide solution (pH 10.5). The composition of buffer B was 5 mM ammonia solution with 90 % Acetonitrile (pH 10.5). After sample loading and washing with 3 % buffer B for 10 min at a flow rate of 300 μl/min, the buffer B concentration was increased from 3 % to 30 % over 55 min and then to 70 % between 55 and 65 min and to 90 % between 65 and 70 min. The eluent was collected every 2 min at the beginning of the gradient and at 1 min intervals for the rest of the gradient.

2.6. 2D ion library construction

SWATH runs (raw files) and 2D information dependent acquisition (IDA) ion library runs (raw) were acquired from the TOF machine. The remaining samples were pooled for High pH reverse phase high performance liquid chromatography fractionation followed by pooling of fractions into 17 fractions. ProteinPilot was used for database searches of IDA data. Group file(s) were converted to text file(s) using PeakView. All text files (1D-IDA s and 2D-IDA s) were zipped. Typically, a seed library is a text file from a 1D-IDA search. The seed library was merged with the 2D-IDA libraries using SwathLibraryMerge on GenePattern.

2.7. SWATH processing

The ion library was imported after quantitation followed by SWATH processing. The multiple comparison of different samples was done using GenePattern server (SwathPairsAndOverall) (Australian Proteome Analysis Facility, Macquarie University).

2.8. Bioinformatic analysis

The significantly Gipie-affected protein interaction network was visualized and analysed using STRING: functional protein annotation networks https://string-db.org/, Cytoscape 3.9.1 www.cytoscape.org, and the database for annotation, visualization, and integrated discovery (DAVID) https://david.ncifcrf.gov/. In STRING, Cytoscape, and DAVID, the installed network statistics application automatically analysed the node degree, degree of distribution, centrality, clustering coefficient, shortest path, and robustness of the network. For gene enrichment analysis, all query genes were first converted to ENSEMBL gene ID or STRING database protein IDs. FDR is calculated based on nominal p-value from the hypergeometric test. Fold enrichment was done after dividing the affected genes belonging to the pathway, to the corresponding percentage in the background. Only pathways that were in the specified size limits were used for enrichment analysis.
Transformation of the p-values (for the y-axis) to -log10, and log2 to the fold change (for the x-axis) was applied. Grey dots represent no significant differential.

3.1. Differential expression of proteins in oral cancer and normal oral cells

We collected cancer cells of oral and salivary gland cells from the 3D cancer-immune co-culture models. Upon discovery phase proteomics analysis to determine the differential protein expressions during the interaction of cancer cells and immune cells, we found OKF6, SCC4, SCC9, SCC25, and CAL27 grouped separately with no cell overlapped with any other cell line (Fig. 1a). Similar findings observed in salivary gland cancer cells (A-253 and UM-HACC-2A) (Supplementary Fig. 7). Further analysis of the proteins of each oral cancer cells compared to OKF6, we found statistically significant overexpressed and underexpressed proteins. Subsequently we analysed the acquired oral cancer SWATH data and screened the statistically significant overexpressed and underexpressed data. For further analysis, only high-fidelity protein data were taken that was based on the assessment of fragmentation pattern of each peptide (matching of the y- and b-ions to the theoretical precursor), additionally, high intensity, and high-quality peaks, with good number of peptides seen. The retention time of all the peptides were appreciable and closely related to each other (when compared across all the cell lines).

A total 1954 proteins identified in oral cancer mass spectrometry data. We selected 86 statistically significant overexpressed proteins in oral cancer cells (consistent data across all oral cancer cell lines) compared to OKF6 (that are both protein and peptide level statistically significant) (Fig. 1b–d) (Supplementary Figs. 1–6). Furthermore, we conducted gene ontology biological process and STRING protein network analysis. We found that the overexpressed proteins are mainly responsible for the proliferation of oral cancer cells. Mitochondrial catabolic process, mitochondrial morphogenesis, negative regulation of catabolic process, post translational regulation of gene expression, peptide metabolic process, cellular amino acid metabolic process, and catabolic processes were the major biological processes that were supported by the overexpressed proteins in oral cancer cells compared to OKF6 (Fig. 1g, Supplementary Fig. 9). Interactive static plot further showed translation, peptide metabolic process, and catabolic process were the most dominant biological process among other processes (Fig. 1i). Upon analysis of protein interaction network, STAT3, EGFR, TEX10, SGT1A were found to closely interacting to each other (Fig. 1h and Supplementary Fig. 8). Whereas when further analysis of the shortest distance of interacting nodes, STAT3 was not found closely interacting with EGFR. EGFR was found to be interacting closely with HFCU1, HUWE1, SARNP, UPF1, HNRNPU, HNRNPM, G3BP1, KHSRP, and RTRAF (Fig. 1l).

3.2. Unique proteins in salivary gland cancer cells that were not present in oral cancer cells

On comparing the salivary gland and oral cancer SWATH data, we found approximately 450 proteins unique to the salivary gland cancer cells, including AKA12, IFI16, MSH2, STAT2, DIDO1, TCRG1, WASC4, HOOK3, TCTP, CD59, MT1A, LAMP1, CCD9B, LAMP2, MTA70, CD9, IFI16, and galectin. Gene ontology enrichment demonstrated biological processes such as the regulation of type I interferon-mediated signalling pathway, regulation of macropage migration, lymphocyte-mediated immunity, regulation of immune system and immune effector processes and activation of cells and leukocytes involved in the immune response (Fig. 2a, Supplementary Fig. 10b). Subsequently, functional annotation clustering and Reactome pathway analysis was conducted. Interestingly, it was found that some proteins inhibited the anti-tumour reactivity of immune cells, including FADD (negative regulation of activation-induced cell death of T-cells, GO:0070,236), CD59 (negative regulation of activation of membrane attack complex, GO:0001971), galectin (negative regulation of T-cell receptor signalling pathway, GO: 0050,860; negative regulation of immune synapse formation, GO: 2,000,521; negative regulation of T-cell activation via T-cell receptor contact with antigen bound to the major histocompatibility complex (MHC) molecule on the antigen-presenting cell, GO: 2,001,189) and interferon-gamma-inducible protein 16 (negative regulation of innate immune response, GO: 0045,824) (Supplementary Figs. 19, 20, 21).

Based on the STRING protein interaction network and the interactive static plot, we further found that leukocyte-mediated immunity, cell activation, leukocyte activation and immune effector processes were the most dominant biological functions. The strongest correlations were found between the immune effector process, leukocyte-mediated immunity, leukocyte activation and leukocyte activation involved in immune process (Fig. 2b, Supplementary Fig. 10a, Fig. 2c, and...
Further, strong correlations were found for the JAK-STAT pathway, PD-L1 expression and PD-1 checkpoint pathway in cancer, Th1 pathway and cancer pathways (Fig. 3d). Differentiation, Th17 cell differentiation, the JAK-STAT signalling expression and the PD-1 checkpoint pathway in cancer, Th1 and Th2 modification of proteins present in cancer cells during their interaction with immune cells. The differential expression of phosphorylated proliferation proteins among oral and salivary gland cancer cells was determined using a proteome profiler phosphokinase array. We used CAL27, A-253 and UM-HACC-2A cells. The phosphokinase array showed significant upregulation and downregulation of 24 phosphorylated proliferation proteins among the oral and salivary gland cancer cells. Of these, RSK, STAT3, YES, HSP60, CREB, WNK, HSP27 and ERK showed the highest relative differences in protein expression among the different cell lines (Fig. 3a).

 STRING protein interaction analysis showed that all proteins, including STAT2, LCK, JUN, STAT5B, AKT, EGFR, MAPK1, CREB1, STAT1 and STAT6, interacted strongly with each other, with JUN, AKT and EGFR showing the strongest interactions (Fig. 3b and c, & Supplementary Fig. 12). Gene ontology enrichment revealed that the most dominant biological processes related to the differentially expressed proteins were PD-L1 expression and the PD-1 checkpoint pathway in cancer, Th1 and Th2 differentiation, Th17 cell differentiation the JAK-STAT signalling pathway and cancer pathways (Fig. 3d & Supplementary Fig. 13).

Additionally, the interactive static plot showed strong correlations between PD-L1 expression and PD-1 checkpoint pathway in cancer, Th1 and Th2 differentiation, Th17 cell differentiation and necroptosis. Further, strong correlations were found for the JAK-STAT pathway, PD-L1 expression and PD-1 checkpoint pathway in cancer and pathways in cancer (Fig. 3e, Supplementary Figs. 13 and 14).

4. Discussion

STAT3 overexpression and activation in OSCC cells contributes to proliferation, anti-apoptosis, metastasis, angiogenesis, radiotherapy resistance and immune evasion [8]. STAT3 activation negatively affects the Th1 immune response and enhances immune-suppressor cells such as myeloid-derived suppressor cells and regulatory T-cells [9]. Similarly, STAT3 overexpression was consistently observed in all oral cancer cells, unlike normal oral cells. EGFR overexpression was also observed. Similar to STAT3, EGFR also participates in the immune evasion of cancer cells [10]. 

pH-dependent anti-EGFR antibody resulted in improved cancer cell selectivity, penetration and anti-tumour activity of immune cells [11]. Additionally, it was found that the FDA-approved EGFR inhibitor cetuximab improved anti-tumour recognition of CD4 helper T-cells and upregulation of MHC class II protein expression [12]. Based on the above observation, we initially predicted that the presence of high EGFR and STAT3 may lead to immune evasion, regulation of the immune effector system, and other biological processes related to immune cell attack on cancer cells. Paradoxically, the gene ontology analysis showed that the major processes in which the proteins participated were the negative regulation of catabolic processes and post-translational regulation of gene expression. After protein interaction network analysis, STAT3 was not found to closely interact with EGFR or any immune-related proteins. EGFR interacted closely with E3 ubiquitin protein ligase, ribonucleoprotein, transcription–translation and transport factor proteins and GTPase-activating protein. Thus, we can conclude that the overexpressed proteins in oral cancer cells may not strongly inhibit the anti-tumour reactivity of immune cells.

Surprisingly, a large set of unique proteins were identified in the salivary gland cancer cells that are not found in oral cancer cells, including FADD, CD59, gamma interferon inducible protein 16, STAT2, CD9, N6-adenosine methyltransferase catalytic subunit, galectin, and lysosome-associated membrane glycoprotein 1. All these proteins affect immune cell function [13–15]. Gene ontology biological process analysis showed that these proteins affected macrophage migration, leukocyte response, immune effector processes and other immune functions. Interestingly, functional annotation clustering and Reactome pathway analysis showed that FADD, CD59, galectin, and interferon-gamma-inducible protein 16 may have hindered the immune response to salivary gland cancer cells. This provides the first rationale for impaired anti-tumour reactivity of immune cells to salivary gland cancer. While CD9 expression in immune cells improves cancer prognosis and confers longer disease-free survival, its presence in cancer cells impedes immune-mediated tumour cell destruction [16]. TripleTOF mass spectrometry failed to detect STAT2 in oral cancer cells, possibly because they were low in protein expression; however, high-intensity peaks were observed in salivary gland cancer cells because of their high protein expression. The interferon-gamma–STAT2 axis was possibly participating in the immune checkpoint blockade [17]. However, quantitative proteomics analysis to determine the expression of interferon gamma was not conducted. Additionally, a co-expression assay of STAT2/IFNγ should be run to determine whether the predicted axis affects immune evasion.

Annotation clustering and Reactome pathway analysis provided a list of proteins that may be involved in reducing the anti-tumour reactivity against salivary gland cancer. Of these proteins, galectin is key and has been previously found to negatively affect T-cell receptor signalling and immune synapse formation, unfavourably disturbing the cancer–immune axis [18] (Supplementary Fig. 22). 

FADD upregulation is associated with poor prognosis, reduction in anti-tumour immune response and resistance to immunotherapy [19]. These recent findings were found for lung adenocarcinoma [13]. Interestingly, interferon-gamma-inducible protein 16 regulates interferon activity and the response of interferon to antigenic stimulation. Additionally, IFI16 confers resistance to chemotherapy and immunotherapy and has been found to induce secretion of pro-inflammatory cytokines from breast cancer cells, resulting in progression of breast cancer. IFI16 has also been found in the minor salivary glands, which is the site of collection of UM-HACC-2A cells. In some autoimmune disorders, IFI16 has been found to initiate and perpetuate inflammatory reactions [14]. Therefore, the biological action of IFI16 in salivary gland cancer remains unclear given that we were unable to find its binding partners in the protein interaction network analysis (Supplementary Fig. 22).

Protein interaction network analysis showed strong interactions between CD9, CD59, lysosomal-associated membrane proteins (LAMPS), and transport factor proteins and GTPase-activating protein. Thus, we can conclude that the overexpressed proteins in oral cancer cells may not strongly inhibit the anti-tumour reactivity of immune cells.
and TPT proteins. CD59 plays a role in the complement pathway, helping to bind C8 and C9, thereby preventing the formation of a membrane attack complex and limiting cancer cell damage [19]. LAMPS 1 and 2 are membrane glycoproteins that are also found in salivary gland cancer cells. LAMPS have been found to actively participate in lysosomal biogenesis and autophagy [20]; however, their role in cancer cells is yet not fully understood. The presence of LAMPS in dendritic cells is a positive prognostic marker in melanoma [21]. Translationally controlled tumour protein (TPT1) is involved in microtubule stabilisation [22], although its molecular mechanism regulating immune function is largely unknown.

Post-translational modifications such as phosphorylation may either activate or deactivate pathway proteins [23]. Therefore, it was imperative to determine whether any differences in phosphorylated protein expression existed between oral and salivary gland cancer cells. Based on the discovery phase results, we conducted a proteome profiler assay to assess the phosphorylated proliferation mechanism.

We observed an array of differentially expressed proteins found in the salivary gland cancer cells compared with the oral cancer cells. Many of the proteins participated in metabolic or proliferation pathways, helping to sustain the growth of the salivary gland cancer cells. However, a large set of set of proteins also actively assisted in the PD-1/PDL1 checkpoint pathway and immune cell differentiation, ultimately affecting the anti-tumour response of immune cells and immunotherapy. Interestingly, our protein interaction network analysis showed that phosphorylated EGRF, JUN, AKT, CREB, PRA540, STAT5, GSK3β/β and STAT1 were more highly expressed in salivary gland cancer than in oral cancer. EGRF plays an active role in various cancer pathways and has been found to influence the anti-tumour reactivity of immune cells. EGRF inhibitors augment MHC class I and II molecules, helping to slow the progression of cancer. Similarly, AKT inhibition is favourable for immune alterations in the tumour microenvironment [24]. Statistically significant expressions of JUN suggest the presence of immunosuppressive proteins in salivary gland cancer cells, affecting differentiation and cytokine release of immune cells [25]. Similar results have been found for beta catenin pathway proteins, CREB, PRA540 and STAT5 [26]. However, the highly expressed protein LCK in the salivary gland promotes an anti-tumour immune response [27].

Despite our interesting early results, it is important to note that the profiler used had limited phosphorylated protein markers for the threonine, serine, and tyrosine sites. Other phosphorylation sites exist in each protein. Phosphorylation at those sites may alter the biological function of the protein, which is beyond the capacity of the prediction tools to assess.

5. Conclusion

Overall, the results based on the proteomics approach indicated a reduced immunotherapy response to salivary gland cancer. However, we cannot make conclusions about the biological activity of proteins that were differentially expressed in salivary gland and oral cancer cells. In future, protein–protein interaction assays, co-localisation assays and specific gene silencing experimental work is needed to understand the molecular biological aspect of cancer–immune cell interactions in salivary gland cancer.

Ethics

The entire work was done following the biosafety approval by the Macquarie University Biosecurity committee (Mammalian Cell Culture 5215).

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Data availability

The datasets generated and analysed during the current study are available in the figshare repository, https://doi.org/10.6084/m9.figsh are.24558565.

CRediT authorship contribution statement

Rajdeep Chakraborty: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft. Charbel Darido: Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. Aidan Tay: Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. Thiri Zaw: Data curation, Formal analysis, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – review & editing. Fei Liu: Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing. Giuseppe Palmisano: Formal analysis, Investigation, Methodology, Project administration, Resources,
Software, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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