SHORT COMMUNICATION

Immune-Related Gene Expression Profile at Peritumoral Tonsillar Tissue Is Modified by Oropharyngeal Cancer Nodal Status

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The prevalence of oropharyngeal cancers (OPCs) is increasing, largely due to rising numbers of human papillomavirus (HPV)-positive cancers. Worldwide, the most frequently used treatment of patients with OPC of the tonsil is radiotherapy, with and without chemotherapy. However, this approach is not without long-term risks, including radionecrosis, sclerosis, postoperative complications in salvage surgery, local failure, and radiation-induced carcinomas. Therefore, in early-stage or small-volume OPCs of the tonsil, transoral lateral oropharyngectomy is advocated by head and neck surgeons. In patients with early-stage n0 OPCs, lymphatic metastasis develops in 20% to 30% of cases and is associated with decreased survival. To correctly stage the disease node status, sentinel lymph node biopsy and/or prophylactic neck dissection is often used. However, these procedures can cause significant morbidity, including surgical scars, shoulder dysfunction, and chyle leakage.

Under physiological conditions, naïve lymphocytes continuously traffic between blood and secondary lymphoid organs (SLOs), such as the lymph nodes and tonsils, through the walls of specific papillary venules called high endothelial venules. The adaptive immune response against...
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cancer occurs in SLOs, wherein mature DCs migrate from
tumor sites and present major histocompatibility complexes
to CD4⁺ and CD8⁺ T cells. Upon antigen binding in pri-
mary follicles, B cells are activated in the SLOs, which then
receive help from CD4⁺ T cells to proliferate, forming a
secondary follicle that progressively becomes a germinal
center. These steps allow lymphocyte proliferation and
differentiation into effector T cells and B memory cells. Thus,
the induction of naïve T- and B-cell infiltrations at
SLOs, such as lymph nodes and tonsils, appears to poten-
tiate an antitumor response by the antigen-specific stimu-
lation of these lymphocytes. These lymphocytes are then
recirculated into the primary tumor site, leading to attack of
the tumor cells. Therefore, in OPCs, it was speculated that
the immunologic processes occurring in peritumoral tonsil
tissue, as an SLO, are crucial for effective antitumor im-
une responses, which facilitates tumor control with
diminished or eliminated disease progression. In addition, it
was hypothesized that understanding the regional immune
system at this site would elucidate mechanisms of disease
progression, such as lymphatic spread, and enable its risk
stratification in OPCs. Recent studies have reported
immune-based classifications of tumor-infiltrating immune
cells according to immune scores and gene expression
profiles, successfully stratifying patients by prognosis. However, immunology profiles of peritumoral tonsil tissue
in OPCs are still poorly characterized.

To evaluate the molecular modifications occurring at the
tonsil tissues associated with lymphatic spread, an explor-
atory study was designed to analyze the immune-related
transcription profile of peritumoral tonsil tissue according to
OPC node status. These data and this analytical approach
are expected to assist studies of the role of the immune
system in metastatic spread and enable the prediction of
node status in patients with OPCs.

Materials and Methods

Patient Population

Data on a total of 43 patients with OPCs originating at the
palatine tonsil and who underwent radical resection of
oropharyngeal primary tumor between 2012 and 2019 were
obtained from the pathology files of Kanazawa University
(Kanazawa, Japan). Cancer staging at diagnosis was per-
fomed according to the Union Internationale Contre le
Cancer’s TNM Classification of Malignant Tumours, 7th Edition. Ten cases were excluded from the analysis: eight
cases without sufficient tonsil tissue for subsequent analysis;
one case with positive surgical margin at the primary site,
followed by postoperative radiotherapy; and one case with
recurrence at the primary site. Thus, in this study, 33 of 43
patients were enrolled and divided into two cohorts based on
whether their samples were assessed using whole-
transcriptome atlas (WTA) analysis (Cohort 1) or micro-
array analysis (Cohort 2). The detailed characteristics of
these 33 patients are listed in Table 1. After primary surgery,
6 patients received adjuvant radiation treatment with and
without concurrent chemotherapy, due to plural metastasis-
positive lymph nodes.

In Cohort 1 (n = 6), 3 patients had metastasis-positive
lymph nodes, and 3 had metastasis-negative lymph nodes. In
Cohort 2 (n = 27), 20 patients had metastasis-positive
lymph nodes, and the other 7 had metastasis-negative
lymph nodes. Patients classified as having lymph node
metastasis included all patients with lymph node metastases at
any stage of the clinical course, including diagnosis, path-
ology diagnosis after surgery, and during the follow-up
period.

The study protocol was approved by the Bioethics
Committee of Kanazawa University (number 2016-033). Written informed consent was obtained from all patients
enrolled in this study.

WTA Analysis

The GeoMx Digital Spatial Profiler platform (NanoString
Technologies, Seattle, WA) facilitates high-pixel profiling at
the protein and RNA levels, providing spatial and temporal
assessment of tumors in frozen or formalin-fixed paraffin-
embedded (FFPE) limited tissue samples. Through this
platform, WTA analysis was used for evaluating over
18,000 protein-coding genes based on the Human Genome

Table 1  Clinical Characteristics of Patients with Oropharyngeal Cancer Included in the Gene Expression Study

<table>
<thead>
<tr>
<th>Factors</th>
<th>Cohort 1 (n = 6)</th>
<th>Cohort 2 (n = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), years</td>
<td>64.5 (45–84)</td>
<td>62 (43–88)</td>
</tr>
<tr>
<td>Sex, no.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>Female</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>HPV, no.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Stage, no.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IVA</td>
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<td>0</td>
</tr>
<tr>
<td>T classification, no.</td>
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<td></td>
</tr>
<tr>
<td>T1</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>T2</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>T3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Lymph node metastasis, no.</td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>3 (0*)</td>
<td>20 (3*)</td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>M0 classification, no.</td>
<td>6</td>
<td>27</td>
</tr>
</tbody>
</table>

Cohort 1 (n = 6) samples were analyzed with whole-transcriptome atlas using the GeoMx Digital Spatial Profiler (NanoString Technologies, Seattle, WA). Cohort 2 (n = 27) samples were analyzed with microarray analysis. *Cases with delayed lymph node metastasis.
Figure 1  Analysis of lymphoid and tumor regions by GeoMx whole-transcriptome atlas (WTA). A and B: Analysis of a surgical specimen resected by transoral lateral oropharyngectomy. 

A: Low-power view of the specimen with fluorescent staining of pan-cytokeratin (green), CD45 (red), smooth muscle actin (yellow), and DNA (blue). Regions of interest (ROIs) 001 to 006, outlined in white, are lymphoid areas within the specimen, while ROIs 007 to 012 are tumor areas. B: High-power views of ROIs 001 to 006, lymphoid areas, and ROIs 007 to 012, tumor areas. C and D: Volcano plots of differentially expressed genes (DEGs) in metastasis-negative (Met−) and metastasis-positive (Met+) oropharyngeal cancers (OPCs) in WTA analysis. Blue dots in the red and blue boxed regions indicate up-regulated and down-regulated genes in Met− cases compared with Met+ cases, respectively. Statistical significance was defined as $|\log_2| \geq 1$ and $-\log_{10} P > 1.3$. The red and green dashed lines indicate $|\log_2| \leq 1$ and $-\log_{10} P > 1.3$, respectively. C: Analysis of lymphoid regions of interest (ROIs) adjacent to tumor tissues of OPCs reveals a total of 239 genes, including 237 up-regulated and 2 down-regulated genes. D: Only 1 gene is significantly up-regulated when tumor ROIs are analyzed. E and F: Volcano plots of DEGs in human papillomavirus–positive (HPV+) and HPV-negative (HPV−) OPCs in WTA analysis. Blue dots in the red and blue boxed regions indicate up-regulated and down-regulated genes in HPV+ cases compared with HPV− ones, respectively. Statistical significance was defined as $|\log_2| \geq 1$ and $-\log_{10} P > 1.3$. The red and green dashed lines indicate $|\log_2| \leq 1$ and $-\log_{10} P > 1.3$, respectively. E: Analysis of lymphoid ROIs adjacent to tumor tissues of OPCs reveals a total of 17 up-regulated genes. F: No gene is differentially expressed when tumor ROIs are analyzed.
Organisation’s (HUGO) Gene Nomenclature Committee database (https://www.genenames.org) cross-referenced with available mRNA sequences in the National Center for Biotechnology’s RefSeq database (https://www.ncbi.nlm.nih.gov/refseq). The human whole transcriptome was measured in each region of interest (ROI) to identify biological changes at specific tissue locations. In ROI selection, the morphology markers pan-cytokeratin (panCK), CD45, and smooth muscle actin (SMA) were used to distinguish tumor cells, immune cells, and mesenchymal cells, respectively.

The six samples from Cohort 1 were submitted for WTA analysis. Figure 1, A and B, demonstrate fluorescence staining and the strategy for selecting ROIs in an OPC tissue sample. In each sample, 12 ROIs were selected, of which 6 were lymphoid regions, and the other 6 were tumor regions. In each ROI, WTA analysis was used to measure the expression levels of over 18,000 protein-coding genes. The WTA procedures are described in the literature.

A total of 36 ROIs in the lymphoid and tumor regions were classified as metastasis or HPV, and then subclassified as metastasis negative or positive, and as HPV negative or positive. A linear mixed model was applied to detect differentially expressed genes (DEGs) between the two groups. Statistical significance was defined as $|\log_2|$ of fold-change $>$1 and $-\log_{10} P > 1.3$.

Microarray and Computational Analysis

Total RNA from FFPE blocks was isolated using the RNeasy FFPE Kit (Qiagen, Tokyo, Japan). The RNA quality and quantity were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). In Cohort 2, total RNA samples from FFPE tissues of the peritumoral tonsil tissue (Supplemental Figure S1A), not including tumor area, were prepared and macrodissected from whole FFPE tissues on slide glass.

Reverse transcription and amplification of 100 pg of high-quality total RNA were performed using the GeneChip WT Pico Kit (Applied Biosystems, Foster City, CA). After linear amplification, the cRNA obtained was converted to biotinylated sense-strand cDNA targets for hybridization. After hybridization in a GeneChip Hybridization Oven (Applied Biosystems), the array was washed, stained, and scanned. Data obtained after the scan were analyzed using Transcriptome Analysis Console software version 4.0.2.15 (Thermo Fisher Scientific). The expression data were processed using a robust multichip average normalization algorithm. The data sets are available at the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo; accession number GSE228432).

The nCounter PanCancer Immune Profiling Panel (NanoString Technologies, Seattle, WA), a highly multiplexed gene expression panel, was used to quantify 730 genes related to immune-cell profiling and function across the innate and adaptive immune systems and tumor-specific antigens. The 730 genes were used to detect DEGs between metastasis-negative and metastasis-positive lymph nodes. The criteria for selecting the DEGs were an adjusted $P < 0.05$ and a $\log_2$ fold-change $>0$.

Gene Ontology Pathway Enrichment Analysis of DEGs and Screening of Hub Genes

In Cohort 2, ClueGO, a widely used plug-in for Cytoscape software version 3.9.1 (https://cytoscape.org/) that allows the visualization of nonredundant biological terms for large clusters of DEGs in a grouped network, was used to decipher functionally grouped gene ontology (GO) pathway annotation associated with the immune system process. A protein–protein interaction network of all DEGs was plotted using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org) to evaluate all protein–interaction associations. The protein–protein interaction network was visualized using Cytoscape. To identify high-level genes that play key roles in the protein–protein interaction network, hub gene analysis was performed using the NetworkAnalyzer tool in Cytoscape. The top 10 genes ranked by degree were considered hub genes.

Gene Set Enrichment Analysis and Principal Component Analysis

Gene Set Enrichment Analysis software version 4.1.0 (GSEA; Broad Institute, Cambridge, MA) was used for DEG profiling of tonsil tissues between metastasis-negative and metastasis-positive cases. The nCounter panel, with 730 immune-related genes, was used as an enrichment pathway. Then, data on the top 20 genes as ranked by GSEA metric score were extracted. Principal component analysis (PCA) was performed using the top 20 genes, then the first principal component was extracted to serve as the gene signature score, and a PCA score for each patient was calculated. SPSS software version 28.0.1.0 (IBM, Armonk, NY) was used to perform PCA, and receiver operating characteristic analysis of PCA scores. $P < 0.05$ was regarded as statistically significant.

Results

Analysis of Gene Expression Profiles at Lymphoid ROIs by WTA-Identified DEGs Between Metastasis-Negative and Metastasis-Positive OPCs

In Figure 1, C and D, volcano plots illustrate the DEGs between metastasis-negative and metastasis-positive cases in lymphoid and tumor ROIs, respectively. A total of 237 up-regulated and 2 down-regulated genes were identified in lymphoid ROIs; in contrast, only 1 gene was up-regulated in tumor ROIs.
Figure 2  Gene ontology (GO) enrichment analysis of the immune system process to identify hub genes. A and B: GO analysis of differentially expressed genes (DEGs) in microarray analysis in Cohort 2. A: The interaction network of GO terms in the metastasis-negative group compared with the metastasis-positive group presented by the ClueGO plug-in for Cytoscape software version 3.9.1. GO terms describing molecular interactions among targets are represented as nodes, and node size represents the term enrichment significance. The most significant term in each group is highlighted. B: The percentage of each GO term in the metastasis-negative group compared with the metastasis-positive group. C and D: Analysis using the Search Tool for the Retrieval of Interacting Genes (STRING) network. C: The protein–protein interaction for DEGs to depict the interaction profile. D: The top 10 hub genes are PTPRC, TLR4, CD80, CD40, STAT3, CD28, CD40LG, CD44, CCR7, and IL7R, which are suggested to be central in suppressing lymph node metastasis in oropharyngeal cancers.
Analysis of DEGs between HPV-positive and HPV-negative cases showed only 17 up-regulated genes in HPV-positive cases compared with HPV-negative ones in lymphoid ROIs (Figure 1, E and F). No DEGs in tumor ROIs were found between HPV-positive and HPV-negative samples. These results suggest that DEG analysis is more sensitive in lymphoid ROIs than in tumor ROIs in corresponding samples. The number of DEGs between HPV-positive and HPV-negative lymphoid ROIs was limited. This finding encouraged an evaluation of the peritumoral tonsil tissue to identify DEGs and the associated immune mechanisms of lymph node metastasis in OPCs.

Detection of DEGs, and GO Enrichment Analysis of the Immune System Process, to Identify Hub Genes

In Cohort 2, 27 RNA samples, prepared from FFPE tissues from peritumoral tonsil areas in OPCs, were analyzed for the expression levels of the 730 genes listed in the nCounter panel. As expected, many genes were differentially expressed (192 up-regulated and 22 down-regulated genes in metastasis-negative versus metastasis-positive cases) (Supplemental Figure S1B). GO enrichment analysis of the 192 up-regulated genes was performed, and ClueGO was used to visualize the results of a GO enrichment analysis of the immune system process. The GO analysis results are summarized in Figure 2, A and B. Positive regulation of T-cell activation, T-cell selection, T-cell activation, z-beta T-cell activation, and positive regulation of T-cell proliferation were the top five significantly enriched GO terms in metastasis-negative cases. A protein–protein interaction network of DEGs in these enrichment pathways was constructed to identify hub genes (Figure 2C). According to the degree ranking, the top 10 hub genes were PTPRC, TLR4, CD80, CD40, STAT3, CD28, CD40LG, CD44, CCR7, and IL7R, as visualized in Figure 2D.

Prediction of Node Status by GSEA and PCA Score

On GSEA, the gene set from the nCounter panel was significantly enriched in the metastasis-negative tonsil tissues compared with the metastasis-positive ones (normalized enrichment score, 1.668; nominal P = 0.002; false-discovery rate q = 0.002) (Figure 3A). These results indicate that the immune-related genes in the gene set participate in suppressing lymph node metastasis in OPCs. Data on the top 20 genes as ranked by GSEA metric score were extracted and applied to PCA, with a PCA score calculated in each patient. Two principal components were detected (Supplemental Table S1), with the first principal component (approximately 73.199% variance) used to represent a patient’s PCA score. According to the receiver operating characteristic analysis, 27 OPCs were segregated into two groups by PCA score: <0.4134 versus >0.4134. The relationship between patients’ lymph node status and PCA score is visualized in Figure 3B (area under the receiver operating characteristic curve, 1; sensitivity, 1; specificity, 1). These results suggest that the top-20 gene signature is useful for stratifying node status without surgical intervention such as sentinel lymph node biopsy or prophylactic neck dissection.
Discussion

SLOs, as initial sites of antitumor immune response, serve as an interface between the immune system and tumor cells. 14, 15 Interestingly, in OPCs originating in the palatine tonsil, surgical specimens from lateral oropharyngectomy contained not only tumor tissue but also peritumoral tonsil tissue, which is an SLO. Therefore it was hypothesized that, in OPCs, an analysis of the gene expression profile of peritumoral tonsil tissue resected en bloc with primary tumors would elucidate their role in both tumor control and disease progression, including lymph node metastasis.

As expected, analysis of lymphoid ROIs with WTA and peritumoral tonsil tissue with microarray revealed abundant DEGs between metastasis-negative and metastasis-positive cases. These results support the hypotheses that peritumoral tonsil tissue has an important role in influencing lymphatic spread, and indicate that their analysis can facilitate the classification of patients as being at high or low risk for lymph node metastasis. In metastasis-negative cases, DEGs of immune-related genes were enriched in the activation of T-cell responses. Hub genes, screened by protein–protein interaction network analysis, were also involved in the activation of immunity reaction and inflammation pathways. The discovery of these specific immune-gene signatures in peritumoral tonsil tissue revealed a mechanism for suppressing potential lymph node metastasis, and encouraged the proposal of a gene-scoring system for predicting node status. This revolutionary idea is to analyze macrodissected peritumoral tonsil tissue to elucidate the mechanism of lymphatic spread and to predict node status.

This study had several limitations. First, the sample number was small, consisting of only 33 FFPE samples of tonsil tissue for gene expression analysis. However, despite the small patient number in each cohort, the findings consistently demonstrated the potential of peritumoral tonsil tissue analysis in the detection of DEGs. In previous reports analyzing tumor-infiltrating lymphocytes, transcriptome profiles and clinical metadata were retrieved from public databases, such as The Cancer Genome Atlas database. 16, 17 Unfortunately, no suitable databases of tonsil tissue were available for gene expression profile analysis in the current study. Second, the patient cohort appeared to have had clinical cases positive for lymph node metastasis at diagnosis, which may have led to interpretation bias of the data in discussing the indication of prophylactic neck dissection. Third, when the volume of tonsil sample was insufficient due to a limited surgical margin, the presented procedures could not be applied. However, these data strongly support further large-scale validation studies. To establish and validate the significance of the present peritumoral tonsil tissue analysis, a multi-institutional study is necessary for the collection of sufficient tonsil samples of cN0 OPCs at diagnosis.

In summary, gene expression analysis of peritumoral tonsil tissue is proposed to elucidate the immune mechanism of lymph node metastasis and to predict node status without surgical intervention of the neck in OPCs.

Supplemental Data

Supplemental material for this article can be found at https://doi.org/10.1016/j.ajpath.2023.04.010.

References


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