Immune delineation of laryngeal papilloma reveals enhanced neutrophil associated gene profile

Laryngeal papilloma (LP) is a rare benign but devastating disease characterized by a high rate of recurrent multisite papillomas that affects the respiratory tract [1]. When the lesions reappear, the condition is called recurrent respiratory papillomatosis (RRP). The membranous vocal folds are frequently involved, but the lesions may have distribution in supraglottic as well as subglottic areas of the larynx [1]. LP usually, occur during two periods of life: early childhood (<12 years, juvenile-onset) and adult life (onset from 20 to 40 years).

Among human papillomavirus (HPV), the low-risk types such as HPV6 and HPV11, have been indicated as a viral causative factor of LP [2]. In addition to the standard surgical treatment of RRP, many adjuvants like prophylactic and therapeutic vaccination, antiviral medication, and most recently immunotherapeutic drugs, have been added as treatment possibilities [3]. Immune checkpoint blockade, specifically the programmed death (PD-) axis, is considered for RRP therapy, primarily due to its clinical efficacy on oropharyngeal cancer, another HPV-driven malignancy [3, 4].

There is limited information about the overall composition of immune cell types infiltrating LP as compared to healthy control tissue. To enhance our understanding of the ongoing immune responses in the LP microenvironment, we have compared the transcriptional profiles of immune- and tumor-related biomarkers in LP samples and paired unaffected adjacent laryngeal tissue. Moreover, to identify novel targets for the treatment of LP, gene expression analysis was combined with immunoprofiling.

The laryngeal papillomatosis of the patients was classified as aggressive or nonaggressive by modification of Doyle’s classification system that categorizes nonaggressive disease as less than three surgical interventions within 1 year [5]. Overall, 88% (15/17) of the included patients, both in RNA and flow cytometry analysis, had non-aggressive LP with less than three surgeries per year.

For RNA analysis, five LP samples were positive for HPV6 and two for HPV11 (Supporting Information Table S1). The same HPV types were detected in all the unaffected adjacent tissue samples, except one sample that was HPV negative (Supporting Information Table S1). Transcriptional analysis of the 14 samples, demonstrated that 113 transcripts out of 494 included in the nCounter PanCancer Immune Profiling Panel, displayed a significantly different expression profile (DE). In this comparative analysis, 37 genes were expressed at a higher level in the LP compared from (cf.) HL samples, whereas 76 genes were expressed at lower levels (fold change, FC ≥ 2).

DE genes in LP cf. HL were visualized by hierarchical clustering for HPV types (Figure 1A and B). Genes with a high fold-change in expression levels in the LP cf. HL included markers of the S100-family (A7, A8, and A12), interleukin signaling molecules (IL1RN, IL1RAP TOLLIR IL23A, IL4R) and chemokines (CXCL6, IL8), suggesting an immune profile skewed towards neutrophil recruitment and regulation of inflammatory responses (Figure 1A). Out of the 37 transcripts significantly overexpressed in LP, 7 of these had previously been identified in LP samples using microarray analysis (S100A7, S100A12, CXCL6, CXCL8, IL23A, CDKN1A, VEGFA) [6]. In contrast, the transcripts with lower fold change in LP cf. HL included markers of T cells (CD8A, CD4, LAG3), B cells (CD79A, CD79B) and NK cells (KLRK1) and chemokines involved in the recruitment of these cells (CCL21, CCL19, CXCL12) (Figure 1B).

To confirm the NanoString results, qRT-PCR was performed on 12 representative genes among the DE genes in LP cf. HL and the results confirmed a similar FC direction in 11 mRNAs. Higher expression of S100A7, S100A12, CDKN1A, VEGFA, IL8, and FOS, as well as lower expression levels of CXCL12, CCL14, CCL21, CD79A, and F13A1 were verified (Figure 1C).

Next, a demarcated pathway enrichment was carried out for DE genes, resulting in 12 and 54 significant pathways (FDR 1%). The pathways involved genes with higher DE in LP cf. HL, revealed pathways of IL signaling and neutrophil degranulation (Supporting Information Table S2). Strikingly, the calcium-binding S100 proteins (A7, A8, and A12) in the LP were associated with the neutrophil degranulation pathway. Previous studies
Figure 1. Transcriptional profiling of 7 paired LP and HL samples using the nCounter PanCancer Immune Profiling Panel and qrt-PCR. Heatmap for DE transcripts arranged in decreasing order of FC, with significantly (A) increased and (B) decreased transcripts in LP, cf. HL, samples. The hierarchical clustering was performed for HPV types 6 (yellow) and 11 (blue) and one HPV negative sample (pink). The LP and HL are numbered according to clinical information as seen in Supporting Information Table S1. Filtering of genes was performed using a two-tailed t-test, Qlucore Omics Explorer 3.6 and FC analysis (p-value < 0.05, FC ≥ 2). (C) Bar graphs depicting the difference in fold change values obtained via qPCR and NanoString for selected genes, considering p-value = 0.05 and fold change ≥ 2 as the significant cut-off. Normalization for qPCR was done using the GAPDH as the housekeeping gene. (D) Bar graphs showing the difference between cell scores in LP cf. HL (multiple unpaired t-test, p-value < 0.05, FDR 1%), * = <0.01. FDR was calculated without assuming consistent standard deviation using the two-stage linear step-up procedure by Benjamini, Krieger and Yekutieli. The error bars represent the standard error of means and centered values being the means.). Data for transcriptomics and qPCR were generated from one experiment run, respectively with seven paired samples per experiment.
Figure 2. Flow cytometric analysis on 10 LP and 8 HL samples (A) Representative flow cytometry gating strategy used for immunoprofiling. After doublets exclusion, viable immune cells (CD45$^+$) were divided into T cells (CD3$^+$) and B cells (CD19$^+$CD20$^+$). The T cells were further separated into CD8$^+$ and CD4$^+$ T cells. The CD3$^-$CD19$^-$CD20$^-$ cells were grouped into HLA-DR$^+$ DCs, macrophages (HLA-DR$^+$CD14$^+$) and HLA-DR$^{low}$CD14$^{int}$ cells. Neutrophils were defined as HLA-DR$^{low}$CD14$^{int}$CD15$^+$ cells. The first three dot plots are visualized in a linear scale, and the remaining dot plots are in the log scale for both the x- and y-axis. Relative percentage expression of (B) T cells, CD8$^+$ T cells, B cells and Neutrophils in the 10 LP and 8 HL samples (Two-way ANOVA, with error bars representing standard error of means and centered values being the means). Data are from 10 experiments with fresh paired donor samples per experiment, except donors 7 and 8, where only LP samples were analyzed per run.
have demonstrated enhanced neutrophilic profile and chemotaxis with increased gene expression of $\text{S100 (A7, A8, and A12)}$ [7]. For lower DE genes, associated pathways consisted of, e.g., adaptive immune system, the regulatory interactions between a lymphoid and a non-lymphoid cell, IFN-alpha/beta and gamma signaling (Supporting Information Table S3).

In this study, immunoprofiling was based on the Danaher method [8] for mRNA data, to virtually assess the composition of specific cell populations in LP cf. HL. In contrast to previous studies, we observed an evident increase of a neutrophilic profile and a decrease in overall profiles of T cells, cytotoxic T cells, and CD8 T cells (Figure 1D). To follow up on the cell score profiling, a flow cytometric analysis was performed on a set of fresh samples (Supporting Information Table S4), in which the CD45$^+$ leukocyte populations were assessed for frequencies of T cells, CD8$^+$ T cells, B cells, and neutrophils (Figure 2). Due to superficial lesions in two of the LP patients for whom no healthy samples could be collected, an unpaired statistical analysis was conducted for the flow cytometric analysis. The results were based on relative distribution of the cells in immune compartment. In contrast to previous studies, we observed an evident increase of a neutrophilic profile and a decrease in overall profiles of T cells, cytotoxic T cells, and CD8 T cells in LP cf. HL.

Interestingly, El Achkar et al. recently reported that the presence of neutrophils had a positive correlation with the laryngoscopic Derkay-scale and that the presence of neutrophils and CD15$^+$ cells were observed in the epithelium and connective tissue of the most severe cases of RRP [9]. This coincides with our findings of relatively high CD14$^+$ CD15$^+$ neutrophils in myeloid cells in the LP (cf. HL). Furthermore, experimental studies showed that neutrophils in high frequency, in the tumor microenvironment, could inhibit the activity of T cells [10]. We observed a similar association between neutrophils and CD8 T cells. This association along with insight on the associated DE genes might shed some light on frequent re-occurrences of LP lesions.

Previously, the studies undertaken to define the gene expression in LP have focused on the T-cell associated profile, and on the shift of balance between T-helper 1 (Th1) to T-helper 2 (Th2) cells [6]. However, none of our patients showed IFN-$\gamma$ mRNA in the LP-bioptries, where the vast majority of patients had mild disease. Thus, our result supports the absence of IFN-$\gamma$ mRNA in LP as previously reported by DeVoti and colleagues [6]. There is also evidence of infiltrate on of a suppressive CD4$^+$/CD25$^+$/Foxp3$^+$/CD127$^+$ low Treg population [4]. Meanwhile, the given lack of differentially expressed genes associated with Tregs (FOXP3, IL10 and TGF$\beta$) or NK cells (Killer-cell Ig-like receptors, KIRs), we do not observe any difference in Treg mediated suppression or NK infiltration in LP cf. HL samples.

Furthermore, PD-L1 has previously been demonstrated to be elevated in LP compared with control laryngeal tissues, suggesting an associated immune suppression [4]. On the contrary, the genes involved in the PD-1 signaling pathway (e.g., PDCD1LG2, CD4, LCK) as part of the adaptive immunity pathway, exhibited lower expression in LP with no significant PD1/PD-L1 in our NanoString gene expression data. Keeping in mind, the difference in expression of PD-1/PD-L1 in laryngeal samples between studies, suggests the need for another immunotherapeutic target than the PD-L1 inhibitor for the treatment of LP is warranted.

In summary, this study highlights the transcriptional activity in laryngeal papilloma, and we demonstrate enhanced gene expression of neutrophil-associated transcripts and reduced gene expression associated with CD8 T cells and B cells. Our finding of increased neutrophils of the LP suggests that future studies should be performed to describe the spectrum of immune cells within the microenvironments of LP to further explore the complex immune profile of LP.

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Abbreviations: FC: fold change · HPV: human papillomavirus · LP: laryngeal papilloma · PD: programmed death · RRP: recurrent respiratory papillomatosis

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