

Expression Profiling of Tumorigenesis Genes in Patients of Laryngeal Papillomatosis

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Abstract

Recurrent Laryngeal papillomatosis (RLP) is a disease of the aero-digestive junction superimposed at the upper respiratory tract. Low risk human papillomavirus type 6 and 11 are infectious agents in RLP. Mainstay of treatment for RLP is CO₂ laser vaporization for debulking of papilloma. Understanding of the gene changes at expression level might prove helpful for RLP management.

The study was designed to evaluate the changes in tumorigenesis genes in RLP cases to mark the severity and aggression at gene expression level for disease management.

Tissue samples and peripheral blood samples were acquired from clinically diagnosed RLP patients of age < 12 years undergoing CO₂ laser vaporization. Control samples were taken from the normal thyroid tissue of individuals undergoing thyroidectomy. Relative quantitation of gene expression was done by real time polymerase reaction using cDNA samples.

Up regulation of CXCL12/ CXCR4 signaling axis, parallel increase in GLUT-1 and over expression of IGF-I, EGFR and ER- α gene product was observed in RLP. On the other hand HIF-1 α levels not discerned elevated along with VEGF, PIK3 and AKT gene expression profile. The expression analysis has provoked the possible strategies which can be planned to treat RLP by adjuvant and tailored medication to reduce multiple surgeries.

Introduction

Patients suffering from this disease develop multiple benign papilloma of the respiratory tract that have a particular predilection for the true vocal cord. On the other hand malignant progression is rare, it appears to be more common in those papilloma harboring high-risk (HR) HPV subtypes. The course of human papilloma virus (HPV)-induced recurrent laryngeal papillomatosis is variable and unpredictable. Some patients experience spontaneous remission after one or two surgical procedure while others suffer from recurrent aggressive growths with dire consequences.

The intensity of nuclear staining of TLR4 was significantly lower in laryngeal papillomatosis transforming into laryngeal squamous cell carcinoma (LSCC) as compared to Laryngeal Papillomatosis have not been malignantly transformed (1).

HPV-6 viral load in adult-onset laryngeal papilloma decreased gradually to zero following several surgeries and intralesional cidofovir therapy. These findings provide evidence that relapses can occur if latent laryngeal HPV reservoirs are not eradicated and HPV replication might re-initiate from those sites after the end of therapy (2).

The human physiology is ardent to respond against or pro benign tumors. Similarly in context of RLP, spontaneous molecular changes may be involved in neoplasia. Therefore, underlying genetic mutations at individual level are contributory in the onset and progression of the disease. A mutation from C to T at

codon 273 of the P53 gene at *CpG* dinucleotide was associated with the integration of HPV-11 in histologically malignant lesions in a 28 year old symptomatic papillomatosis patient (3).

As a consequence of the laryngeal papillomatosis, different signaling pathways are deregulated.

Therefore, deregulated underlying molecular mechanisms were studied in the clinically diagnosed patients in the present study. Expression levels of different tumor suppressors and oncogenes were investigated to understand if overexpression or under expression of target genes modulated the disease severity.

Materials And Methods

Subjects:

The study was approved for use of tissue biopsy sample by the ethical review committee, KRL Hospital, Islamabad, Pakistan. Experimental protocols were approved by Institute of Biomedical and Genetic Engineering, Islamabad, Pakistan. All the methods were done in accordance with relevant regulations and guidelines. Patients, aged < 12 years presenting to ENT department of tertiary care hospital with respiratory distress and airway blockage due to RLP were requested to participate in this study. Patients present with hoarseness, respiratory distress and wart like tumorous growth on surface vocal cords and other laryngeal sites. Biopsies of the laryngeal papilloma of the patients undergoing CO2 laser vaporization surgery (Figure 1) were collected after informed consent. The patients presented with heavy papillomatosis growth which restricted the isolation of normal biopsy from the larynx. The control tissue samples were ascertained from normal thyroid tissue of patients undergoing thyroidectomy. The samples were stored at -80°C till further use.

RNA extraction and cDNA synthesis:

Pursuance of the molecular screening was done as with the isolation of total RNA from the tissue samples. Total RNA was extracted by GeneJET RNA purification Kit (K0731; Thermo Fisher Scientific, USA). RNA quantification was done First strand cDNA was synthesized from the extracted RNA templates by following instructions as per kit RevertAid First Strand cDNA Synthesis Kit (K1621; Thermo Fisher Scientific, USA).

Expression profiling of Tumorigenesis Genes:

Genetic expression profiling was carried out for target genes EGFR, ER- α , CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1a, VEGF, ERK1/2, PIK3 and AKT. GAPDH gene expression was determined for relative quantitation of target genes. RT-PCR was done using all genes mRNA specific primers, Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific, Lithuania) and cDNA in standard cycling program for SYBR Green on SLAN96 RT-PCR Machine (Sansure, China).

A fold change in expression of the target genes in disease relative to normal tissue was calculated. The calculation was done by adjusting the GAPDH expression level (as house-keeping) for target gene expression in disease and normal tissues using double delta cT method.

Results And Discussion

Patients suffering from RLP belong to age less than 10 years. Laryngeal obstruction due to extensive growth in larynx and in some cases trachea and hypopharynx was caused respiratory distress and swallow difficulty. The RLP obstruction was removed with CO2 laser ionization under general anesthesia.

Expression profiling for 12 genes along with the house keeping gene was quantitated. The relative fold gene expression analysis between the disease and normal tissue showed following discrete post translational gene expression changes.

i) Epidermal growth factor receptor (EGFR)

Association of Epidermal growth factor receptor (EGFR) has been well established in many tumors. Likewise EGFR translational availability has been implicated in patients of this study. EGFR inhibition in RRP with EGFR inhibitors as an adjuvant therapy has proven to lower RRP operative frequency and improvement in the modified Derkay scores and general disease (4)

ii) Estrogen Receptor Alpha (ER- α)

About 67% patients showed overexpression of Estrogen Receptor alpha (ER- α) which shows their ER positivity. Estrogen Receptors are hormone activated transcription factors with an important role in carcinogenesis (5). ER α -positive cases are not only responsive to endocrine therapies, but also sensitive to CDK4/6 inhibitors (6, 7). ER α -negative tumors, on the other hand, are more aggressive and metastatic (8). Treatment choices of ER positive cases by reported evidence for other cancers or malignancies can be opted for laryngeal papillomatosis.

iii) Chemokine Receptor and its Ligand (CXCL12/CXCR4)

Overexpression of chemokine CXCL12/CXCR4 signaling axis was observed. This favors virus production and HPV induced proliferation. This axis has been verified by gain of function mutation in CXCR4. Expression of CXCR4 promoted stabilization of HPV oncoproteins thus disturbing cell cycle progression and proliferation at the expense of the ordered expression of the viral genes required for virus production (9).

iv) Glucose transporter 1 (GLUT-1) & Insulin-like growth factor 1 (IGF-1)

High grade neoplasm observed in majority of RLP cases in the present study. An increased metabolite uptake by the proliferating cells is demanded, and tumor cells need to improve the nutrient uptake from the environment/serum. Glucose up-take may be regarded is the rate limiting step in neoplastic growth

and tumor cell metabolism. Since glucose does not merely drawn across the lipid-bilayer, three specialized categories of cell membrane protein transporters for sugar have been identified in mammals as Glucose

Transporter 1 (GLUT-1), Sodium-sugar linked Transporters (SGLT) and Sugars will eventually be exported transporter (SWEET) (10, 11, 12, 13). Among these GLUT-1 facilitates glucose transport the concentration gradient dependent glucose transport without requiring the primary or secondary ATP hydrolysis (14). In our study group up-regulation of the GLUT1 gene has been noted in 90 percent of patients. GLUT-1 overexpression can be considered as a strategy to increase glucose uptake for unprecedented cell growth like as other cancer cells hallmark feature. As there has been a parallel increase in IGF-I has been seen in present study. Mechanism of GLUT1- mediated glucose uptake has also been documented in the adrenomedullary cells. And an enhanced glucose transport in response to IGF-I associated activation of IGF receptor type 1 and GLUT1 translocation (15).

v) Hypoxia-inducible Factor (HIF-1a) - Vascular Endothelial Growth Factor (VEGF), Extracellular Signal-Regulated Protein Kinase 1/2 (ERK1/2) and Phosphatidylinositol-4 (PIK3), 5-bisphosphate 3-kinase-Protein Kinase B (AKT)

Enhanced angiogenesis through HIF-1a dependent VEGF expression is hallmark of tumor vascularization and growth. In context of the high-risk HPV type 16; E6 and E7 oncoproteins induced an increase in accumulation of HIF-1a protein and subsequently Hif1-a triggered VEGF expression by ERK1/2 and PIK3/AKT. These were suggestive findings observed for increased Hif1- α and VEGF expression in tumor growth of cervical cancer (16). E6 and E7 oncogenes also inhibit tumor suppressor p53 and Rb (17). The RLP patient's expression profiling presented a relatively surprising data in case of Hif1a, VEGF, AKT and PIK3 in the present study. Only in about 2 percent of patients the said genes were overexpressed while the rest of the cases did not show any aberrant expression.

The RLP patient papillomas cause airway obstruction, which leads to fall in oxygen fall in blood oxygen levels in spontaneously breathing patient. In this event patient also retain CO₂ so that their blood CO₂ rises. Hypoxia (low oxygen) and hypercapnia (high carbon dioxide) are concurrently present in tissue microenvironment in variety of pathophysiological conditions due to respiratory diseases e.g. obstructive sleep apnea syndrome, pneumonia and chronic obstructive pulmonary disease (COPD) (18, 19). The hypercapnia has been well studied *in vivo* and *in vitro* to counter-regulate and suppress hypoxia induced Hif1- α pathway activation. The mechanism involves CO₂ dependent pH reduction which assists non-canonical lysosomal degradation of Hif1- α protein (20). This can be best hypothetical model for the pathophysiology for current study where Hif1- α and its target genes expression was not overexpressed in presence of high CO₂ retention in airway obstructed patients.

Conclusion

The extract of the study highlights important molecular based targeting mechanisms for RLP and to put a barrier in papillomatosis development. The key targets as understood in the genetic expression profiling

include; Inhibition of chemokine receptors through targeted therapy, adjuvant therapy by EGFR inhibitors, ER α antagonist Tamoxifen, which is structurally similar to estrogen is used for patients with ER α over expression. And therapies for inhibition of Hif1- α may not prove efficient in this disease. A quick expression profiling for cell proliferation and oncogenes in RLP patients can reveal the molecular targets to treat with available and or adjuvant therapy. Furthermore antivirals still stand to compensate the disease. Further insights at the level of protein analysis may facilitate new treatment regime formulation.

Declarations

Conflict of Interest

The authors did not declare any conflict of interest.

References

1. Ilmarinen, T. *et al.* Low expression of nuclear Toll-like receptor 4 in laryngeal papillomas transforming into squamous cell carcinoma. *Otolaryngol Head Neck Surg.* **151(5)**, 785-90 (2014).
2. Mikolajczak, S. *et al.* The impact of cidofovir treatment on viral loads in adult recurrent respiratory papillomatosis. *Eur Arch Otorhinolaryngol.* **269**, 2543–48 (2012).
3. Rady, P. L., Schnadig, V. J., Weiss, R. L., Hughes, T. K. and Tying, S. K. Malignant transformation of recurrent respiratory papillomatosis associated with integrated human papillomavirus type 11 DNA and mutation of p53. *Laryngoscope.* **108(5)**, 735-40 (1998).
4. Logotheti, S. *et al.* Progression of Mouse Skin Carcinogenesis Is Associated with Increased Era Levels and Is Repressed by a Dominant Negative Form of Era. *PLOS ONE* **7(8)**: e41957 (2012).
5. Moldan, M. M., Bostrom, B. C., Tibesar, R. J., Lander and T. A., Sidman, J. D. Epidermal growth factor receptor inhibitor therapy for recurrent respiratory papillomatosis. **2-202**, (2013).
6. Brufsky, A. M. and Dickler, M. N. Estrogen receptor-positive breast cancer: exploiting signaling pathways implicated in endocrine resistance. **23**, 528–39 (2018).
7. Xu, H. *et al.* Recent advances of highly selective CDK4/6 inhibitors in breast cancer." *Journal of hematology & oncology.* **10, 1 97**, (2017).
8. Dunnwald, L. K., Rossing, M. A., Li, C. I. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. *Breast Cancer Res.* **9**, R6 (2007).
9. Meuris, F. *et al.* The CXCL12/CXCR4 Signaling Pathway: A New Susceptibility Factor in Human Papillomavirus. *PLoS pathogens.* **12(12)**, e1006039 (2016)
10. Mueckler, M., Thorens, B. The SLC2 (GLUT) family of membrane transporters. *Aspects Med.* **34**, 121–138 (2013).
11. Chen, L. Q., Cheung, L. S., Feng, L., Tanner W., Frommer, W. B. Transport of Sugars. *Rev. Biochem.* **84**, 865–894 (2015).

12. Feng, L. and Frommer, W. B. Structure and function of Semi SWEET and SWEET sugar transporters. *Trends Biochem. Sci.* **40**, 480–486 (2015).
13. Deng, D. and Yan, N. GLUT, SGLT, and SWEET: Structural and mechanistic investigations of the glucose transporters. *Protein Sci.* **25**, 546–558 (2015).
14. Zambrano, A., Molt, M., Uribe, E., Salas, M. Glut 1 in Cancer Cells and the Inhibitory Action of Resveratrol as A Potential Therapeutic Strategy. *Int J Mol Sci.* **20(13)**, 3374 (2019).
15. Fladeby, C., Bjønness, B., Serck-Hanssen, G. GLUT1-mediated glucose transport and its regulation by IGF-I in cultured bovine chromaffin cells. *J Cell Physiol.* **169(2)**, 242-7 (1996).
16. Tang, X. *et al.* Overexpression of human papillomavirus type 16 oncoproteins enhances hypoxia-inducible factor 1 alpha protein accumulation and vascular endothelial growth factor expression in human cervical carcinoma cells. *Clinical cancer research: an official journal of the American Association for Cancer Research.* **13(9)**, 2568-76 (2007).
17. Moody, C. A., Laimins, L. A. Human papillomavirus oncoproteins: pathways to transformation. *Nat Rev Cancer.* **10(8)**, 550-60 (2010)
18. Krieger, J. *et al.* Pulmonary hypertension, hypoxemia, and hypercapnia in obstructive sleep apnea patients. **96**, 729–737 (1989).
19. Budweiser, S., Jörres, R. A., and Pfeifer, M. Treatment of respiratory failure in COPD. *J. Chron. Obstruct. Pulmon. Dis.* **3**, 605–618 (2008)
20. Selfridge, A. C. *et al.* Hypercapnia Suppresses the HIF-dependent Adaptive Response to Hypoxia. *The Journal of biological chemistry.* **291(22)**, 11800-8 (2016).

Figures

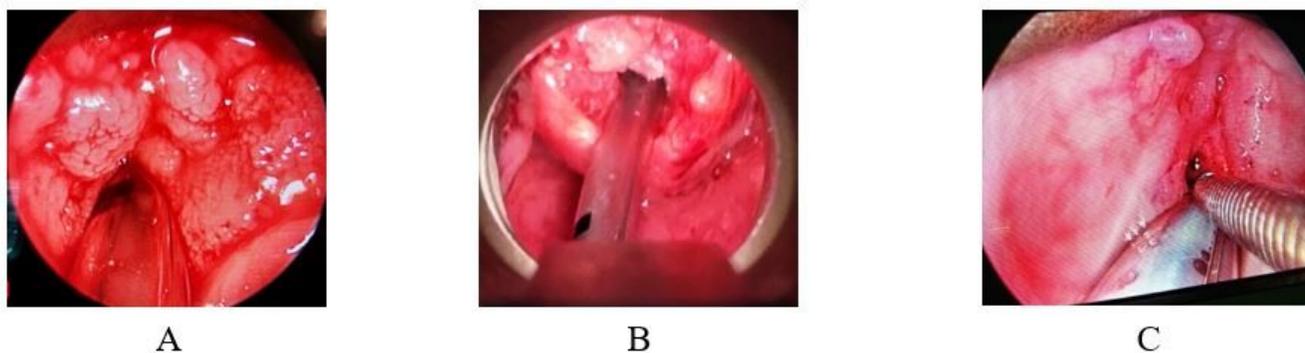


Figure 1

Blocked Larynx of the Laryngeal Papillomatosis patients A, B, C at the time of laser excision surgery.