*Proceedings of the Pakistan Academy of Sciences: B*
*Life and Environmental Sciences* 61(S): 117-123(2024)

ISSN (Print): 2518-4261; ISSN (Online): 2518-427X
http://doi.org/10.53560/PPASB(61-sp1)1015

Pakistan Academy of Sciences

Research Article

# Expression Profiling of Genes Associated with the Pathogenesis of Recurrent Laryngeal Papillomatosis

**Muhammad Yasir Khan[1], Zahra Sarwar[1], Mavra Sarwar[1], Saif Ullah Khan[1], Muhammad Sarwar Khan[1*], Rashida Khan[2], and Qaisar Mansoor[2*]**

[1]Department of ENT, KRL Hospital, Islamabad, Pakistan
[2]Institute of Biomedical & Genetic Engineering (IBGE), Islamabad, Pakistan

**Abstract:** Recurrent Laryngeal Papillomatosis (RLP) affects the aero-digestive intersection with a predilection for the glottis. It is predominantly a juvenile-onset disease. The main infectious agents are type 6 and 11 low-risk human papillomaviruses. Understanding the genetic changes associated with the pathogenesis of RLP might prove helpful to mark the severity and aggression of the disease and lead to better clinical management. Clinically diagnosed RLP children below the age of 12 years along with a control sample of healthy tissue from age and gender-matched children undergoing tonsillectomy and thyroidectomy were collected. All the samples were processed for total RNA extraction followed by first strand cDNA synthesis. Real-time PCR was done to determine the relative gene expression of EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PI3K and AKT genes along with GAPDH as gene of reference. An increase in the transcriptional level expression of the genes CXCL12/CXCR4, GLUT-1, EGFR, ER-α, and IGF-I was observed in the cases in comparison to the controls. The expression of HIF-1α, VEGF, PI3K, and AKT genes was not noticeably elevated. The gene expression analysis may open the avenues for possible strategies that can be employed to treat RLP more effectively.

**Keywords:** Gene Expression Profiling, Human papillomavirus (HPV), Laryngeal Papillomatosis, Transoral Laser Microsurgery (TLM)

## 1. INTRODUCTION

Patients suffering from Recurrent Laryngeal Papillomatosis (RLP) develop multiple benign papilloma of the respiratory tract that have a particular predilection for the true vocal cord. RLP is the commonest form of benign mesenchymal neoplasm of the larynx categorized by hyperplastic stratified squamous epithelium with fibrovascular core in aerodigestive tract of kids. Moreover, it is the toughest to treat histologic conditions because of high recurrence and its spread in the surrounding respiratory tract [1]. Malignant progression is rare but can be found in papilloma harboring high-risk (HR) human papillomavirus subtypes. The course of human papillomavirus (HPV)-induced recurrent laryngeal papillomatosis is variable and unpredictable. Some patients experience spontaneous remission after one or two surgical procedures while others suffer from recurrent aggressive growths with dire consequences. RLP is a multifaceted ailment and presents with substantially discrete outcomes. Administration through ancillary-assisted therapies has shown remarkable treatment outcomes that reportedly help eradicate or reduce the lesion size and less frequent surgeries [5].

The contagious nature of the disease has only been documented through contact with laser smoke on the laser surgeons and supporting staff at the time of the patient's surgery. HBV 6 and 11 had been found adequately on the gel foams drenched in the operation theater of RLP. However, the virulent viral load was way smaller to cause infectious transmission. The precautionary measures are in line with the SOPs of the surgical area while handling laryngeal papilloma [2].

The modes of transmission for the juvenile-onset include; at the time of childbirth, primigravida, recently acquired genital warts, and longer stages

Received: October 2023; Revised: December 2023; Accepted: January 2024
*Corresponding Authors: Qaisar Mansoor <qmibge@gmail.com; qaisar@ibge.edu.pk>

of the mother's labor during first baby birth. While in the adult onset of the disease, the speculated transmission mode is oral foreplay and sex [3].

The incidence of laryngeal papillomatosis has been reported as 4.3 and 1.8 per 100,000 in children and adults respectively [4]. Zumaeta-Saavedra et al. reported a case of a 13-year-old male with laryngeal papillomatosis right from the age of 2 years. The hallmark manifestations presented for the disease included respiratory distress and multiple stenosis nodules in the larynx and trachea along with pulmonary cysts. A single dose administration of bevacizumab 400mg and respiratory therapies followed by excision of papillomatous and tracheostomy; yielded promising treatment outcomes without disease relapse in follow-up [6]. On a malignancy comparative evaluation between laryngeal squamous cell carcinoma (LSCC) as compared to Laryngeal Papillomatosis; it was seen that the intensity of nuclear staining of TLR4 was significantly lower in LSCC as compared to Laryngeal Papillomatosis that did not undergo malignant transformation [7]. Another study revealed that HPV-6 viral load in adult-onset laryngeal papillomatosis decreased gradually to zero following several surgeries and intralesional cidofovir therapy. This provides evidence that relapses can be avoided if latent laryngeal HPV reservoirs are eradicated [8]. In RLP, spontaneous molecular changes may be involved in neoplasia. Therefore, underlying genetic mutations contribute to the disease initiation and progression. A mutation from C to T at codon 273 of the P53 gene at CpG dinucleotide was reported for integrating HPV-11 in histologically classified malignant lesions in a 28-year-old symptomatic papillomatosis patient [9].

It is pertinent to mention that there is very little gene expression data available for RLP; though targeted cancer drugs have been used to lessen the papillomatous growth. As a consequence of the HPV infection, different signaling pathways are deregulated.

The present work aims to investigate the deregulated genetic signaling mechanisms underlying the clinically diagnosed RLP children of age less than 12 years. The study focuses on the signaling pathways associated with cell survival and proliferation. Various cell signaling pathways give growth advantage to the rapidly proliferating cells. In accordance with this concept; EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PI3K and AKT in RLP patients were investigated. This likely deregulation in these gene expressions will help to understand if the overexpression or underexpression of target genes modulates the disease and its severity.

## 2. MATERIALS AND METHODS

### 2.1. Subjects

The study was approved for use of tissue biopsy samples of the patient and normal tissue specimens by the ethical review committee (Reference ERC-19/03/02), KRL Hospital, Islamabad, Pakistan. All the study subjects provided the informed consent to participate in the study.

The inclusion criteria comprised of children less than 12 years of age either presenting for the first time or recurrent laryngeal papillomatosis to the ENT department with symptoms of respiratory distress, airway obstruction, and hoarseness of voice who on flexible nasendoscopy had papillomatous growths on the surface of the vocal cords and other laryngeal sites were included in the study. The patients without any clinical hallmark of papillomatous growth on the larynx or vocal cords were excluded from the study.

Biopsies of the laryngeal papilloma of the 19 RLP patients undergoing $CO_2$ laser evaporation (Figure 1, showing a representation of various stages of surgery from complete obstruction to normal laryngeal inlet) were collected. The control tissue samples, however, were obtained from the larynges of the of age and gender-matched individuals undergoing surgeries like thyroidectomy or tonsillectomy. The samples were stored at -80°C till further use.

### 2.2. Isolation of RNA And First Strand cDNA Preparation

Complete RNA of the patients' and normal tissue biopsies was isolated by GeneJET RNA isolation kit (Thermo Fisher Scientific, USA) as per the manufacturer's instruction. RNA quantification

**Fig. 1.** (A) Severe respiratory obstruction caused by papillomas. (B) During $CO_2$ laser-assisted evaporation. (C) View at the end of surgery.

was done and First-strand cDNA was synthesized from the extracted RNA by the RevertAid First Strand cDNA Synthesis Kit (K1621; Thermo Fisher Scientific, USA).

### 2.3. Expression Profiling of Cell Survival Genes

Transcription level relative expression profiling was carried out for target genes EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PIK3, and AKT along with GAPDH as housekeeping gene. First-strand cDNA was employed for relative gene quantification in RT-PCR kit Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Lithuania) using gene-specific primers according to the standard cycling program described in the user manual of the kit. The RT PCR was carried out on an SLAN96 RT-PCR Machine (Sansure, China).

### 2.4. Expression Level and Statistical Calculations

The change in the expression of target genes in terms of "fold change" for disease samples relative to normal samples was calculated. The calculation was done by adjusting the GAPDH expression level (as housekeeping) for the target gene expression in disease and normal tissues using the double delta cT method [10]. The positive value indicates an increase in the expression of gene(s) and a negative fold change indicates a decrease in the expression. The fold change value for cases and controls was represented in actual (Figure 2-5) and in logarithmic scale (base 2) log2 (Figure 6). Chi-square was calculated to determine the significance of differential expression in patients as compared to controls. A *p-value* <0.05 was taken as significant for differential expression.

## 3. RESULTS AND DISCUSSION

Laryngeal obstruction due to extensive growth in the larynx and in some cases, trachea and hypopharynx caused mainly respiratory distress and dysphonia and only occasionally led to swallowing difficulty. The RLP obstruction was removed with transoral $CO_2$ laser microsurgery under general anesthesia. Expression profiling for EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PI3K and AKT genes along with the housekeeping gene was quantitated. The relative gene expression analysis between the disease and normal samples showed discrete transcriptional level gene expression changes as presented below:

### 3.1. Epidermal Growth Factor Receptor (EGFR) and Estrogen Receptor Alpha (ER-α)

Aggressive overexpression of EGFR in 64% and ER-α in 67% of patients was observed in the present study (Figure 2). Association of EGFR has been well-established in many tumors. Likewise, EGFR translational availability has been implicated in patients of this study. EGFR inhibition in Recurrent Respiratory Papillomatosis (RRP) with EGFR inhibitors as an adjuvant therapy has been shown to lower RRP operative frequency and improve the modified Derkay scores and general disease [11].

Estrogen receptors are hormone-activated transcription factors with an important role in carcinogenesis [12]. ERα-positive cases are not only responsive to endocrine therapies but also sensitive to CDK4/6 inhibitors [13-14]. Whereas, ERα-negative tumors are more aggressive and metastatic [15]. Treatment choices of ER-positive cases by reported evidence for other cancers or malignancies can be opted for laryngeal papillomatosis.

### 3.2. Chemokine Ligand and Its Receptor (CXCL12/CXCR4)

Overexpression of the CXCL12/CXCR4 signaling axis was observed (Figure 3) This favors virus

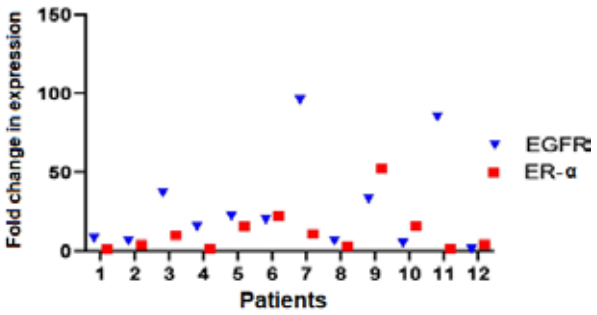


**Fig. 2.** Relative gene expression of EGFR and ER-α.

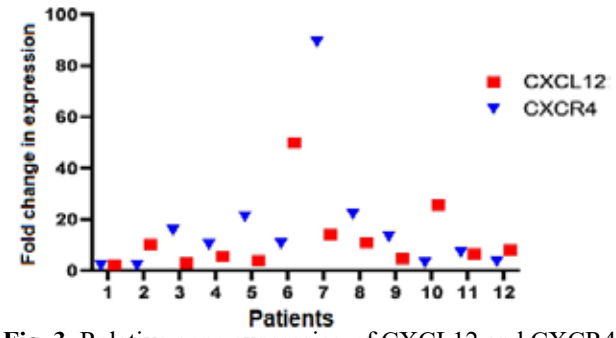


**Fig. 3.** Relative gene expression of CXCL12 and CXCR4.

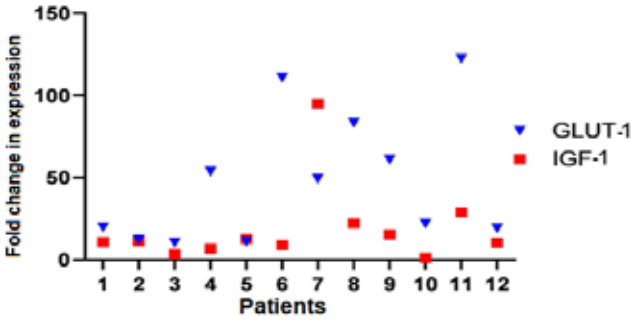


**Fig. 4.** Relative gene expression of GLUT-1 and IGF-1.

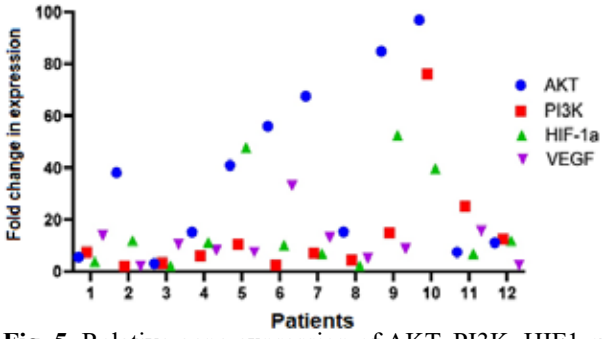


**Fig. 5.** Relative gene expression of AKT, PI3K, HIF1-α and VEGF.

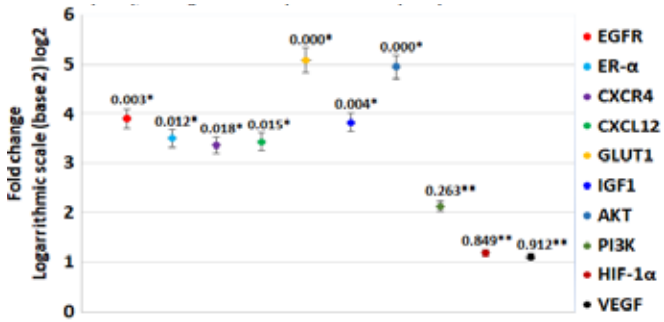


**Fig. 6.** Comparison of the studied genes in RLP patients and controls.

production and HPV-induced proliferation. This axis has been verified by the gain of function mutation in CXCR4. CXCR4 promotes HPV oncoproteins stabilization; derailing the development and propagation of the host cell cycle, mechanistically knitted with viral genes expression responsible for virus survival [16].

### 3.3. Insulin-Like Growth Factor 1 (IGF-1) And Glucose Transporter 1 (GLUT-1)

High-grade neoplastic activity was observed in the majority of RLP cases in the present study. An increased metabolite uptake by the proliferating cells is demanded, and tumor cells require the grasping of the nutrients from the environment/ serum. The critical threshold step might be regulated by glucose uptake during the neoplasm and cancer cells metabolic activity for growth. Glucose cannot simply pass the cell membrane; protein channels in the cell membrane namely Sodium-Glucose linked transporters (SGLT), Sugars will eventually be exported transporters (SWEET) and GLUT-1 were identified in mammals. [17-20]. Interestingly the gradient-dependent glucose transport in the absence of ATP hydrolysis is enabled by GLUT-1 only [21]. The current work identified an increased expression of the GLUT-1 gene in 90 percent of patients. GLUT-1 overexpression can be considered as a strategy to increase glucose uptake for unprecedented cell

growth like other cancer cells' hallmark features. A parallel increase in IGF-I has been seen (Figure 4). An evident increase in the transportation of glucose in response to IGF-I-linked stimulation of IGF1R and translocation of GLUT1 has been reported [22].

### 3.4. Hypoxia-inducible Factor (HIF-1α) – Vascular Endothelial Growth Factor (VEGF), Extracellular Signal-Regulated Protein Kinase 1/2 (ERK1/2) and Phosphatidylinositol-4 (PI3K), 5-Bisphosphate 3-Kinase-Protein Kinase B (AKT)

Enhanced angiogenesis through HIF-1α dependent VEGF expression is the hallmark of tumor vascularization and growth. In the context of the high-risk HPV type 16; E6 and E7 oncoproteins induced an increase in accumulation of HIF-1α protein and subsequently, HIF-1α triggered the expression of VEGF by ERK1/2 and PIK3/AKT. These were suggestive findings observed for increased HIF-1α and VEGF expression in tumor growth of cervical cancer [23]. E6 and E7 oncogenes also inhibit tumor suppressor p53 and Rb [24]. The RLP patient's expression profiling presented relatively surprising data in the case of HIF-1α, VEGF, AKT, and PI3K in the present study (Figure 5). Only in about 2 percent of patients, the said genes were overexpressed while the rest of the cases did not show any aberrant expression.

In RLP, the papilloma causes airway obstruction, which leads to a fall in oxygen saturation levels in spontaneously breathing patients. In this event, patient also retains $CO_2$ so that their blood $CO_2$ concentration rises. Hypoxia (low oxygen) and hypercapnia (high carbon dioxide) are concurrently present in the tissue microenvironment in a variety of pathophysiological conditions due to respiratory diseases e.g., obstructive sleep apnea syndrome, pneumonia and chronic obstructive pulmonary disease (COPD) [25-26]. The hypercapnia has been well studied in vivo and in vitro to counter-regulate and suppress hypoxia-induced HIF-1α pathway activation. The mechanism involves $CO_2$-dependent pH reduction which assists in non-canonical lysosomal degradation of HIF-1α protein (27). This can be the best hypothetical model for the pathophysiology of a current study where HIF-1α and its target genes expression were not overexpressed in the presence of high $CO_2$ retention in airway-obstructed patients.

### 3.5. Comparison of Studied Genes in RLP with Controls

The relative gene expression of the oncogenes targeted in the present study revealed concerning disturbances in the genetic transcription levels in RLP in comparison to controls. Expression levels of GLUT1, AKT, EGFR, ER- α, CXCL12/CXCR4, and IGF-1 were found to be upregulated (Figure 6). The overexpression of these genes in laryngeal papillomatosis in the present study suggests that they work to enhance cell growth, survival, and proliferation. However, the expression profiles of HIF-1α, VEGF, and PI3K were not found to be different from the controls. Hence there is a need for controlling the upregulation of specifically GLUT1, AKT, EGFR, ER-α, CXCL 12/ CXCR4, and IGF-1.

## 4. CONCLUSİONS

This study highlights the important molecular-level mechanisms involved in the progression of RLP. In-depth knowledge of these may prove helpful in limiting the disease. The key targets as understood in the genetic expression profiling include the inhibition of chemokine receptors through targeted therapy, adjuvant therapy by EGFR inhibitors, and ER-α antagonist agents (like Tamoxifen), which are structurally similar to estrogen and used for patients with ER-α overexpression. While the study of expression profiling for cell proliferation and oncogenes in RLP patients revealed the molecular targets to treat, further insight at the level of protein analysis is required to achieve new treatment regimens in the management of this disease condition.

## 5. ACKNOWLEDGEMENTS

The authors would like to acknowledge Ms. Tehreem Tahir Scientific Officer IBGE and operation theatre staff, KRL Hospital, Islamabad, Pakistan for their elementary support in sample collection. Authors express deep gratitude and highly acknowledge the patients and normal individuals who contributed to this research work.

## 6. CONFLICT OF INTEREST

The authors declared no conflict of interest associated with this article.

## 7. REFERENCES


1. G.A. Rivera, F Morell. Laryngeal Papillomas. Treasure Island (FL). StatPearls Publishing Treasure Island, FL, USA (2023).
2. H.K. Kashima, T. Kessis, P. Mounts, and K. Shah. Polymerase chain reaction identification of human papillomavirus DNA in $CO_2$ laser plume from recurrent respiratory papillomatosis. *Otolaryngology–Head and Neck Surgery* 104(2): 191-195 (1991).
3. M. Carifi, D. Napolitano, M. Morandi, and D. Dall'Olio. Recurrent respiratory papillomatosis: current and future perspectives. *Therapeutics and Clinical Risk Management* 11: 731-738 (2015).
4. C.S. Derkay. Recurrent Respiratory Papillomatosis. *The Laryngoscope* 111(1): 57-60 (2001).
5. E.Z. Saavedra, C.C. Chilet, J.M. Afaray, and M.L. Vilchez. Recurrent laryngeal papillomatosis with pulmonary involvement: case report. *Revista Peruana de Medicina Experimental y Salud Pública* 40(1): 111-114 (2023).
6. S.A. Ballestas, S. Shelly, R.M. Soriano, and A. Klein. Trends in recurrent respiratory papillomatosis treatment. *Acta Otorrinolaringologica (English Edition)* 72(2): 109-120 (2021).
7. T. Ilmarinen, J. Hagström, C. Haglund, E. Auvinen, I. Leivo, A. Pitkäranta, and L.M. Aaltonen. Low expression of nuclear Toll-like receptor 4 in laryngeal papillomas transforming into squamous cell carcinoma. *Otolaryngology–Head and Neck Surgery* 151(5): 785-90 (2014).
8. S. Mikolajczak, G. Quante, S. Weissenborn, A. Wafaisade, U. Wieland, J.C. Lüers, J.P. Klussmann, and D. Beutner. The impact of cidofovir treatment on viral loads in adult recurrent respiratory papillomatosis. *European Archives of Oto-Rhino-Laryngology* 269: 2543–2548 (2012).
9. P.L. Rady, V.J. Schnadig, R.L. Weiss, T.K. Hughes, and S.K. Tyring. Malignant transformation of recurrent respiratory papillomatosis associated with integrated human papillomavirus type 11 DNA and mutation of p53. *The Laryngoscope* 108(5): 735-740 (1998).
10. K.J. Livak, and T.D. Schmittgen. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* 25(4): 402-408 (2001).
11. M.M. Moldan, B.C. Bostrom, R.J. Tibesar, T.A. Lander, and J.D. Sidman. Epidermal growth factor receptor inhibitor therapy for recurrent respiratory papillomatosis. *F1000Research* 2: 202 (2013).
12. S. Logotheti, D. Papaevangeliou, I. Michalopoulos, M. Sideridou, K. Tsimaratou, I. Christodoulou, K. Pyrillou, V. Gorgoulis, S. Vlahopoulos, and V. Zoumpourlis. Progression of Mouse Skin Carcinogenesis Is Associated with Increased Erα Levels and Is Repressed by a Dominant Negative Form of Erα. *PLoS One* 7(8): e41957 (2012).
13. A.M. Brufsky, and M.N. Dickler. Estrogen receptor-positive breast cancer: exploiting signaling pathways implicated in endocrine resistance. *The Oncologist* 23(5): 528–539 (2018).
14. H. Xu, S. Yu, Q. Liu, X. Yuan, S. Mani, R.G. Pestell, and K. Wu. Recent advances of highly selective CDK4/6 inhibitors in breast cancer. *Journal of Hematology & Oncology* 10(1): 97 (2017).
15. L.K. Dunnwald, M.A. Rossing, and C.I. Li. Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients. *Breast Cancer Research* 9(1): R6 (2007).
16. F. Meuris, L. Carthagena, A.J. Ros, F. Gaudin, P. Cutolo, C. Deback, Y. Xue, F. Thierry, J. Doorbar, and F. Bachelerie. The CXCL12/CXCR4 Signaling Pathway: A New Susceptibility Factor in Human Papillomavirus. *PLoS Pathogens* 12(12): e1006039 (2016).
17. M. Mueckler, and B. Thorens. The SLC2 (GLUT) family of membrane transporters. *Molecular Aspects of Medicine* 34(2-3): 121–138 (2013).
18. L.Q. Chen, L. S. Cheung, L. Feng, W. Tanner, and W.B. Frommer. Transport of Sugars. *Annual Review of Biochemistry* 84: 865–894 (2015).
19. L. Feng, and W.B. Frommer. Structure and function of Semi SWEET and SWEET sugar transporters. *Trends in Biochemical Sciences* 40(8): 480–486 (2015).
20. D. Deng, and N. Yan. GLUT, SGLT, and SWEET: Structural and mechanistic investigations of the glucose transporters. *Protein Science* 25(3): 546–558 (2015).
21. A. Zambrano, M. Molt, E. Uribe, and M. Salas. Glut 1 in Cancer Cells and the Inhibitory Action of Resveratrol as A Potential Therapeutic Strategy. *International Journal of Molecular Sciences* 20(13): 3374 (2019).
22. C. Fladeby, B. Bjønness, and G.S. Hanssen. GLUT1-

mediated glucose transport and its regulation by IGF-I in cultured bovine chromaffin cells. *Journal of Cellular Physiology* 169(2): 242-247 (1996).

23. X. Tang, Q. Zhang, J. Nishitani, J. Brown, S. Shi, and A.D. Le. Overexpression of human papillomavirus type 16 oncoproteins enhances hypoxiainducible factor 1 alpha protein accumulation and vascular endothelial growth factor expression in human cervical carcinoma cells. *Clinical Cancer Research* 13(9): 2568-2576 (2007).
24. C.A. Moody, and L.A. Laimins. Human papillomavirus oncoproteins: pathways to transformation. *Nature Reviews Cancer* 10(8): 550-560 (2010).
25. J. Krieger, E. Sforza, M. Apprill, E. Lantpert, E. Weitzenblum, and J. Ratomaharv. Pulmonary hypertension, hypoxemia, and hypercapnia in obstructive sleep apnea patients. *Chest* 96(4): 729–737 (1989).
26. S. Budweiser, R.A. Jörres, and M. Pfeifer. Treatment of respiratory failure in COPD. *International Journal of Chronic Obstructive Pulmonary Disease* 3(4): 605–618 (2008).
27. A.C. Selfridge, M.A.S. Cavadas, C.C. Scholz, E.L. Campbell, L.C. Welch, E. Lecuona, S.P. Colgan, K.E. Barrett, P.H.S. Sporn, J.I. Sznajder, E.P. Cummins, and C.T. Taylor. Hypercapnia Suppresses the HIF-dependent Adaptive Response to Hypoxia. *Journal of Biological Chemistry* 291(22): 11800-11808 (2016).