

# A Comprehensive Review of Biomarkers of Hypoxia in Oral Squamous Cell Carcinoma

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## Abstract

Areas of hypoxia in squamous cell carcinomas of the oral cavity lead to various adaptive mechanisms like anaerobic glycolysis, altered gene expression and deviations in the cellular proteome. These mechanisms not only thwart cellular death but enable rapid progression of growth, culminating in poor prognosis. Amongst a wide number of biomarkers stimulated by hypoxic conditions in oral squamous cell carcinoma (OSCC), the most notable ones are hypoxia-inducible factors (HIF), vascular endothelial growth factors (VEGF) and aquaporins (AQP). In several cancers, including OSCC it has been observed that HIF plays the role of a crucial transcriptional activator. This leads to the downstream expression of several genes that control the process of tumour angiogenesis. The most significant of these genes is VEGF, which has a dominant role as a regulatory gene controlling angiogenesis. VEGFs are a diverse family consisting of which primarily VEGF-A has an essential role in regulating angiogenesis in specific vascular endothelial cells. This is critical for the cellular functions of cancerous tissues to adapt to hypoxic conditions. In addition, VEGF has an immune-regulatory property, thereby modulating immune cell antitumour activity. Another potential biomarker is AQP, a family of transmembrane water channel proteins, that plays a role in transcellular and transepithelial water transport. Unlike AQP1, AQP2, AQP4, AQP5, and AQP8, which exclusively transport water, AQP3, AQP7, AQP9, and AQP10 transport glycerol and other small solutes, and so are termed as "aqua-glyceroporins". Studies have implicated AQP in the molecular mechanism of oncogenesis across many cancers, where they help in the proliferation of cells, cellular migration, invasiveness and angiogenesis.

**Keywords:** Biomarkers; Hypoxia; Early Diagnosis; Oral Carcinoma.



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## INTRODUCTION

Malignancies of the oral cavity are a global burden and feature in the top ten, with approximately 0.3 million fresh cases annually. As per WHO: GLOBOCAN 2020, the common variety (approximately 90%) is squamous cell cancers, which account for approximately 0.17 million deaths per year. It is estimated that between 2020 and 2040, there would be 55-60% rise in the number of cases of OSCC. It is alarming to note that India accounts for nearly one out of four of all cases of



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OSCC, with 77 thousand new cases and 52 thousand deaths annually.<sup>1-3</sup> The alarming magnitude of this problem is reflected by the fact that more than 65% of patients attending Head and Neck Cancer Clinic of any tertiary care institute in India have locally advanced disease, and due to the late stage of detection, 5-years survival rate is at a dismal 20%.<sup>4-6</sup> OSCC originates at the upper aerodigestive tract mucosal lining epithelium. Being highly aggressive these cause severe destruction above the clavicle, developing lymph node metastasis early and distant metastasis over time even after therapy. Physiological hypoxia is responsible for a multitude of changes such as promoting angiogenesis, erythropoiesis, and glycolysis. The cellular responses are a result of the formation of reactive oxygen species at the mitochondrial level. Damaged mitochondria can also induce mitophagy and mitochondrial dysfunction can have an impact on several biological processes and has emerged as a prominent signature of metabolic, cardiovascular, inflammatory, and neurodegenerative diseases; cancer; and many age-related diseases.<sup>7</sup> Data obtained in the past two decades have shown that 50% to 60% of locally advanced solid tumours exhibit hypoxic or anoxic areas<sup>8</sup> that result from an imbalance between the supply and consumption of oxygen. Hypoxia leads to chaotic vasculature, blood flow is affected, and abnormalities in tumour microvessels over an excessively aggressively growing tumour. A hypoxic environment shapes the tumour microenvironment also playing a major role in tumour growth and progression. Epithelial mesenchymal transition is mediated by HIF-1 which activates various transcription repressors.<sup>9</sup> The hypoxic TME triggers varied molecular responses. These responses promote tumour progression and confer radiation resistance and chemoresistance to tumours.<sup>10</sup> The predominant molecules that are associated with hypoxia research are the hypoxia-inducible factors (HIFs).

### ***Hif-1 System***

Structurally HIF-1 is a heterodimer consisting of HIF-1 $\beta$  subunit along with one HIF-1 $\alpha$  subunit. It has two basic helix-loop-helix proteins (bhlh) of the PAS family (PER, AHR, ARNT and SIM family).<sup>11-12</sup> HIF system consists of  $\alpha$  and  $\beta$  subunits, with three isoforms of the  $\alpha$  subunit, viz. HIF-1 $\alpha$ , which is ubiquitously expressed in all cells, and plays a dominant role under hypoxic conditions. In contrast, HIF-2 $\alpha$  and HIF-3 $\alpha$  have tissue-specific expression. HIF-2 $\alpha$  is an upregulator of genes involved in controlling redox metabolism, unlike HIF-3 $\alpha$ ,

which downregulates through a HIF-1 $\alpha$  inhibitor mediator.<sup>13</sup> Under normoxic conditions, HIF prolyl hydroxylases domain-containing enzymes (PHD) inactivate HIF-1 $\alpha$ . In contrast, under hypoxia, PHD is inhibited which increases the level of HIF-1 $\alpha$  located on chromosome 14 (14q21-q24) and then drives it to heterodimerise with HIF-1 $\beta$  for the aryl hydrocarbon receptor nuclear translocator (ARNT) and binds to hypoxia response elements (HREs) in the genome.<sup>14</sup> This stabilisation of HIF-1 $\alpha$  activates multiple genes, including VEGF, the downstream effect of which controls redox homeostasis and thereby angiogenesis and vascular remodelling, along with survival, growth, metabolism, extracellular matrix function and tumour immunity. A major event in metastasis involves epithelial-mesenchymal transition (EMT) in OSCC. HIF-1 $\alpha$  and  $\beta$ -catenin are the downstream mediators of the EMT process of OSCC.<sup>15</sup> A disturbed extracellular matrix regulated by HIF-1 $\alpha$  is an important factor in the EMT of tumour cells. A prognosticator role for HIF-1 $\alpha$  as well as HIF-2 $\alpha$  isoforms for OSCC development and metastasis was suggested by Qian and co-workers.<sup>16</sup> The over expression of HIF-1 $\alpha$  could be used as a marker for distinguishing healthy mucosa, precursor lesions and OSCC. Oral epithelial dysplastic lesions with an increased HIF-1 $\alpha$  expression had a greater risk of malignant transformation. This immediately, suggested that HIF-1 $\alpha$  is expressed early in oral carcinogenesis. miRNAs also have been identified which have been associated with OSCC and might serve as a potential biomarker.<sup>17-18</sup> miR-21 is upregulated and it affects snail, vimentin expression while significantly decreasing Ecadherin expression in OSCC,<sup>19</sup> suggesting that miR-21 is not only HIF-associated but is also a relevant initiator for EMT. There exists a complex crosstalk between HIF-1 $\alpha$  dependent and HIF-1 $\alpha$  independent pathways with a role of miRNA in hypoxia-induced cellular metabolic changes.

### ***Vascular Endothelial Growth Factor (VEGF)***

Structurally VEGF is a 34-42 kDa dimeric glycoprotein and is a diverse group consisting of VEGF-A to D along with placental growth factor.<sup>20</sup> Unlike VEGF-A and VEGF-B, which help in the growth of new blood vessels in tumour cells, VEGF-C promotes the generation of lymphatic vessels.<sup>21</sup> VEGF brings about angiogenesis by increasing the formation of collateral vessels or neovascularization, as seen in ischemia of the myocardium and retina, and also in the growth of cancer cells. It enhances the permeability of vascular

channels and also increases the proliferation of endothelial cells.<sup>22</sup> Higher expression of VEGF has been seen in pancreas<sup>23</sup>, colorectum<sup>24</sup>, breast<sup>25</sup> and gastric malignancies.<sup>26</sup> VEGF protein or mRNA may also be a good marker for the prediction of oral cancer progression and prognosis. VEGFmRNA expression was shown to be high by real-time PCR.<sup>27</sup> Another group demonstrated a positive correlation between VEGF expression in OSCC tissue and lymph node metastasis.<sup>28</sup> An Indian study has compared the serum levels of VEGF-A in OSCC (1264.08±1216.70 pg/ml) with premalignant oral lesions (462.54±344.76 pg/ml) and controls (187.91±106.75 pg/ml). It highlights that VEGF-A levels of OSCC patients were comparatively higher than normal.<sup>29</sup> The receptors for VEGFA are VEGFR1 and VEGFR2. The tyrosine kinase activity of VEGFR1 is weak compared to VEGFR2.<sup>30</sup> The signalling pathways activated by VEGFR include extracellular signal-regulated kinase/mitogen-activated protein kinase and phosphoinositide 3 kinase/AKT.<sup>31</sup> Under hypoxic stress, HIF-1 $\alpha$  drives the generation of VEGF levels,<sup>32-33</sup> thus any alteration either of the HIF-1 $\alpha$  gene or of the downstream HIF-1  $\alpha$  pathway tends to diminish VEGF less, which cascades into the suppression of neovascularisation and also tumour growth and spread.<sup>33</sup>

### Aquaporins

AQPs are part of a large family of major intrinsic proteins (MIPs).<sup>34</sup> AQPs belong to a family of membrane channel proteins. AQP1 was the first to be characterised as a membrane water channel.<sup>35</sup> Electrophysiology and site-directed mutagenesis have proven useful in defining the molecular domains involved in gating and modulation of AQP ion channel activity. AQP1 central pore has a four-fold axis of symmetry in the tetramer for cation conduction. Water fluxes occur by separate distinct pores that are located in each of the subunits of the tetramer.<sup>36</sup> Human AQP1 is a non-selective monovalent cation channel activated by intracellular cyclic GMP. AQPs have an important role in maintaining the cellular environment by facilitating the transport of water, molecules, and ions. Aquaporins are present in various tissues throughout the body, including kidneys, lung airways, eyes, brain, glands, and vascular systems. Out of 17, 13 AQPs have been identified.<sup>37</sup> Based on their permeability properties these have been grouped into two major categories. AQP 0, 1, 2, 4, 5, 6 & 8, are water selective channels but are permeable to gases,

urea, ammonia, hydrogen peroxide, and ions.<sup>38</sup> The other category includes aquaglyceroporins AQP3,7,9 and 10. They are permeable to glycerol, small solutes, and water.<sup>39</sup> A highly conserved pair of sequences of asparagine-proline-alanine (NPA) boxes, form a pore. However, this may not be present in some unorthodox AQPs. These cannot be grouped with the other two categories classical AQPs and aquaglyceroporins. Super-aquaporins (AQP 11 & 12), distinguished for their subcellular localization and atypical permeability properties, are less homologous with other aquaporins.<sup>40</sup> Some AQPs are related to water transport such as fluid secretion, fluid absorption, and cell volume regulation, and the others are not directly related to water transport such as cell adhesion, cell migration, cell proliferation, and cell differentiation. The first AQP to be reported in mammals is AQP-1, which was initially observed in erythrocytes and tubules of the kidney,<sup>41</sup> and later in a multitude of locations like the endothelium of cornea, spinal cord (in the C-fibres), the choroidal plexus and in vascular endothelium.<sup>42</sup> Structurally AQP-1 is a monomer of 28 kDa protein made of 6 titled alpha-helical domain (H1-H6) linked by 5 loops (A-E), which in turn forms a tetramer across the plasma membrane. Water and solutes are carried across the water channel of AQP-1 formed by loops located in Loop B and E, while gas and ions travel across the central pore of the homotetramer.<sup>43-44</sup> Tumour cells showing irregular patterns of growth and metastasis have modified cellular metabolic pathways that depend largely on AQPs for osmotic water flux. Expression of AQP4 is seen in normal astrocytes, but little or no AQP1 and AQP9. There is now substantial evidence from different investigators that AQP1, AQP4 and AQP9 are strongly expressed in astrocytomas.<sup>45</sup> AQP expression is upregulated in epithelial ovarian tumours. AQP7 and AQP9 expression in malignant and borderline tumours is much more compared with benign tumours and normal ovarian tissue with AQP9 expression level positively correlating with tumour grade. AQP 1 and AQP4 have a role in brain tumour oedema in astrocytomas.<sup>46</sup> AQP over expression is also seen to facilitate cell migration towards chemotactic stimulus. AQPs also facilitate the rapid changes in cell shape that take place as a migrating cell squeezes through the tortuous extracellular space.<sup>47</sup> AQPs have been proposed to have an osmotic engine model, causing cell migration through confined micro-spaces without the need for actin depolymerization-polymerization or myosin II mediated contractility.<sup>47</sup> AQP1 is associated with

tumour angiogenesis. By bringing about endothelial cell migration AQP1 plays an important role in neoangiogenesis. AQP3 and AQP5 play a role in tumour cell proliferation. AQP5 might interact with the Ras pathway in colon cancer.<sup>48</sup> AQP4 in cell-cell adhesion was also proposed based on structural considerations.<sup>49</sup> AQP3 was reported to be highly expressed in lung adenocarcinoma,<sup>50</sup> colorectal carcinoma,<sup>51</sup> oesophageal and oral SCC.<sup>52</sup> AQP3 in hypoxic settings is linked to lesser invasive OSCC characteristics.<sup>51</sup> In one of the studies siRNA was used to inhibit AQP3 function implying an innovative role in the OSCC treatment.<sup>53</sup> AQP3 has a role in lung cancer via the VEGF pathway. It also has an oestrogen-receptive component with a role in breast carcinoma, AQP5 play a significant role in carcinogenesis and tumour progression. AQP5 is connected with breast cancer cell proliferation and cell migration.<sup>54</sup> A positive correlation between AQP5 and Ki-67 expression levels and the involvement of lymph nodes in cervical cancer, was observed. AQP5 suppression was also found to impede cell proliferation in a tongue SCC cell line. Prospective therapeutics would involve specific monoclonal antibodies against AQP, gene therapy, and specific AQP inhibitors. The role of aquaglyceoporins in glycerol trafficking in cell growth as well as oncogenesis also makes them potential targets for therapeutics.

## CONCLUSION

Overall, a multi-dimensional approach is required to comprehend the complex interaction between HIF, VEGF and AQP and understand the mechanism of angiogenesis in OSCC, under a hypoxic milieu, and also to assess interactions with other pathways of tumour biology, like cell adhesion, cellular proliferation, and cellular migration. A comprehensive understanding of these would accelerate the identification of biomarkers for the early detection of OSCC, which would not only make cancer diagnosis more affordable and accessible to the masses of the country but also form the foundation for developing therapeutic targets.

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