Hypoxia and its Master Regulator HIF-1α Regulate the Expression of Calprotectin in Carcinoma Cells

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SHOROUK ELNAGDY

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Adviser: Dr. Mark C. Herzberg
Co-Adviser: Dr. Flavia Lakschevitz

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common cancer in the head and neck region. The severity of OSCC has been correlated inversely with the levels of a protein complex called calprotectin in carcinoma cells. Intracellular expression of calprotectin in carcinoma cells has anti-tumor properties such as apoptosis, arrest of cell cycle at G2/M phase, and reduction of size of tumor *in vivo*, thus contributing to better tumor outcomes. However, in OSCC, there is significant downregulation of calprotectin protein and mRNA levels leading to unfavorable tumor prognosis. Solid tumors such as human OSCC are characterized by abnormal cell growth leading to reduced oxygen availability in the tissue. This creates a state of pathologic hypoxia that has been shown to modify gene transcription in tumor cells that favors the growth and survival of cells. The transcriptional change is regulated by hypoxia inducible factors (HIFs) that selectively upregulate transcription of genes that are essential for the growth and survival of tumor cells. Available evidence suggests an association between hypoxia, its master regulator HIF-1α and calprotectin in several diseases such as inflammatory bowel disease and prostate cancer. The aim of this *in vitro* study was to determine the effect of hypoxia on the fate of calprotectin in squamous carcinoma cell lines. In addition, we wanted to determine whether there was an association between calprotectin and HIF-1α mRNA and/or protein levels in carcinoma cells. Squamous carcinoma cell lines (TR146 cells and KB HeLa-like cells) were treated with cobalt chloride to induce hypoxia. Treated cells were compared to control cells under normoxia. Our results suggest that intracellular calprotectin protein and mRNA expression levels appear to be independent of hypoxia and HIF-1α mRNA expression. Nevertheless, calprotectin and HIF-1α levels may be co-regulated indirectly through RAGE signaling pathway when calprotectin is released extracellularly and acts as ligand for RAGE receptor activation. In conclusion, the human OSCC microenvironment is a complex network in which many regulatory pathways coexist making it a challenge to reproduce complex conditions in *in vitro* experiments. Under the experimental conditions used, calprotectin expression on the protein and transcriptional levels is independent of hypoxia-induced pathways. Calprotectin and HIF-

1α mRNA may be co-regulated, however, through intracellular and extracellular signaling pathways.

Key words: HNSCC, hypoxia, HIF-1α, inflammation, S100A8/A9

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BCA……………………………………….Bicinchoninic acid

CA………………………………………...Carbonic anhydrase

DMEM……………………………………Dulbecco's Modified Eagle Medium

ECM………………………………………Extracellular matrix

EPOR……………………………………..Erythropoietin receptor

FBS……………………………………….Fetal bovine serum

GLUT…………………………………….Glucose transporter

HIFs……………………………………...Hypoxia-inducible factors

HNSCC…………………………………Head and Neck squamous cell carcinoma

HPRT……………………………………Hypoxanthine guanine phosphoribosyl transferase

HRE……………………………………..Hypoxia response elements

IBD………………………………………Inflammatory bowel disease

MAPK……………………………………Mitogen-activated protein kinase

MEM……………………………………. Minimal Essential Medium

MMP-2…………………………………...Matrix metalloproteinase 2

mRNA……………………………………Messenger RNA

NF-κB……………………………………Nuclear factor kappa B

ODDD……………………………………Oxygen-dependent degradation domain

OSCC…………………………………….Oral squamous cell carcinoma

PBS……………………………………….Phosphate-buffered saline

pVHL……………………………………..Von Hippel-Lindau protein

qPCR……………………………………...Quantitative polymerase chain reaction

RAGE……………………………………..Receptor for advanced glycation end products

TGF-α…………………………………….Transforming growth factor-α

TLR-4……………………………………..Toll-like receptor-4

TME………………………………………Tumor microenvironment

Introduction

To date, there is still no definitive cure for cancer; treatment is difficult and affected patients have reduced life expectancy **(Bray et al, 2021).** Cancer biologists are changing the outlook on cancer from simply a cancer cell-centric disease to a more complex disease that is made up of a network of stromal cells comprised of fibroblasts, vascular cells and inflammatory immune cells collectively forming the tumor microenvironment (TME) **(Nie et al, 2016; Huang et al, 2015).** Within the TME, oxygen availability is decreased, and pathologic hypoxia is a common feature of solid tumors **(Zander et al, 1985).** The adaptation of cancer cells to a hypoxic environment is mainly achieved by the upregulation of hypoxia-inducible protein factors (HIFs) **(Petrova et al, 2018)**. HIFs are major transcriptional regulators of genes in tumor cells that enhance survival and growth under hypoxia **(Jun et al, 2018).**

In HNSCC, an important inflammatory protein complex called calprotectin (S100A8/A9) has been shown to be significantly downregulated in squamous carcinoma cells compared to normal squamous epithelial cells **(Funk et al, 2015; Khammanivong et al, 2016; Kong et 2004)**. Intracellular expression of calprotectin in squamous carcinoma cells reflects anti-tumor properties such as decreases in cell growth and tumor size *in vivo*, induction of apoptotic signaling, and arrest of the cell cycle at G2/M phase **(Argyris et al, 2018).** Therefore, the loss of the protective role of calprotectin in HNSCC has been associated with poor tumor prognosis **(Argyris et al, 2018)**.

Calprotectin and hypoxia master regulator HIF-1α levels are associated in inflammatory bowel diseases (IBD) **(Vavricka et al, 2016)** and prostate cancer **(Gebhardt et al, 2012).** HIF-1α protein binds to the hypoxia response elements (HRE) present on the promoter region of the calprotectin genes (S100A8 and S100A9) **(Gebhardt et al, 2012)**. Given the evidence that calprotectin can be regulated by hypoxia-associated pathways, the aim of this *in vitro* study was to determine whether hypoxia and its master regulator HIF-1α can affect the fate of calprotectin in oral squamous carcinoma cell lines. Additionally, we aimed to determine whether there was an association between calprotectin and HIF-1α in squamous carcinoma cell lines.

Tumor Hypoxia

One of the characteristic features of cancer is the uncontrollable proliferation of cancer cells **(Fares et al, 2020).** The oxygen needs of proliferating cells can exceed angiogenic capacity within the tumor tissue, creating an environment deficient in oxygen and a state of pathologic hypoxia **(Zander et al, 1985).** Hypoxia upregulates transcriptional proteins named hypoxia-inducible factors (HIFs). HIF proteins are heterodimeric transcription factors made of two subunits: an oxygen-sensitive alpha subunit (HIF-1α), and oxygen-independent beta subunit (HIF-1β) **(Greer et al, 2012).**

In HNSCC, endogenous specific protein markers are upregulated in response to hypoxia in tumor tissue. Hypoxia markers include carbonic anhydrase (CA), glucose transporter (GLUT), HIF-1α, and the erythropoietin receptor (EPOR) and can be used as indicators of disease severity **(Kunder et al, 2021).** Of those markers, HIF-1α and EPOR have been significantly correlated with poor disease-specific and disease-free survival rates in HNSCC **(Unwith et al, 2013; Uehara et al, 2009; Winter et al, 2006).**

Increased levels of HIF-1α have been evident in various human tumors such as oral squamous cell carcinoma, breast, lung and kidney cancers **(Talks et al, 2000; Zhong et al, 1999)**. High levels of HIF-1α protein in solid tumors has been associated with aggressive tumor behavior due to the upregulation of metastasis-promoting proteins such as vimentin, fibronectin, keratins 14, 18, 19, matrix metalloproteinase 2 (MMP-2), and TGF-α **(Semenza et al, 2003)**, and the decrease in tumor suppressor proteins such as E-cadherin **(Esteban et al, 2006).** Therefore, hypoxic tumors become more aggressive than better-oxygenated ones, and negatively impact patient outcomes in human cancers.

Fate of HIF-1α

The oxygen-sensitivity of HIF-1α is attributed to the presence of an oxygen-dependent degradation domain (ODDD) in the protein **(Chan et al, 2005).** Within the ODDD, there are proline residues **(Ivan et al, 2001)** and lysine residues **(Geng et al, 2012)** that are often targets of enzymatic degradation by HIF-1 prolyl hydroxylase (**Bruick et al, 2001)** and lysyl acetyl transferase (**Jeong et al, 2002),** enzymes that are activated under aerobic conditions. Consequently, modified by enzymatic activity when subjected to normoxic conditions, HIF-1α protein is recognized by von Hippel-Lindau protein (pVHL), a tumor suppressor protein that is a member of the E3 ubiquitin protein ligase family (**Maxwell et al, 1999; Kamura et al, 2000),** which ultimately leads to the proteasomal degradation and ubiquitination of HIF-1α **(Jaakkola et al, 2001; Masson et al, 2001)**. In solid tumors, therefore, hypoxia favors the stabilization and accumulation of HIF-1α in the cytoplasm of tumor cells. With the help of nuclear localization signals **(Depping et al, 2008)**, stable HIF-1α under hypoxia migrates into the cell nucleus where it binds with its coactivator, the beta subunit, to form a heterodimeric complex (HIF-αβ) **(Chan et al, 1999)**. This complex binds to hypoxia response elements (HRE) in the promoter region of HIF target genes **(Wenger et al, 2005),** promoting transcriptional upregulation of selected genes that enhance the adaptation and survival of tumor (e.g., angiogenesis, metastasis, invasion, and energy metabolism) **(Majmundar et al, 2010; Kallio et al, 1998)**. Among the first genes found to be upregulated by HIF-αβ complex is the erythropoietin (EPO) gene that is responsible for the formation of new red blood cells to sustain adequate oxygen supply for tissue survival **(Semenza et al, 1992).**

Calprotectin (S100A8/ S100A9)

Calprotectin is an antimicrobial protein complex made of two protein subunits S100A8 and S100A9 that belong to a family of low-molecular weight S100 proteins **(Srikrishna et al, 2012).** Calprotectin is released primarily by phagocytes in response to inflammation and plays a major role in innate immunity when released into the extracellular environment **(Roth et al, 2003, Cheng et al, 2008; Sinha et al, 2008; Goyette et al, 2011).** When released into the extracellular space, calprotectin interacts with cell-surface receptors such as TLR4 **(Xia et al, 2018)** and RAGE **(Wang et al, 2018).** Engagement of these receptors activate the NF-kB pathway, inducing the production of pro-inflammatory cytokines and chemokines that attract neutrophils, macrophages, and monocytes to the site of inflammation **(Gebhardt et al, 2008).** Therefore, calprotectin is used an inflammatory biomarker and used to determine the severity and treatment outcomes, for example, of inflammatory bowel diseases (IBD) **(Vavricka et al, 2016)**. In addition to its pro-inflammatory role when released extracellularly, calprotectin exhibits anti-microbial activity upon release from neutrophils and epithelial cells at the site of infection **(Zygiel et al, 2019)**. The mechanism of antimicrobial function involves sequestration of metal ions (Zn2+, Mn2+, etc.) from the extracellular space thereby reducing essential metal nutrients (i.e., nutritional immunity) and inhibiting the growth of species such as *Staphylococcus aureus* **(Corbin et al, 2008).** Intracellular calprotectin also has an antimicrobial function. Intracellular calprotectin contributes to the effectiveness of the epithelial barrier thereby preventing the invasion of oral epithelial cells by invasive oral pathogens such as *Porphyromonas gingivalis* that is associated with periodontal disease **(Nisapakultorn et al, 2001).** Therefore, depending on the cellular context, calprotectin has pro-inflammatory and antimicrobial functions making it important in innate immunity.

Calprotectin in tumors

In the context of tumors, calprotectin plays a unique role in the behavior and fate of tumors depending on its cellular localization **(Argyris et al, 2018).** Intracellular expression of calprotectin in oral squamous cell carcinoma (OSCC) is associated with important anti-tumor functions such as downregulation of MMP-2, deceleration of cancer proliferation by controlling the cell cycle at G2/M phase, and induction of apoptosis by carcinoma cells **(Khammanivong et al, 2013; 2016; Silva et al, 2014).**
In contrast, when calprotectin is released from tumor cells to the extracellular

environment, it interacts with TLR4 and RAGE and promotes inflammation-associated carcinogenesis as seen in prostate cancer cell lines (**Gebhardt et al, 2012).** In addition to the cellular localization of calprotectin, another factor that determines the fate of tumors is the expression levels of calprotectin in cancer cells compared to normal epithelial cells **(Argyris et al, 2018).** Normal cells of the pancreas and breast do not normally express calprotectin, but in neoplasms originating from these tissues, collectively known as adenocarcinomas, calprotectin expression is increased. In contrast, normal oral, esophageal, cervical, and pharyngeal squamous cells express intracytoplasmic calprotectin in health, which is significantly suppressed in oral, oropharyngeal and cervical squamous cell carcinomas **(Argyris et al, 2018).** When compared to normal squamous epithelial cells, the downregulation of intracellular calprotectin in oral carcinoma cell lines appears to reduce the protective role against OSCC **(Argyris et al, 2018).** The restoration of the intracellular levels of calprotectin in squamous carcinoma cells could improve the prognosis of HNSCC. The rationale for restoration of calprotectin levels to suppress HNSCC is based on our understanding of the underlying regulatory pathways associated with the loss of calprotectin expression in squamous carcinoma cells. Correlation between calprotectin and hypoxia has been shown in several diseases such as IBD **(Vavricka et al, 2016)**, cerebral ischemic disease **(Ziegler et al, 2009)** and prostate cancer **(Gebhardt et al, 2012)**. A feature of solid tumors such as OSCC, hypoxia may influence the level of calprotectin in squamous carcinoma cell lines and the possible correlation between calprotectin and hypoxia master regulator HIF-1α.

Aims

The aims of this *in vitro* study were to: (1) determine the expression of HIF-1α as a function of hypoxia; (2) determine the expression of HIF-1α under hypoxia in the presence and absence of calprotectin; and (3) determine the expression of calprotectin as a function of hypoxia.

Materials and Methods

Cell culture

Four different cell lines were used to achieve the goals of this in vitro study. Wild type KB cells are HeLa-like tumor cell line of extra-oral origin **(Ogura et al, 1993).** These cells lack expression of S100A8/A9 genes **(Zou et al, 2013)**. $KB^{A8/A9+}$ cells are KB cells that were transfected with S100A8 and S100A9 genes to achieve S100A8 and S100A9 protein expression **(Nisapakultorn et al, 2001).** Wild type TR146 cells are a well-differentiated human oral squamous cell carcinoma (OSCC) cell line that expresses calprotectin and HIF-1α genes **(Rupniak et al, 1985)**. $TR146^{A8/A9-}$ cells are OSCC cells with knocked-down (silenced) S100A8/A9 genes **(Sorenson et al, 2012)**.

Wild type KB cells were maintained in Minimal Essential Medium (MEM) (Thermo Scientific™) supplemented with 10% FBS (Thermo Scientific™) and $KB^{S100A8/A9+}$ cells were maintained in MEM supplemented with 10% FBS and the antibiotic, Geneticin (Gibco™) (350μg/mL). Wild type TR146 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Scientific™) supplemented with 10% FBS and $TR146^{S100A8/A9-}$ cells were maintained in DMEM supplemented with 10% FBS and antibiotic Geneticin (350μg/mL)**.** Control cells were cultured under normoxic conditions consisting of 5% CO2 and 95% air in a humidified atmosphere at 37°C. For hypoxic conditions, cells were treated with 150μmoL cobalt chloride for 8 hours in a humidified incubator at 37°C at an atmospheric pressure of 5% (v/v) CO2/air **(Harrison et al, 2018).**

Cell harvesting in hypoxic conditions

Cells were grown to a confluency rate of 60-70%. Cultured cells were rinsed with PBS to remove any dead or detached cells followed by a 5-minute treatment with trypsin in an incubator at 37°C. After trypsin-treatment, the remaining adherent cells detached from the surface of the flasks and counted using a hemocytometer using (1:1 cells and dye). The cell counts were adjusted, and 200,000 cells were added per well to each well of the 6-well plate. Using 6-well plates, 3 biological replicates of each cell type were treated under normoxia and 3 biological replicates treated under hypoxia using cobalt chloride.

After incubation at 37°C for 8 hours with cobalt chloride, cells were harvested and lysed in an anaerobic chamber to maintain hypoxic conditions at all times.

Cell lysis

To collect the cell lysates from samples, cells were rinsed with cold phosphate-buffered saline (PBS) on ice, scraped, and lysed using a lysis buffer that is maintained on ice. The lysis buffer contained Halt™ Protease Inhibitor (100X) (Thermo Scientific™) to prevent the proteolytic degradation during cell lysis and protein extraction, Dimethyloxalylglycine (DMOG 20X, a HIF-hydroxylase inhibitor) (Santa Cruz) and RIPA lysis buffer (1X) (Thermo Scientific™) that enables the extraction of membrane, nuclear and cytoplasmic proteins from cultured cells. Pierce® BCA Protein Assay Kit was used for accurate determination of protein concentration of the purified samples.

Western blotting

Samples were then mixed with 4X Laemmli sample buffer (Bio-Rad) that has been supplemented with 1.4mM of mercaptoethanol before mixing with the samples. 4X Laemmli sample buffer was diluted to 1X by mixing 4μL of sample buffer with 12μL of protein samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and heated at 95°C for 10 minutes. The sample buffer functions as a color indicator to monitor the migration of proteins during gel electrophoresis. The protein samples were loaded on SDS polyacrylamide gels and electrophoresed to separate the protein bands based on their molecular weights. After electrophoresis was completed, protein bands were transferred from the gel onto a nitrocellulose membrane for antibody staining and detection. To do so, nitrocellulose membranes, filter papers and the electrophoresis gels were soaked in 1X Transfer Buffer (25mM Tris base, 192mM glycine, 10% methanol) for 10 minutes prior to assembly of the transfer "sandwich". Assembly of the transfer sandwich included layering filter papers as the base, followed in order by the electrophoresis gel, the nitrocellulose membrane, and another layer of filter papers on the top. Any air bubbles between the gel and membrane were removed using a roller as bubbles may inhibit the transfer of proteins to the membrane. The transfer sandwich was then placed into transfer cassette. Transfer was performed by passing a current across the gel to the membrane (120V) for 1 hour. Immediately after membrane transfer, total protein staining (Revert™ 520 Total Protein Stain for Western Blot Normalization) was performed to ensure the success of protein transfer from gel to membrane and allow subsequent normalization of the target proteins with the total protein per lane. The membranes were then dried and kept in a light-sensitive container overnight. Blocking buffer (Licor) supplemented with PBS (1:1) was added to the membranes for 1 hour at room temperature to enhance the accuracy of the target protein detection by eliminating any non-specific protein binding by the primary antibodies. After blocking, the membranes were incubated overnight at 4°C with mouse monoclonal primary IgG antibodies against HIF-1α and calgranulin B (Santa Cruz). Following incubation, the membranes were rinsed with wash buffer solution to remove any unbound primary antibodies, and blots were resolved using IRDye® 680RD Goat anti-Mouse IgG secondary antibody (LI-COR). Protein bands were quantified using densitometry and imageJ software.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from all cell lines using the RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA with the ProtoScript® II First Strand cDNA Synthesis Kit. qPCR was performed using PerfeCTa® qPCR FastMix® (QuantaBio) and a Chromo4 Real-Time Detector (Bio-Rad, Munchen, Germany). Relative mRNA expression was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) **(Gebhardt et al, 2012).** All reactions were performed in triplicate with 2 technical replicates for each of the 3 biological replicates. Normalization of mRNA levels between samples for accurate comparison of mRNA transcription was achieved using a stable housekeeping gene. Under the experimental conditions used, the most stable housekeeping gene was hypoxanthine guanine phosphoribosyl transferase (HPRT) and was chosen for normalization. Sequence of primers used was as follows: S100A9 forward primer: 5'-AAA AGG TCA TAG AA C ACA TCA TGG-3'; S100A9 reverse primer: 5'-GAA GCT CAG CTG CTT GTC CTG-3'; HIF-1α forward primer: 5'-TTC ACC TGA GCC TAA

TAG TCC-3'; HIF-1α reverse primer: 5'-CAA GTC TAA ATC TGT GTC CTG-3'; HPRT forward primer: 5'-GAA GAG CTA TTG TAA TGA CCT-3'; HPRT reverse primer: 5'-GCG ACC TTG ACC ATC TTT-3'.

Statistical analysis

The results are reported as a mean of three independent experiments. Variation between groups were statistically evaluated using the two-tailed homoscedastic Student's *t*-test. Statistical significance was set at a p-value < 0.05.

Results

HIF-1α mRNA expression under hypoxia

This experiment determined the levels of HIF-1α under hypoxic conditions in the model cell lines representing oral squamous cell carcinoma. Upon treating the cells with $CoCl_2$ for 8 hours to induce hypoxia, transcription of HIF-1α tended to be greater in hypoxic than normoxic cells (Fig. 1). Differences between the two conditions reached statistical significance in KB wild type cells, $KB^{A8/A9+}$ cells and TR146 A8/A9-knocked down cell lines but not in TR146 wild type cells. Although the results appeared to trend towards greater HIF-1α in the cells without than with calprotectin, the data suggest that HIF-1α expression under hypoxia is independent of calprotectin.

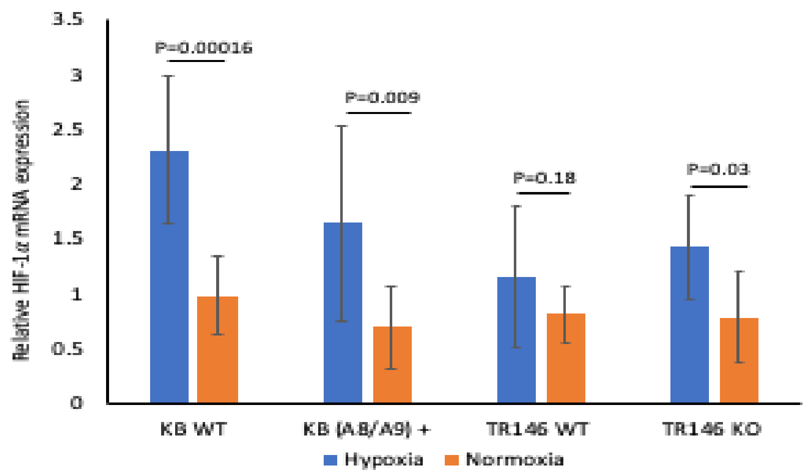

Figure 1. Hypoxia induces HIF-1a mRNA expression. HIF-1α mRNA expression in KB$^{(A8/A9)+}$, TR146 wild type, TR146$^{(A8/A9)-}$ and KB wild type cells after 8 hr hypoxia treatment. Expression levels were normalized on mRNA expression of housekeeping gene HPRT and related to normoxia incubated control. Data represent mean +/- SD of three independent experiments; $p < 0.05$ (Student's t-test, compared to respective treated control).

S100A9 protein and mRNA expression under hypoxia versus normoxia in carcinoma cells

To determine whether hypoxia affected the expression of S100A9, cells were incubated under normoxic and hypoxic conditions for 8 hours. Western blot analysis was performed on cells that naturally express calprotectin (TR146 wild type), and cells that were engineered to express calprotectin (KB$^{A8/A9+}$ cells). The results from one representative Western Blot show that there is likely a higher expression of S100A9 protein in TR146 cells compared to KB$^{A8/A9+}$ cells under hypoxia (Fig. 2a). However, when replicates of these experiments were done and quantitative analysis was performed, it appeared that the S100A9 protein expression is independent of hypoxia in both cell lines (Fig. 2b). Similar to S100A9 protein levels, the transcription of S100A9 mRNA appears to be independent of hypoxia (Fig. 2c). Therefore, under the experimental conditions using cobalt chloride to induce hypoxia in squamous carcinoma cell lines, the expression of S100A9 protein and mRNA appear to be regulated by hypoxia-independent pathways.

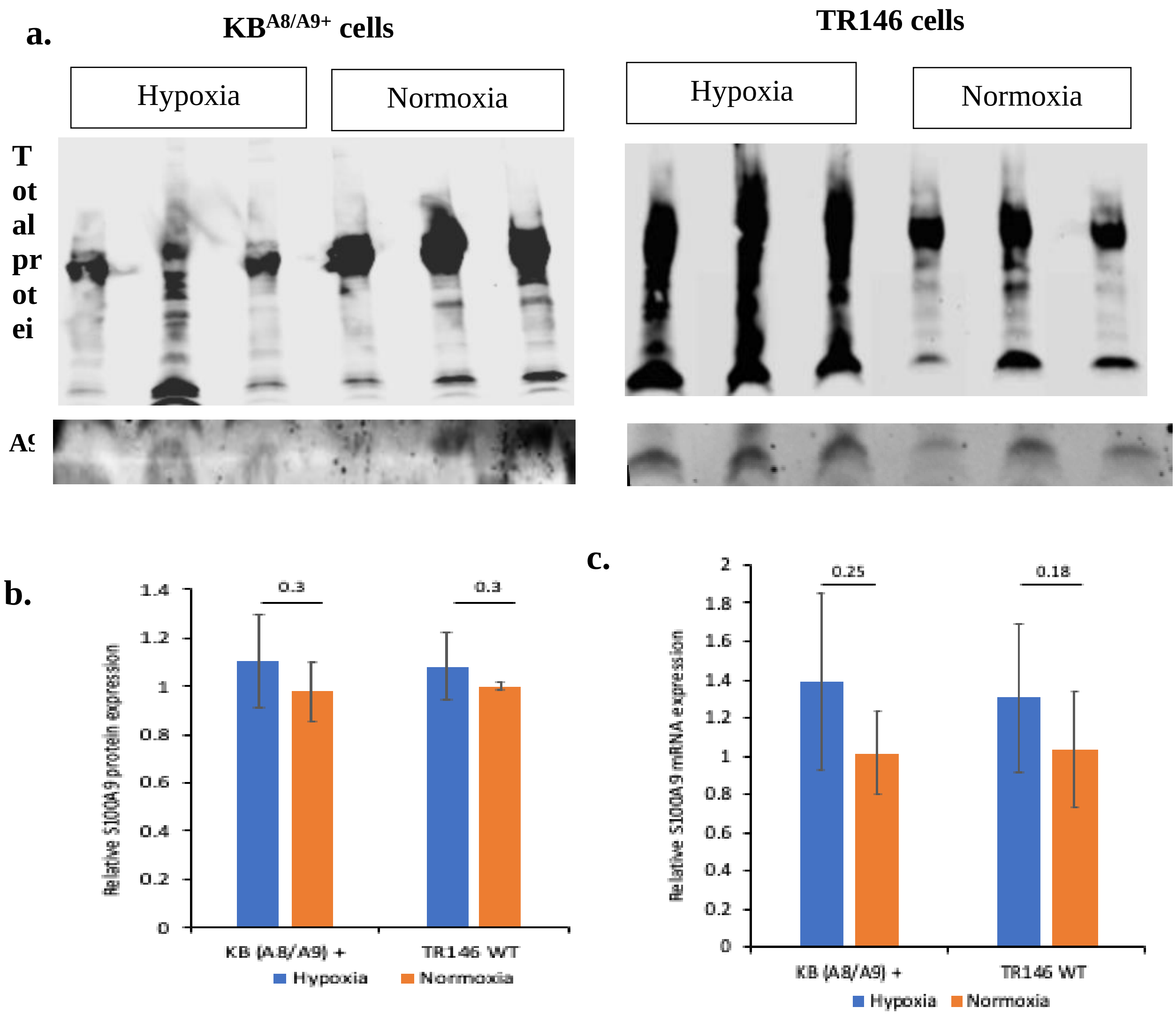

Figure 2. S100A9 protein and mRNA expression under hypoxia. (a) Western blot analysis of S100A9 protein levels in KB$^{(A8/A9)+}$ and TR146 wild type cells after 8 hr of hypoxic and normoxic treatment. Total protein staining was used for normalization. (b) Quantitative analysis of S100A9 protein level after 8 hr of hypoxia. Results represent mean +/- SD of three independent experiments; $p < 0.05$ (Student's t-test, compared to respective normoxia treated control). (c) S100A9 mRNA expression in KB$^{(A8/A9)+}$ and TR146 wild type cells after 8 hr hypoxia incubation. Expression levels were normalized on mRNA expression of housekeeping gene HPRT and related to normoxia incubated control. Data represent men +/- SD of three independent experiments; $p <0.05$ (Student t-test, compared to respective normoxia treated control).

Discussion

Decreased oxygen concentration (hypoxia) is a major stressor that challenges the survival of tissues. Hypoxia can be a feature of bacterial infections, wounds, cardiovascular disease, and cancer **(Lee et al, 2019)**. “Physiological hypoxia” refers to the transient drop in oxygen levels that is usually restored to normal levels by vasodilation, increasing blood flow to the tissues **(Michiels 2004)**. Different tissues have different oxygen sensitivity levels, and some can function normally under physiologic hypoxia, e.g., intestinal mucosa, renal medulla, placenta, bone marrow etc. **(Taylor et al, 2017).** This physiological hypoxia generally falls in the range of 2-6% oxygen, since the normal oxygen level of tissues generally is 3-7% **(McKeown et al, 2014)**. Another feature of physiological hypoxia is the temporary stabilization of hypoxia inducible factors (HIFs), that are responsible for the transcription of genes needed for adaptation and survival (**Lee et al, 2020; Jun et al, 2018).** Maximal expression of HIF-1α was found at 0.5% oxygen levels which was reduced by half at 1.5 - 2% oxygen levels, and was significantly low at oxygen levels above 4%, confirming that HIF-1α is sensitive to high oxygen levels **(Jiang et al, 1996; Lee et al, 2020).**

Cancer is no longer viewed as only a bulk of malignant cancer cells. The tumor microenvironment (TME) is composed of various cells including fibroblasts, immune cells and cancer cells that interact with one another with the help of secreted cytokines and chemokines within the extracellular matrix (ECM) **(Nie et al, 2016; Huang et al, 2015).** Increased extracellular level of calprotectin in TME has been correlated with inflammation-associated carcinogenesis in prostate **(Hermani et al, 2006),** bladder **(Sahin et al, 2019)**, and pancreatic **(Grebhardt et al, 2012)** cancers through its extracellular role in activating TLR4 and RAGE signaling pathways in cancer cells. TLR4 and RAGE signal mitogen-activated protein kinase (MAPK) to upregulate the NF-κB pathway and downstream proinflammatory gene expression **(Hermani et al, 2006; Hiratsuka et al, 2008; Gebhardt et al, 2006)**. In contrast, the intracellular expression of calprotectin in oral squamous cell carcinoma (OSCC) is associated with important anti-tumor functions such as downregulation of MMP-2, deceleration of cancer proliferation by controlling the cell cycle at G2/M phase and inducing apoptosis of carcinoma cells **(Khammanivong et al, 2013; Khammanivong et al 2016; Silva et al, 2014).** Therefore, the dual life of calprotectin in the intracellular and extracellular environment could determine the fate of a tumor by either promoting apoptosis of tumor cells or supporting tumor development **(Ghavami et al, 2009).**

Calprotectin and tumor hypoxia both have important roles in determining the severity and prognosis of tumors, and there is evidence of an association between hypoxia and calprotectin in chronic inflammatory diseases such as inflammatory bowel disease and prostate cancer **(Grebhardt et al, 2012)**. However, there is lack of evidence on the association between hypoxia and calprotectin in oral squamous carcinoma cells. For this reason, we were interested in investigating the role of hypoxia in determining the fate of calprotectin in squamous carcinoma cell lines and whether HIF-1α response to hypoxia is affected by calprotectin.

In this study we used four human squamous carcinoma cells. The human HNSCC cell line TR146 naturally expresses calprotectin and HIF-1α genes **(Rupniak et al, 1985).** Identical in all other ways, the S100A8/A9 knocked down TR146 cells ($TR146^{A8/A9-}$) have suppressed calprotectin which is achieved by treating the cells with short hairpin RNA (shRNA) that specifically silences target S100A8 and S100A9 genes **(Sorenson et al, 2012)**. Similarly, the human carcinoma cell line (KB wild type) fails to express S100A8 and S100A9 mRNAs **(Zou et al, 2013)**. To engineer this cell line to express S100A8 and S100A9, KB cells were transfected to express S100A8/A9 ($KB^{S100A8/A9+}$) **(Nisapakultorn et al, 2001).**

Using these isogenic pairs of carcinoma lines that vary in their expression of calprotectin, hypoxia was induced using hypoxia mimetic agent cobalt chloride ($CoCl_2$) **(Sánchez et al, 2019).** $CoCl_2$ chemically stabilize HIF-1α by competitively binding to iron ions that are essential for the activity of the prolyl hydroxylase enzyme involved in HIF-1α degradation **(Nandal et al, 2011).**

Consistent with previous studies **(Zhong et al, 1999)**, HIF-1α mRNA transcription tends to be greater under hypoxia than normoxia in all cell lines in this study. Statistical significance was reached in KB wild type, $KB^{S100A8/A9+}$ and $TR146^{A8/A9-}$ but not in TR146 wild type cells. A trend was observed towards a higher expression of HIF-1α mRNA in cell lines not expressing calprotectin compared to cells expressing calprotectin. These results suggest a possible association between calprotectin and HIF-1α in the cells.

HIF-1α protein is only stabilized at O_2 concentrations below 5%, presenting a challenge to harvest and extract cell proteins in hypoxic conditions in preparation for Western Blot analysis. In the presence of oxygen, HIF-1α is rapidly degraded within 5-8 minutes in both nuclear and cytoplasmic compartments **(Moroz et al, 2009; Jewell et al, 2001).** Although we attempted to maintain anaerobic conditions, we were unable to consistently detect HIF-1α protein. Better sample preparation in an appropriately anaerobic environment may ensure Western Blot success. Another challenge with the Western Blot analysis of HIF-1α is that the size of the protein may differ depending on the form of HIF-1α that was produced at the time of analysis. Degraded HIF-1α has molecular weight of 40-80 kDa; post-translationally modified HIF-1α has molecular weight of 110-130 kDa and the heterodimer form with HIF-1β has a molecular weight of about 200 kDa **(Moroz et al, 2009).** Therefore, the variation in the location of the HIF-1α bands between experiments, possibly due to lack of reproducibility of experimental hypoxia, made it difficult to make conclusions about the HIF-1α protein levels.

Given these experimental challenges, all our cell lines were treated similarly. Our results suggested the expression of intracellular calprotectin protein and mRNA were independent of hypoxia. In carcinoma cells, calprotectin expression did not appear to be influenced by hypoxia pathways. Since calprotectin is downregulated as carcinoma progresses and becomes increasingly severe, we considered that epigenetic mechanisms may contribute.

Epigenetics have been correlated with many disease processes including cancer **(Lu et al, 2020).** The most common epigenetic process identified is DNA methylation, which has been linked to several human cancers and other disease processes such as autoimmune diseases and metabolic diseases **(Jin et al, 2018).** Epigenetic mechanisms can change expression and activity of genes without causing mutations in the DNA sequence **(Biswas et al, 2019).** In hypoxic tumors, it has been shown that increased DNA methylation is attributed to hypoxia-induced suppression of oxygen-dependent TET enzyme that regulates DNA de-methylation **(Thienpont et al, 2017).** Evidence from the Cancer Genome Atlas (TCGA) RNA-Seq data from human HNSCC samples, suggest a strong correlation between the downregulation of S100A9 and DNA methylation at the S100A9 promoter region **(Khammanivong et al, 2016).** Although our results indicate that S100A9 expression is independent of hypoxia, evidence from the literature suggest that hypoxia may play a role in downregulation of S100A9 in cancer cells through increased DNA methylation.

In addition to the intracellular antimicrobial role of calprotectin, which attenuates bacterial invasion into oral epithelial cells **(Nisapakultorn et al, 2001),** its intracellular anti-tumor roles in oral squamous cell carcinoma **(Khammanivong et al, 2013; Khammanivong et al, 2016; Silva et al, 2014)** include the cytoplasmic to nuclear translocation noted in breast epithelial cells **(Song et al, 2021).** In the nucleus, calprotectin functions as a transcriptional coactivator of target genes and contributes to the malignant transformation of the cells **(Song et al, 2021).** Although nuclear translocation of calprotectin has been shown in normal breast epithelial cells, no evidence of such translocation has been shown in squamous carcinoma cells. Nevertheless, the observation that HIF-1α transcriptional level is affected by the presence and absence of calprotectin in carcinoma cells as seen in this study, indicates that if it occurred in our cells, nuclear translocation may explain the expression pattern of HIF-1α. Hence, there is a need for future studies to determine the potential nuclear translocation of calprotectin in squamous carcinoma cell lines $KB^{A8/A9+}$ cells and TR146 wild type cells and its possible role in transcriptional regulation of HIF-1α.

The use of $CoCl_2$ may confound our study of hypoxia in cells. $CoCl_2$ may cause dose- and time-dependent cytotoxicity during hypoxia induction in *in vitro* studies as has been shown in human HaCaT keratinocytes and HSG submandibular gland cell lines after 4, 6 and 48 hours of exposure **(Gault et al, 2010; Klasson et al, 2021; Akita et al, 2007).** Cobalt chloride may be generally more toxic on cancer cell lines than non-cancerous cell lines **(Mahey et al, 2016).** Therefore, with the experimental design used in this study, we would expect some cell death upon treatment with $CoCl_2$ for 8 hours and the subsequent release of calprotectin into the extracellular space. In the extracellular environment, calprotectin acts as a cytokine that engages with RAGE receptors to initiate intracellular signaling pathways and activate NF-kB transcriptional factor that initiate the transcription of genes involved in inflammation including HIF-1α and S100A8/A9 **(Gebhardt et al, 2006)**. Therefore, a positive feedback loop could be created through the release of calprotectin from our cells and activation of RAGE **(Taneja et al, 2021).** The potential role of RAGE in the fate of calprotectin and HIF-1α in oral squamous carcinoma cell lines under hypoxia is worth investigating.

To our knowledge, the impact of RAGE on calprotectin and HIF-1α expression in OSCC cells under hypoxia has not been investigated. With the aim of determining the role of RAGE in the expression of calprotectin and HIF-1α under hypoxia in oral carcinoma cells, future studies should investigate oral carcinoma cell lines: TR146 wild type, TR146 with calprotectin knocked down and knocked down TR146 cells that are transfected with siRNA for transient silencing of RAGE expression in the cells. Like the current study conditions, the cells would be treated under hypoxia and normoxia for 8 hours. This experimental model will allow us to determine (1) whether the decreased expression of HIF-1α under hypoxia in the presence of calprotectin observed in our study is dependent on the S100A8A9/RAGE interaction or on a different pathway, and (2) whether the expression of S100A8/A9 under hypoxia is influenced by the presence or absence of RAGE. Improvements on our current *in vitro* study should include the use healthy gingival epithelial cells **(Kubo et al, 2009)** as healthy controls for OSCC cell lines. We should also study hypoxia in tumor models that include the TME. The response to hypoxia may be more evident when all of the cooperating cells, cytokines and extracellular matrix are present. This is important because in our study we used only carcinoma cell lines of oral and non-oral origins, which made it difficult to infer the results to actual tumors.

Conclusion

HNSCC is considered one of the malignancies with the worst patient-related outcomes due low responsiveness to treatment and severe drug resistance **(Filho et al, 2013).** The anti-tumor role of calprotectin may contribute to better disease survival and tumor prognosis in HNSCC. Thus, with the aim to understand the molecular pathway leading to the decrease in calprotectin expression, we were able to suggest that calprotectin protein and mRNA expression are independent of hypoxia and that calprotectin may be influencing the expression of HIF-1α through various regulatory mechanisms. Therefore, further studies will be necessary to substantiate the current results and to expand on the possible regulatory pathways such as HIF-1α/RAGE/ S100A8A9 pathway that may contribute to better understanding of calprotectin regulation in OSCC cell lines. Indeed, we may find that the TME environment is needed to recapitulate the response to hypoxia. It is important to appreciate the limitations of *in vitro* studies as they do not precisely simulate the complex tumor environment in real patients. There may be complex processes on a molecular and transcriptional levels that may not be apparent in an *in vitro* setting but could be contributing to the expression of calprotectin and HIF-1α in the squamous carcinoma cells.

Bibliography

- Akita K, Okamura H, Yoshida K, Morimoto H, Ogawa-Iyehara H, Haneji T. Cobalt chloride induces apoptosis and zinc chloride suppresses cobalt-induced apoptosis by Bcl-2 expression in human submandibular gland HSG cells. *Int J Oncol*. 2007;31(4):923-929.
- Argyris, P. P., Slama, Z. M., Ross, K. F., Khammanivong, A., & Herzberg, M. C. (2018). Calprotectin and the Initiation and Progression of Head and Neck Cancer. *Journal of dental research*, *97*(6), 674–682. https://doi.org/10.1177/0022034518756330
- Bray F, Laversanne M, Weiderpass E, Soerjomataram I. The ever-increasing importance of cancer as a leading cause of premature death worldwide. Cancer. 2021;127(16):3029-3030.
- Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*. 2001;294(5545):1337-1340.
- Chan, D. A., Sutphin, P. D., Yen, S. E., & Giaccia, A. J. (2005). Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 alpha. *Molecular and cellular biology*, *25*(15), 6415–6426.
- Chan, W. K., Yao, G., Gu, Y. Z., & Bradfield, C. A. (1999). Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. Demonstration of competition and compensation. *The Journal of biological chemistry*, *274*(17), 12115–12123.
- Cheng, P., Corzo, C. A., Luetteke, N., Yu, B., Nagaraj, S., Bui, M. M., Ortiz, M., Nacken, W., Sorg, C., Vogl, T., Roth, J., & Gabrilovich, D. I. (2008). Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *The Journal of experimental medicine*, *205*(10), 2235–2249.
- Corbin BD, Seeley EH, Raab A, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science. 2008;319(5865):962-965.
- Depping R, Steinhoff A, Schindler SG, et al. Nuclear translocation of hypoxia-inducible factors (HIFs): involvement of the classical importin alpha/beta pathway. *Biochim Biophys Acta*. 2008;1783(3):394-404.
- Esteban, M. A., Tran, M. G., Harten, S. K., Hill, P., Castellanos, M. C., Chandra, A., Raval, R., O'brien, T. S., & Maxwell, P. H. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. *Cancer research*, *66*(7), 3567–3575. https://doi.org/10.1158/0008-5472.CAN-05-2670
- Fares, J., Fares, M.Y., Khachfe, H.H. et al. Molecular principles of metastasis: a hallmark of cancer revisited. Sig Transduct Target Ther 5, 28 (2020).
- Funk, S., Mark, R., Bayo, P., Flechtenmacher, C., Grabe, N., Angel, P., Plinkert, P. K., & Hess, J. (2015). High S100A8 and S100A12 protein expression is a favorable prognostic factor for survival of oropharyngeal squamous cell carcinoma. *International journal of cancer*, *136*(9), 2037–2046.
- Gault N, Sandre C, Poncy JL, Moulin C, Lefaix JL, Bresson C. Cobalt toxicity: chemical and radiological combined effects on HaCaT keratinocyte cell line. *Toxicol In Vitro*. 2010;24(1):92-98.
- Gebhardt C, Németh J, Angel P, Hess J. S100A8 and S100A9 in inflammation and cancer. *Biochem Pharmacol*. 2006;72(11):1622-1631.
- Gebhardt C, Riehl A, Durchdewald M, et al. RAGE signaling sustains inflammation and promotes tumor development. *J Exp Med*. 2008;205(2):275-285.
- Grebhardt, S., Veltkamp, C., Ströbel, P., & Mayer, D. (2012). Hypoxia and HIF-1 increase S100A8 and S100A9 expression in prostate cancer. *International journal of cancer*, *131*(12), 2785–2794. https://doi.org/10.1002/ijc.27591
- Geng, H., Liu, Q., Xue, C., David, L. L., Beer, T. M., Thomas, G. V., Dai, M. S., & Qian, D. Z. (2012). HIF1α protein stability is increased by acetylation at lysine 709. *The Journal of biological chemistry*, *287*(42), 35496–35505.
- Ghavami S, Chitayat S, Hashemi M, et al. S100A8/A9: a Janus-faced molecule in cancer therapy and tumorgenesis. *Eur J Pharmacol*. 2009;625(1-3):73-83.

- Goyette, J., & Geczy, C. L. (2011). Inflammation-associated S100 proteins: new mechanisms that regulate function. *Amino acids*, *41*(4), 821–842.
- Greer SN, Metcalf JL, Wang Y, Ohh M. The updated biology of hypoxia-inducible factor. EMBO J. 2012;31(11):2448-2460.
- Harrison H, Pegg HJ, Thompson J, Bates C, Shore P. HIF1-alpha expressing cells induce a hypoxic-like response in neighbouring cancer cells. *BMC Cancer*. 2018;18(1):674.
- Hermani A, De Servi B, Medunjanin S, Tessier PA, Mayer D. S100A8 and S100A9 activate MAP kinase and NF-kappaB signaling pathways and trigger translocation of RAGE in human prostate cancer cells. *Exp Cell Res*. 2006;312(2):184-197.
- Hiratsuka S, Watanabe A, Sakurai Y, et al. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol*. 2008;10(11):1349-1355.
- Huang J, Xie Y, Sun X, et al. DAMPs, ageing, and cancer: The 'DAMP Hypothesis'. *Ageing Res Rev*. 2015;24(Pt A):3-16.
- Ivan M, Kondo K, Yang H, et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science*. 2001;292(5516):464-468.
- Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*. 2001;292(5516):468-472.
- Jeong JW, Bae MK, Ahn MY, et al. Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. *Cell*. 2002;111(5):709-720.
- Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M. Induction of HIF-1alpha in response to hypoxia is instantaneous. *FASEB J*. 2001;15(7):1312-1314.
- Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol. 1996;271(4 Pt 1):C1172-C1180.
- Jin, Z., & Liu, Y. (2018). DNA methylation in human diseases. *Genes & diseases*, *5*(1), 1–8.
- Jun, J. C., Rathore, A., Younas, H., Gilkes, D., & Polotsky, V. Y. (2017). Hypoxia-Inducible Factors and Cancer. *Current sleep medicine reports*, *3*(1), 1–10.
- Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H., & Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *The EMBO journal*, *17*(22), 6573–6586.
- Kamura T, Sato S, Iwai K, Czyzyk-Krzeska M, Conaway RC, Conaway JW. Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc Natl Acad Sci U S A. 2000;97(19):10430-10435.
- Khammanivong A, Sorenson BS, Ross KF, et al. Involvement of calprotectin (S100A8/A9) in molecular pathways associated with HNSCC. *Oncotarget*. 2016;7(12):14029-14047.
- Khammanivong, A., Wang, C., Sorenson, B. S., Ross, K. F., & Herzberg, M. C. (2013). S100A8/A9 (calprotectin) negatively regulates G2/M cell cycle progression and growth of squamous cell carcinoma. *PloS one*, *8*(7), e69395.
- Klasson, M., Lindberg, M., Särndahl, E., Westberg, H., Bryngelsson, I. L., Tuerxun, K., & Persson, A. (2021). Dose- and time-dependent changes in viability and IL-6, CXCL8 and CCL2 production by HaCaT-cells exposed to cobalt. Effects of high and low calcium growth conditions. *PloS one*, *16*(6), e0252159.
- Kong JP, Ding F, Zhou CN, et al. Loss of myeloid-related proteins 8 and myeloid-related proteins 14 expression in human esophageal squamous cell carcinoma correlates with poor differentiation. *World J Gastroenterol*. 2004;10(8):1093-1097.
- Koukourakis, M. I., Giatromanolaki, A., Sivridis, E., Gatter, K. C., & Harris, A. L. (2013). High DLL4 expression in tumour-associated vessels predicts for favorable radiotherapy outcome in locally advanced squamous cell head-neck cancer (HNSCC). *Angiogenesis*, *16*(2), 343–351. https://doi.org/10.1007/s10456-012-9318-5

- Kubo C, Tsutsui TW, Tamura Y, Kumakura S, Tsutsui T. Immortalization of normal human gingival keratinocytes and cytological and cytogenetic characterization of the cells. Odontology. 2009;97(1):18-31.
- Kunder, S., Chatterjee, A., Manna, S., Mahimkar, M., Patil, A., Rangarajan, V., Budrukkar, A., Ghosh-Laskar, S., Agarwal, J. P., & Gupta, T. (2021). Correlation between imaging and tissue biomarkers of hypoxia in squamous cell cancer of the head and neck. *World journal of nuclear medicine*, *20*(3), 228–236.
- Lee P, Chandel NS, Simon MC. Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. Nat Rev Mol Cell Biol. 2020;21(5):268-283.
- Lee, J.W., Ko, J., Ju, C. et al. Hypoxia signaling in human diseases and therapeutic targets. Exp Mol Med 51, 1–13 (2019).
- Lu Y, Chan YT, Tan HY, Li S, Wang N, Feng Y. Epigenetic regulation in human cancer: the potential role of epi-drug in cancer therapy. Mol Cancer. 2020;19(1):79. Published 2020 Apr 27.
- Mahey S, Kumar R, Arora R, et al. Effect of cobalt(II) chloride hexahydrate on some human cancer cell lines. *Springerplus*. 2016;5(1):930. Published 2016 Jun 30.
- Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell. 2010;40(2):294-309.
- Masson N, Willam C, Maxwell PH, Pugh CW, Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J*. 2001;20(18):5197-5206.
- Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature*. 1999;399(6733):271-275.
- McKeown S. R. (2014). Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. The British journal of radiology, 87(1035), 20130676.
- Melo Filho MR, Rocha BA, Pires MB, et al. Quality of life of patients with head and neck cancer. *Braz J Otorhinolaryngol*. 2013;79(1):82-88.
- Michiels C. (2004). Physiological and pathological responses to hypoxia. *The American journal of pathology*, *164*(6), 1875–1882.
- Moroz E, Carlin S, Dyomina K, et al. Real-time imaging of HIF-1alpha stabilization and degradation. PLoS One. 2009;4(4):e5077.
- Muñoz-Sánchez J, Chánez-Cárdenas ME. The use of cobalt chloride as a chemical hypoxia model. *J Appl Toxicol*. 2019;39(4):556-570.
- Nandal, A., Ruiz, J. C., Subramanian, P., Ghimire-Rijal, S., Sinnamon, R. A., Stemmler, T. L., Bruick, R. K., & Philpott, C. C. (2011). Activation of the HIF prolyl hydroxylase by the iron chaperones PCBP1 and PCBP2. *Cell metabolism*, *14*(5), 647–657.
- Nie Y, Yang D, Oppenheim JJ. Alarmins and Antitumor Immunity. *Clin Ther*. 2016;38(5):1042-1053.
- Nisapakultorn K, Ross KF, Herzberg MC. Calprotectin expression in vitro by oral epithelial cells confers resistance to infection by Porphyromonas gingivalis. Infect Immun. 2001;69(7):4242-4247.
- Ogura H, Yoshinouchi M, Kudo T, Imura M, Fujiwara T, Yabe Y. Human papillomavirus type 18 DNA in so-called HEP-2, KB and FL cells--further evidence that these cells are HeLa cell derivatives. Cell Mol Biol (Noisy-le-grand). 1993;39(5):463-467.
- Petrova, V., Annicchiarico-Petruzzelli, M., Melino, G. et al. The hypoxic tumour microenvironment. Oncogenesis 7, 10 (2018).
- Roth J, Vogl T, Sorg C, Sunderkötter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. *Trends Immunol*. 2003;24(4):155-158.
- Rupniak HT, Rowlatt C, Lane EB, et al. Characteristics of four new human cell lines derived from squamous cell carcinomas of the head and neck. *J Natl Cancer Inst*. 1985;75(4):621-635.
- Semenza G. L. (2003). Targeting HIF-1 for cancer therapy. *Nature reviews. Cancer*, *3*(10), 721–732. https://doi.org/10.1038/nrc1187

- Semenza, G. L., & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molecular and cellular biology*, *12*(12), 5447–5454.
- Silva, E. J., Argyris, P. P., Zou, X., Ross, K. F., & Herzberg, M. C. (2014). S100A8/A9 regulates MMP-2 expression and invasion and migration by carcinoma cells. The international journal of biochemistry & cell biology, 55, 279–287. https://doi.org/10.1016/j.biocel.2014.09.007
- Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. *J Immunol*. 2008;181(7):4666-4675.
- Song, R., & Struhl, K. (2021). S100A8/S100A9 cytokine acts as a transcriptional coactivator during breast cellular transformation. *Science advances*, *7*(1), eabe5357.
- Sorenson BS, Khammanivong A, Guenther BD, Ross KF, Herzberg MC. IL-1 receptor regulates S100A8/A9-dependent keratinocyte resistance to bacterial invasion. *Mucosal Immunol*. 2012;5(1):66-75.
- Srikrishna G. (2012). S100A8 and S100A9: new insights into their roles in malignancy. Journal of innate immunity, 4(1), 31–40.
- Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., & Harris, A. L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *The American journal of pathology*, *157*(2), 411–421.
- Taneja S, Vetter SW, Leclerc E. Hypoxia and the Receptor for Advanced Glycation End Products (RAGE) Signaling in Cancer. Int J Mol Sci. 2021;22(15):8153. Published 2021 Jul 29.
- Taylor CT, Colgan SP. Regulation of immunity and inflammation by hypoxia in immunological niches. Nat Rev Immunol. 2017;17(12):774-785.
- Thienpont, B., Steinbacher, J., Zhao, H., D'Anna, F., Kuchnio, A., Ploumakis, A., Ghesquière, B., Van Dyck, L., Boeckx, B., Schoonjans, L., Hermans, E., Amant, F., Kristensen, V. N., Peng Koh, K., Mazzone, M., Coleman, M., Carell, T., Carmeliet, P., & Lambrechts, D. (2016). Tumour hypoxia causes DNA hypermethylation by reducing TET activity. Nature, 537(7618), 63–68. https://doi.org/10.1038/nature19081
- Uehara, M., Sano, K., Ikeda, H., Nonaka, M., & Asahina, I. (2009). Hypoxia-inducible factor 1 alpha in oral squamous cell carcinoma and its relation to prognosis. *Oral oncology*, *45*(3), 241–246. https://doi.org/10.1016/j.oraloncology.2008.05.007
- Unwith, S., Zhao, H., Hennah, L., & Ma, D. (2015). The potential role of HIF on tumour progression and dissemination. *International journal of cancer*, *136*(11), 2491–2503. https://doi.org/10.1002/ijc.28889
- Vavricka, S., Ruiz, P. A., Scharl, S., Biedermann, L., Scharl, M., de Vallière, C., Lundby, C., Wenger, R. H., Held, L., Merz, T. M., Gassmann, M., Lutz, T., Kunz, A., Bron, D., Fontana, A., Strauss, L., Weber, A., Fried, M., Rogler, G., & Zeitz, J. (2017). Protocol for a prospective, controlled, observational study to evaluate the influence of hypoxia on healthy volunteers and patients with inflammatory bowel disease: the Altitude IBD Study. *BMJ open*, *7*(1), e013477. https://doi.org/10.1136/bmjopen-2016-013477
- Wang S, Song R, Wang Z, Jing Z, Wang S, Ma J. S100A8/A9 in Inflammation. *Front Immunol*. 2018;9:1298.
- Wenger, R. H., Stiehl, D. P., & Camenisch, G. (2005). Integration of oxygen signaling at the consensus HRE. *Science's STKE : signal transduction knowledge environment*, *2005*(306), re12.
- Winter, S. C., Shah, K. A., Han, C., Campo, L., Turley, H., Leek, R., Corbridge, R. J., Cox, G. J., & Harris, A. L. (2006). The relation between hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression with anemia and outcome in surgically treated head and neck cancer. *Cancer*, *107*(4), 757–766. https://doi.org/10.1002/cncr.21983
- Xia C, Braunstein Z, Toomey AC, Zhong J, Rao X. S100 Proteins As an Important Regulator of Macrophage Inflammation. *Front Immunol*. 2018;8:1908. Published 2018 Jan 5.

- Zander, R., & Vaupel, P. (1985). Proposal for using a standardized terminology on oxygen transport to tissue. *Advances in experimental medicine and biology*, *191*, 965–970. https://doi.org/10.1007/978-1-4684-3291-6_98
- Zhong, H., De Marzo, A. M., Laughner, E., Lim, M., Hilton, D. A., Zagzag, D., Buechler, P., Isaacs, W. B., Semenza, G. L., & Simons, J. W. (1999). Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer research*, *59*(22), 5830–5835.
- Ziegler, G., Prinz, V., Albrecht, M. W., Harhausen, D., Khojasteh, U., Nacken, W., Endres, M., Dirnagl, U., Nietfeld, W., & Trendelenburg, G. (2009). Mrp-8 and -14 mediate CNS injury in focal cerebral ischemia. *Biochimica et biophysica acta*, *1792*(12), 1198–1204.
- Zou X, Sorenson BS, Ross KF, Herzberg MC. Augmentation of epithelial resistance to invading bacteria by using mRNA transfections [published correction appears in Infect Immun. 2015 Mar;83(3):1226-7]. *Infect Immun*. 2013;81(11):3975-3983.
- Zygiel, E. M., & Nolan, E. M. (2018). Transition Metal Sequestration by the Host-Defense Protein Calprotectin. Annual review of biochemistry, 87, 621–643.