The Effect of Hypoxia on VEGF Expression in Feline Oral Squamous Cell Carcinoma

Madison King, Haili Wang, Luis Garcia, Russ Fraser, and Chelsea Martin

Department of Pathology & Microbiology, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island

INTRODUCTION

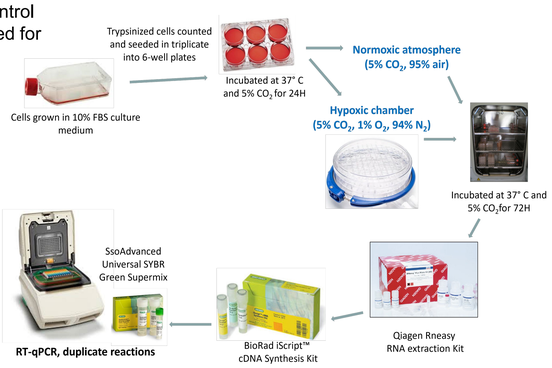
- Feline oral squamous cell carcinoma (FOSCC) is a treatment resistant cancer with poor patient outcomes.
- Solid tumors like FOSCC can outgrow vascular capacity, leading to areas of hypoxia that upregulate angiogenic factors like vascular endothelial growth factor (VEGF).
- VEGF is a biomarker for poor prognosis in human OSCC, but the cancer responds poorly to treatment with anti-angiogenic drugs (Salem 2021).
- VEGF-A is the best studied within the VEGF protein family, but the presence of VEGF-D has also been found in FOSCC tissue samples and cell lines (Harris 2019).

METHODS

1. Cell Culture & Hypoxic Treatment:

Five cell lines were selected to represent the FOSCC tumor microenvironment. Cells were seeded into 6-well plates (2x10^5 cells/well) and incubated at 37° C and 5% CO_2 for 24H. Plates were divided into experimental and control groups, then incubated for an additional 72H.

Cell Line	Tissue	Disease State	Morphology	Patient Information
SCCF1	Larynx	FOSCC	Epithelial	Domestic cat Unreported age Unreported sex
SCCF2	Gingiva	FOSCC	Epithelial	Domestic short-haired cat 7 years old Castrated male
SCCF3	Tongue	FOSCC	Epithelial	Domestic medium-haired cat 12 years old Castrated male
CCL94	Cortical Kidney	Healthy	Epithelial	Domestic cat 12 week old Female
CRL6167	Trachea	Healthy	Fibroblast	Domestic cat Fetus Male



Adapted from W. Nasry, 2024

2. Reverse Transcription- quantitative PCR:

RNA was extracted using a commercial kit. RNA concentration was measured with spectrophotometry and used to synthesize 12.5ng/ul of cDNA. Samples were reverse transcribed for one cycle in a thermal cycler at 25° C for 5 minutes, 46° C for 20 minutes, and 95° C for 1 minute. Primers were designed in triplicate for each target sequence using NCBI'S Primer Blast. Primers were validated using pooled cell line cDNA with positive (feline heart) and negative control samples. Primer conditions were optimized, and PCR products were sent for sequencing (Psomagen, Inc.). Primer efficiency was determined, and mRNA expression was normalized to three endogenous reference genes: GAPDH, RPS182, and TUBB2.

RESULTS

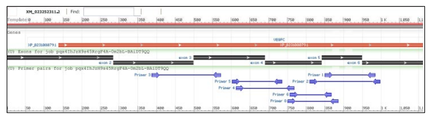


Figure 1. Primers were designed in triplicate using NCBI's Primer-BLAST. Primer pairs were considered if the forward and reverse primer had similar melting temperatures close to 60° C. Primers were prioritized by intron length and amplicon size.

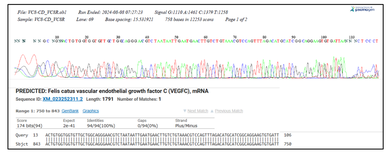


Figure 2. PCR products were sequenced in both directions (see example chromatogram) and their identity determined with NCBI Nucleotide Blast. Products from two VEGF-C primer sets (#6 and #8) were 94-100% identical with predicted feline VEGF-C mRNA.

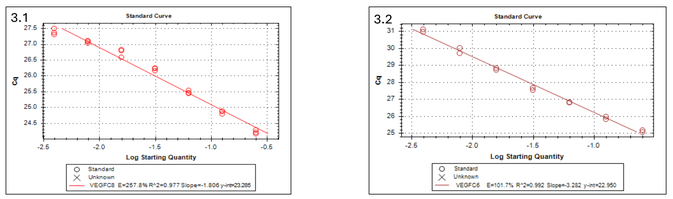


Figure 3 .Serial dilutions of pooled cell line cDNA was used to create standard curves to evaluate reaction efficiencies. In a perfect PCR reaction, the reaction efficiency would be 100%, but efficiencies ranging from 90-110% are acceptable. **VEGF-C primer set #8** (Figure 3.1), showed poor performance (E=257.8%). **VEGF-C primer set #6** (Figure 3.2) met the desired efficiency criteria (E=101.7%).

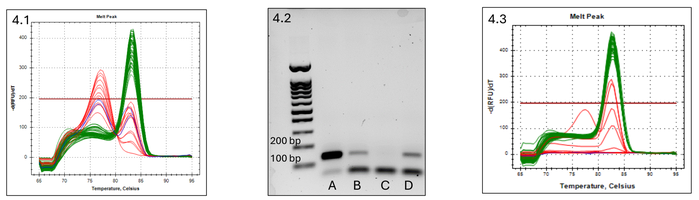


Figure 4. Non-specific amplification was observed with **VEGF-C primer set #8**, interpreted as primer dimer products. Melt curve analysis showed double peaks at low cDNA concentrations in the standard curve (not shown), in no-template control samples (blue lines in 4.1) and in low-expressing SCCF2 cells (red lines in 4.1, overlapping with blue negative controls). PCR products were electrophoresed on a 1% agarose gel (Figure 4.2). CCL94 kidney cells (lane A) yielded a single melt peak and a dominant band at the expected product size (135 base pairs). Amplification from SCCF2 samples (lanes B and C) resulted in no band (or a faint band) at the expected size, with prominent bands below 100 bp indicating primer dimer formation. Negative control (lane D) also showed primer dimer products and possible contamination. **VEGF-C primer set #6** showed far less non-specific amplification in SCCF2 cells (Figure 4.3, red lines) and there was no amplification in negative controls.

References

Harris K, Gelberg HB, Kiupel M, Helfand SC. Immunohistochemical Features of Epithelial-Mesenchymal Transition in Feline Oral Squamous Cell Carcinoma. Vet Pathol. 2019 Nov;56(6):826-839. doi: 10.1177/0300985819859873. Epub 2019 Jul 22. PMID: 31331247.

Nasry WHS, Martin CK. In Vitro Gene Expression of PGE2 Synthase Enzymes, PGE2 Receptors, HIF-1α and VEGFA in Feline Oral Squamous Cell Carcinoma. Journal of Veterinary Diagnostic Investigation, In review.

Nasry WHS, Wang H, Jones K, Dirksen WP, Rosol TJ, Rodriguez-Lecompte JC, Martin CK. CD147 and Cyclooxygenase Expression in Feline Oral Squamous Cell Carcinoma. Vet Sci. 2018 Aug 13;5(3):72. doi: 10.3390/vetsci5030072. PMID: 30104530; PMCID: PMC6163611.

Salem A, Salo T. Vasculogenic Mimicry in Head and Neck Squamous Cell Carcinoma-Time to Take Notice. Front Oral Health. 2021 Mar 31;2:666895. doi: 10.3389/froh.2021.666895. PMID: 35048009; PMCID: PMC8757801.

RESULTS

Target	Sense	Antisense	Amplicon (bp)	Melting Temp (° C)	Annealing Temp (° C)	Efficiency
VEGFA	CGAGGCAGCTTGAGTTAAACG	CGTGGTTTCTGTGTCAGTCC	149	85.5	60.0	95.0
VEGFB	CCAGGCTTGGAGATTCGACC	CTATCAGCGCTGGGAAACGGG	117	90.5	61.4	109.7
VEGFC	CTTCCTGCCGATGCATGTCT	GGCAAGTCTTGTTTGCAGCC	111	83.0	63.3	101.7
VEGFD	GTTTGCGGCCACTTTCTACG	GCTTGAAGAACGTGTCGGTG	137	87.0	63.3	103.9
GAPDH	GGGCGTGAACCACGAGAAGTA	GATGGCATGGACTGTGGTCA	144	86.5	60.0	109.7
RPS18	GTTCTCTCTTCCACAGGAGGC	ACTCGCAAAATGTGCTGGAAC	92	85.5	63.0	102.0
TUBB2	ATTGACCCCACCGGTACCTA	CCCAAAAGGACCTGAGCGAA	159	85.0	60.0	105.0

Figure 5. Final primer information and qPCR conditions

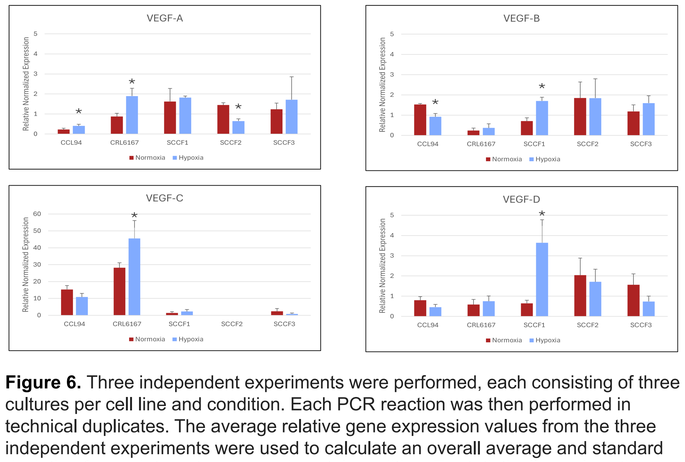


Figure 6. Three independent experiments were performed, each consisting of three cultures per cell line and condition. Each PCR reaction was then performed in technical duplicates. The average relative gene expression values from the three independent experiments were used to calculate an overall average and standard deviation for each target and experimental condition. To statistically analyze the change in gene expression in response to hypoxia, a 2-tailed T test was performed for each cell line and target using GraphPad online software (*p<.05).

CONCLUSION

- **VEGF-A:** Renal epithelial cells and tracheal fibroblasts showed increased mRNA expression in response to 72H of hypoxia while SCCF2 cells showed a decrease in expression. SCCF1 & SCCF3 cells showed a nominal increase in mean VEGF-A expression, but the data was variable, and the results were not statistically significant.
- **VEGF-B:** SCCF1 cells showed a marked increase in hypoxic expression while renal epithelial cells showed a mild decrease in expression.
- **VEGF-C:** Tracheal fibroblasts expressed more mRNA in response to hypoxia and in larger quantities relative to the other cell lines. SCCF2 cells consistently produced little to no VEGF-C mRNA, but there was not a statistically significant change in expression in response to hypoxia.
- **VEGF-D:** SCCF1 had marked upregulation of VEGF-D in hypoxic conditions. This supports the findings of Harris 2019.
- **Significance:** These findings demonstrate how mediators of angiogenesis can be variably expressed between patients and could translate to variable and inconsistent treatment response. Additionally, stromal cells in the tumor microenvironment could be an important source of VEGF-C, even if expression is not apparent in tumor cells.
- **Future Research:** This study shows that these cells exhibit potentially important changes in gene expression after 72H of hypoxia. Future experiments will employ RNA sequencing to characterize changes in the transcriptome-wide response of FOSCC cells to hypoxia.

Acknowledgments

The authors are grateful for the assistance of undergraduate researcher, Lucy Zhang.

Financial support provided by AVC VetSRA (M. King), AVC Internal Research Fund (R. Fraser and C. Martin), and NSERC Discovery Grant (C. Martin).