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Expression of VEGF-A/VEGFR-2 pathway in feline oral squamous cell carcinoma *in vitro* and anti-tumour effect of Bevacizumab in a xenograft model

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ABSTRACT

Vascular endothelial growth factor A (VEGF-A) plays a key role in tumour angiogenesis, proliferation, and metastasis, contributing to malignant transformation. Bevacizumab is a monoclonal antibody (mAb) which binds to VEGF-A, preventing it from binding to and activating VEGF receptor 2 (VEGFR-2) on endothelial and cancer cells, thereby resulting in anti-tumour effects. Bevacizumab is employed in treatment of numerous human malignancies and showed promising results also against head and neck squamous cell carcinoma (HNSCC). Feline oral SCC (FOSCC), the counterpart of HNSCC in cat, is characterized by aggressive behaviour, local invasion and metastasis, and is unresponsive to standard treatments, thus displaying poor prognosis. In this study, we characterized expression of VEGF-A/VEGFR-2 pathway in FOSCC cell lines SCCF1, SCCF2 and SCCF3, and tested the response to Bevacizumab both *in vitro* and in a xenograft mouse model of SCCF3 expressing luciferase. RT-PCR and Western blotting (WB) analysis showed expression and activation of VEGF-A/VEGFR-2 axis at steady-state and serum-starved conditions in the three cell lines, suggesting the presence of a functional autocrine signalling loop. Treatment of cells with Bevacizumab at 50 or 100 μg/mL for 6 h inhibited activation of VEGFR-2 and its downstream mediator AKT, as shown by WB and densitometric analysis. Most importantly, volumetric and bioluminescence analysis demonstrated that Bevacizumab (5 mg/kg, twice a week) suppressed tumour growth in the xenograft model. Our data suggest that Bevacizumab displays anti-cancer activity against FOSCC *in vitro* and *in vivo*, paving the way for translational studies aimed at introducing molecular targeted therapy with mAbs in veterinary oncology.

Introduction

The members of the vascular endothelial growth factor (VEGF) family are specific, highly potent regulators of both physiological and pathological angiogenesis, with the VEGF-A playing a major role (Wang et al., 2020). VEGF-A gene encodes different isoforms generated by alternative splicing, resulting in different molecular species of 121, 165, 189, and 206 amino acids in length, with VEGF-A_{165} showing the highest biological activity (Peach et al., 2018). In solid tumours, cancer cells produce VEGF-A, which then binds to and activates VEGF receptor 2 (VEGFR-2) on endothelial cells in a paracrine fashion. Activated VEGFR-2 is responsible for VEGF-A-mediated pro-angiogenic and pro-metastatic effects, including endothelial cell proliferation, survival and chemotaxis as well as vascular permeability (Haibe et al., 2020; Wang et al., 2020). Beyond its pro-angiogenic functions, VEGF-A secreted by tumour cells fuels an autocrine VEGF-A/VEGFR-2 signalling loop which support their own proliferation, survival and resistance to apoptosis through activation of Ras-mitogen-activated protein kinase-ERK (Ras-MAPK-ERK) and phosphatydil-inhositol-3 kinase (PI3K)/AKT downstream pathways, further enhancing neoplastic

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progression to malignancy (Adamcic et al., 2012; Wang et al., 2020; Wang et al., 2024). Therefore, targeting VEGF-A with specific drugs appears as a rational approach to fight cancer.

In this regard, molecular targeted drugs have changed the paradigm of cancer therapy in recent years, with monoclonal antibodies (mAbs) leading this revolution (Wang et al., 2025). Indeed, the use of mAbs represents a consolidated therapeutic reality in human oncology and many of them are in clinical development to further enrich the anti-cancer arsenal (Wang et al., 2025). Bevacizumab is a recombinant humanized IgG1 mAb which binds to VEGF-A with high affinity, thus blocking activation of VEGFR-2, thereby counteracting tumour growth and metastasis (Wang et al., 2024). Bevacizumab is pivotal in treatment of various human malignancies, including glioblastoma, colorectal, breast, cervical, ovarian, renal and non-small cell lung cancer (Garcia et al., 2020). Interestingly, Bevacizumab has demonstrated a high therapeutic potential also in pre-clinical models of head and neck squamous cell carcinoma (HNSCC) and, most importantly, has provided encouraging results in clinical trials, standing out among the anti-VEGF molecules which are currently under investigation against this class of neoplasms (Micaily et al., 2020; Tamimi et al., 2023).

Feline oral SCC (FOSCC) represents the counterpart of HNSCC, accounting for 60–70% of oral malignancies in cat (Altamura and Borzacchiello, 2019). It is locally aggressive, potentially metastatic and resistant to conventional treatments (surgery, radio/chemotherapy), thereby displaying extremely poor clinical outcome (Tutu et al., 2025). FOSCC mimics HNSCC in terms of biological behaviour, histopathological features, disease mechanisms and molecular markers with potential therapeutic targetability (Tutu et al., 2025). Much like HNSCC, FOSCC expresses pro-angiogenic factors, including members of VEGF family, in correlation with malignant features like angiogenesis, invasion and metastasis (Harris et al., 2019; Kabak et al., 2020; Nasry et al., 2025). Feline VEGF-A shares high percentage homology with human counterpart and VEGF-A_{163}, a feline analogue of human VEGF-A_{165} has been identified (Koga et al., 2002; Muellerleile et al., 2019). Moreover, previous research demonstrates that feline VEGF-A conserves the binding region to Bevacizumab and, consistently, that it is bound by this latter (Michishita et al., 2016; Muellerleile et al., 2019); thus, applying anti-VEGF-A targeted therapy in felines appears challenging.

Although mAbs have recently entered the veterinary practice for the therapy of inflammatory diseases (i.e. osteoarthritis, dermatitis), no such drug has been introduced yet in veterinary oncology; the only exception is Gilvetmab, a mAb raised against the immune-checkpoint inhibitor PD-1, provisionally approved for treatment of canine cancer (Wang et al., 2025). However, recent pre-clinical studies demonstrate that the anti-epidermal growth factor receptor (EGFR) mAb Cetuximab, approved for clinical use in HNSCC patients, displays anti-cancer activity against FOSCC, suggesting that mAbs can be successfully transferred from human to feline oncology (Altamura and Borzacchiello, 2022; Altamura et al., 2024).

Based on this background, the aim of this investigation was to characterize expression of VEGF-A/VEGFR-2 pathway in FOSCC cell lines and to assess the anti-cancer effect of Bevacizumab both *in vitro* and in a xenograft model to hypothesize a new mAb-based therapeutic approach.

Materials and methods

Cell lines and treatments conditions

FOSCC cell lines SCCF1 (laryngeal SCC), SCCF2 (gingival SCC) and SCCF3 (tongue SCC), developed in the Rosol laboratory, were a kind gift from Professor T.J. Rosol (The Ohio State University) and have been cultured as described elsewhere (Martin et al., 2012; Martin et al., 2010; Tannehill-Gregg et al., 2001). Bevacizumab-sensitive cell line CAL-27 (human tongue SCC) was cultured according to product datasheet (Wu et al., 2015).

For protein and gene expression experiments at steady state, cells were subjected to trypsinization and viable counting with Trypan Blue using the Cell Counter apparatus (Bio-Rad Laboratories, Milan, Italy) and plated at 1.5×10^6 in 60 mm dishes in complete DMEM. After 24 h (h) of culture, cells were harvested by trypsinization to allow RNA and protein extraction. For comparative analysis on steady state *vs* starved cells, medium was replaced with fresh DMEM with or without fetal bovine serum (FBS) for additional 24 h, and then cells were harvested to perform protein extraction.

For treatment with Bevacizumab, cells were seeded at 3×10^5 in 6-well plates in complete DMEM. After 24 h of culture, cells were starved in DMEM without FBS for further 24 h and then incubated with Bevacizumab (#A2006, Selleckchem, Houston, Texas, USA) or non-specific human immunoglobulin G of the same isotype (hIgG1, #A2051, Selleckchem, Houston, Texas, USA) as control, diluted in fresh DMEM without FBS at doses of 25, 50 and 100 μg/mL for the times of 1, 3 and 6 h. At the end of the treatments, cells were collected and subjected to protein extraction.

Stably expressing luciferase SCCF3 cell line (SCCF3 Luc, #T6448) was purchased from Applied Biological Materials Inc. (Richmond, BC, Canada). Briefly, cells were cultured in PriGrow III medium (#TM0003, Applied Biological Materials Inc., Richmond, BC, Canada), supplemented with 10% FBS (#A52566701, Gibco, Fisher Scientific Italia, Milan, Italy) and 1% L-Glutamine (#25030024, Gibco, Fisher Scientific Italia, Milan, Italy). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. After the first passage, 0,4 mg/mL Geneticin/G418 (#G271, Applied Biological Materials Inc. Richmond, BC, Canada) were added to the culture medium for selection. Cells were routinely tested for mycoplasma (Mycoalert, #LT07–318, Lonza Bioscience, Basel, Switzerland).

Western blotting and antibodies

Protein extraction, protein quantification, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting (WB), protein bands detection and densitometric analysis were performed as previously described (Altamura and Borzacchiello, 2022). Detection of AKT, phospho-AKT (pAKT), ERK, phospho-ERK (pERK) and β-actin was guaranteed by using antibodies already validated in feline cell lines, employed as previously reported (Altamura and Borzacchiello, 2022). Anti-VEGFR-2 (#E-AB-70080, Elab science, Houston, Texas, USA) and phospho-VEGFR-2 (pVEGFR-2, #NBP3–16893, Novus Biologicals, Centennial, Colorado, USA) antibodies were used at 1:500 and 1:1000 dilution, respectively, and their reactivity against feline antigens was ensured by running feline cells protein extracts along with human samples (CAL-27). Normalization of proteins for β-actin, densitometric data and fold change in treated *vs* control samples were calculated and expressed as described in previous studies (Altamura and Borzacchiello, 2022).

Reverse transcription-PCR

RNA extraction, reverse transcription (RT) and PCR reactions on the obtained cDNAs were performed as previously described (Altamura et al., 2018). Specific amplification of transcripts from feline VEGF-A, VEGF-A_{163} and β2-microglobulin (as house-keeping gene) was carried out using the primers designed elsewhere (Altamura et al., 2018; Gudenschwager-Basso et al., 2022).

Animals and housing

Female athymic mice (6–8 weeks old) were purchased from Charles River Laboratories (strain: Carlene(NCr)-Foxn1^{nu}, Strain Code 490). Animals were housed in individually ventilated cages (IVCs; Tonoplast, Italy) in groups of five under controlled environmental conditions, including temperature (22 ± 2 °C) and relative humidity (55 ± 10%),

with a 12 h light/dark cycle. Standard rodent chow and water were provided *ad libitum*. Environmental enrichment consisted of red plastic mouse houses and nesting paper. All experimental procedures were conducted at CEINGE-Advanced Biotechnology Institute (Naples, Italy), in accordance with institutional guidelines and authorized by the Italian Ministry of Health (Ministry Decree 15/2018-UT).

SCCF3 Luc cells inoculation

Cells were cultured and maintained at CEINGE-Advanced Biotechnology Institute (Naples, Italy), following standard techniques. Briefly, once detached, they were counted and 4×10^6 cells were suspended in 100 µl Phosphate Buffer Saline (PBS, Gibco, Fisher Scientific Italia, Milan, Italy) and injected as quickly as possible after preparation in 22 mice. Mice were anesthetized with 2% isoflurane delivered via a nose-cone mask to ensure adequate sedation during the procedure. Before each injection, a topical anaesthetic ointment was applied to the injection site to minimize pain and discomfort. The cells were then administered subcutaneously using insulin syringes (1 mL, 27 G).

Bevacizumab treatment and randomization

When tumours reached a measurable size (approximately 5 mm in diameter), animals were randomly assigned to treatment or control groups. Randomization was performed to ensure comparable mean tumour size across groups at the start of treatment. Mice in the treatment group received 5 mg/kg Bevacizumab via intraperitoneal injection using insulin syringes (1 mL, 27 G). Injections were administered twice a week. Control group of animals received an equivalent volume of saline, under the same experimental conditions. The endpoint of the study was set at 24 days after treatment began. Tumour growth was monitored throughout the study in all the enrolled animals using digital calliper, implementing bioluminescence imaging (BLI) in a subset of them. Animals were humanely euthanized for tumour collection and subsequent analyses.

Tumour volume monitoring

Tumour growth was assessed once a week on average, using a combination of *in vivo* (BLI) and digital caliper measurements, to allow comprehensive longitudinal monitoring. For BLI approach, mice (n°10) were intraperitoneally injected with D-luciferin (IVISbrite™ D-Luciferin Potassium Salt, #122799, Revvity,) at the dose of 150 mg/kg. Imaging acquisitions were performed 10 min after luciferin injection, corresponding to the optimal time for substrate kinetics, using the NEWTON 500 FT imaging system (Vilber, Paris, France). Tumour length (the longest dimension) and width (the perpendicular shortest dimension) were recorded, and tumour volume was calculated using the formula:

$$\text{Tumour Volume } (\text{mm}^3) = \frac{\text{Length} \times \text{Width}^2}{2}$$

This dual approach provided complementary quantitative data on tumour progression, from both physical and metabolic perspective. At the endpoint, tumours were excised and weighed to obtain final tumour mass.

Tissue preparation and histopathological analysis

Tissue samples from control animals were collected, fixed in 4% paraformaldehyde for 12–24 h, embedded in paraffin, and sectioned at 5–6 µm. Sections were stained with haematoxylin and eosin (H&E), examined under a light microscope, and digital images were captured for documentation and histopathological analysis.

Statistical analysis

Data about the evaluation of tumour growth in mice by digital caliper, as well as BLI were statistically analysed by using two-way RM ANOVA, with time as repeated measure, followed by Tukey's multiple comparison, when required. All Statistical analysis was performed with GraphPad (version 10.0; La Jolla, CA).

Ethics statement

In vivo experiments were carried out according to protocols approved by the veterinary department of the Italian Ministry of Health (Authorization number: 996/2024-PR), in compliance with the Italian Decree Law No. 26/2014-Implementation of the Directive 2010/63/EU about the protection of animals used for scientific purposes).

Results

Expression of VEGF-A and VEGFR-2 in FOSCC cell lines

The first step of this study was to analyze expression of VEGF- A, the major target of Bevacizumab, in FOSCC cell lines SCCF1, SCCF2, SCCF3. Gene expression analysis by RT-PCR allowed us to identify VEGF-A transcript in all the three feline models (Fig. 1A). Importantly, when using oligos specific to VEGF-A_{163}, the feline equivalent of human VEGF-A_{165}, a successful amplification was also obtained (Figure1A). Amplification of β2-microglobulin as house-keeping gene confirmed the reliability of the results (Fig. 1A). Once we confirmed expression of the ligand, we checked the expression and activation of its receptor by WB, using antibodies anti-VEGFR-2 and its activated form pVEGFR-2, in the three FOSCC cell lines along with human oral squamous cell carcinoma cells CAL-27. The results showed the presence of VEGFR-2 and pVEGFR-2 in all the analysed feline cell lines, although with different expression levels among them (Fig. 1B). The simultaneous use of CAL-27 cells, known to express the active VEGF pathway (Wu et al., 2015), ensured the identity of the bands: specifically, two bands were identified for both VEGFR-2 and pVEGFR-2, with approximative molecular weights of ~230 kDa and ~150 kDa, corresponding to the different isoforms described in the literature (Fig. 1B) (Wang et al., 2020). Detection of β-actin ensured comparable protein loading among samples (Fig. 1B).

To investigate whether VEGFR-2 might be activated by VEGF-A produced by cells and not only growth factors added in culture medium with FBS, feline (SCCF3) and human (CAL-27) tongue squamous cell carcinoma cell lines were analysed by WB for pVEGFR-2 under starvation conditions. It was observed that receptor phosphorylation persisted even in absence of FBS, strengthening this hypothesis (Fig. 1C). The experiment was performed and data confirmed also in SCCF1 and SCCF2 (not shown). However, in the subsequent phases of this study, only the VEGFR-2 isoforms with higher molecular weight will be considered, as they are particularly involved in signal transduction (Wang et al., 2020).

Bevacizumab inhibits VEGFR-2 pathway in FOSCC cell lines

Feline VEGF-A/VEGFR-2/pVEGFR-2 expression data in FOSCC cell lines provided the rationale for studies on the inhibition of this pathway by Bevacizumab. A calibration experiment was run on CAL-27 and SCCF3 cell lines (not shown). Serum-starved cells were treated at 25, 50, and 100 µg/mL for 1, 3 and 6 h with Bevacizumab or a hIgG1 as control, to be then analysed by WB for VEGFR-2, pVEGFR-2, the downstream mediators AKT, ERK and their phosphorylated forms pAKT and pERK. The most impressive reduction in pVEGFR-2 levels in CAL-27 cells was observed after 1 h of treatment at the highest dose (100 µg/mL), accompanied by a pAKT decrease (Fig. 2A). The intermediate dose (50 µg/mL) together with a longer incubation time (6 h) was required to observe the most relevant pVEGFR-2 reduction in SCCF3 cells, yielding

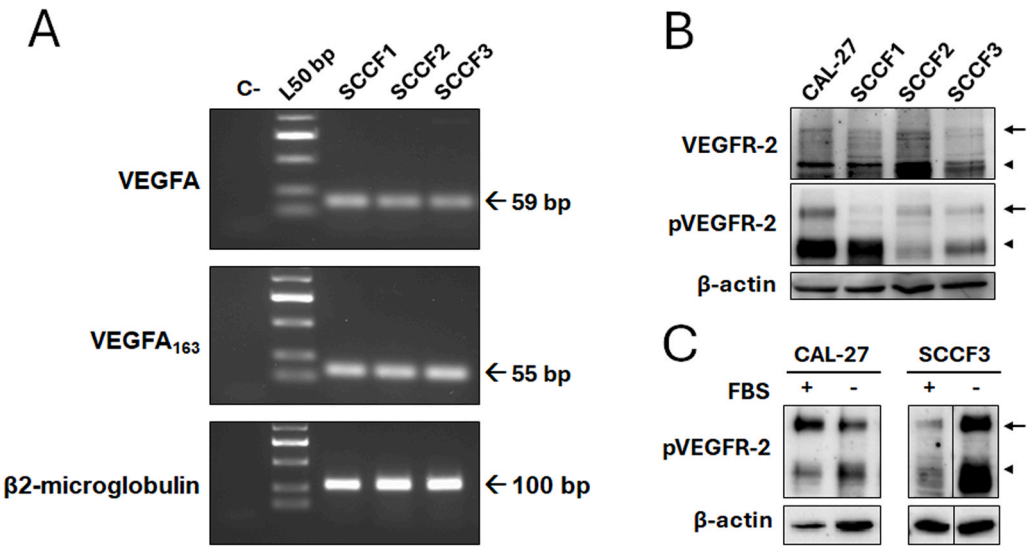

Fig. 1. Analysis of VEGF-A and VEGFR-2 pathway in feline oral squamous cell carcinoma (FOSCC) cell lines. (A) RT-PCR analysis for the expression of the VEGF-A gene (59 bp) and the VEGF-A_{163} isoform (55 bp) in FOSCC cell lines (SCCF1, SCCF2, SCCF3). Amplification of β2-microglobulin (100 bp) is shown as an internal control. C- in the first lane indicates the no template control, a 50 base pairs molecular ladder (L50 bp) is loaded in the second lane. **(B)** Western blotting for the expression of the VEGFR-2 receptor (VEGFR-2) and its phosphorylated form (pVEGFR-2) in human (CAL-27) and FOSCC cell lines. Two bands of approximately 230 (black arrow) and 150 kDa (black arrowhead) corresponding to VEGFR-2 isoforms, were correctly identified in all cell lines. β-actin is shown as a loading control. **(C)** Expression of pVEGFR-2 in presence (+) and absence (-) of FBS in CAL-27 and SCCF3. β-actin is shown as a loading control.

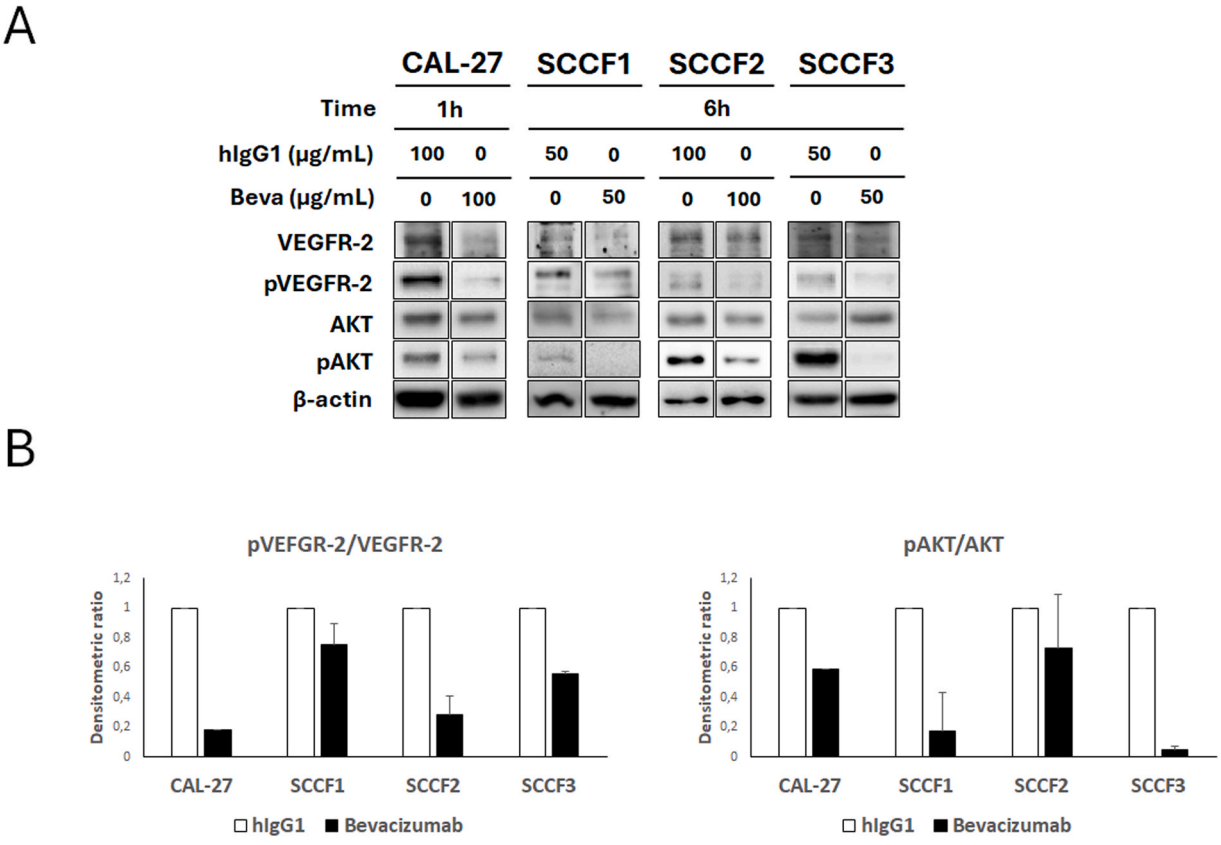

Fig. 2. Inhibition of VEGFR-2 pathway by Bevacizumab in feline oral squamous cell carcinoma cell lines. (A) SCCF1, SCCF2, and SCCF3 cell lines were incubated with Bevacizumab (Beva) at the indicated doses and times and analysed by Western blotting (WB) for VEGFR-2, phospho-VEGFR-2 (pVEGFR-2), AKT and phospho-AKT (pAKT). Results from calibration experiments in Bevacizumab-sensitive CAL-27 cells are shown. The treatment induced reduction of pVEGFR-2 levels in all cell lines with respect to control (hIgG1). Impairment of pVEGFR-2 by Bevacizumab was accompanied by a decrease of pAkt levels in all cell lines. WB for β-actin antibody ensured comparable protein loading and allowed normalization. **(B)** Densitometric analysis of pEGFR and pAkt expressed as densitometric ratio pVEGFR-2/VEGFR-2 and pAKT/AKT, respectively. Standard deviations are from two repeated, independent experiments.

also a consistent downregulation of pAKT levels. Therefore, experiments were performed and repeated in all feline models exclusively at the most

effective conditions, providing the following data: similarly to SCCF3, SCCF1 treated for 6 h at 50 μg/mL showed lower pVEGFR and pAKT levels compared to control, whilst the dose of 100 μg/mL was necessary to obtain the same result in SCCF2 (Fig. 2A). Densitometric analysis of VEGFR-2 and AKT phosphorylation, expressed as pVEGFR-2/VEGFR-2 and pAKT/AKT ratios, confirmed WB visual results (Fig. 2B). However, when analyzing pERK, a negligible effect was observed in CAL-27 (Supplementary Fig. 1 A), which became more evident after 3 h of treatment, appearing delayed with respect to pVEGFR-2 inhibition (not shown). As far as feline cell lines were concerned, a substantial down-regulation of pERK was seen only in SCCF1 but not in SCCF2, where a surprising upregulation was yielded upon treatment, nor in SCCF3, where a clear trend among experimental replicates could not be identified (Supplementary Fig. 1 A). Data from densitometric measures of ERK phosphorylation, expressed as pERK/ERK ratio, confirmed visual WB results (Supplementary Fig. 1B).

Establishment of FOSCC xenograft tumours

Athymic nude mice (n = 11/treatment) were injected with 4×10^6 SCCF3 Luc cells. Tumour growth was observed on the injected flank, confirming that engraftments were successful. When neoplastic mass reached a size of approximately 5 mm in diameter, animals were subjected to treatments as described in methods and data collected providing the results described below.

Effects of Bevacizumab in FOSCC xenograft model

According to the results *in vitro*, we assessed the effect of the intraperitoneally injected Bevacizumab (5 mg/kg) upon tumour growth in FOSCC xenografted mice. Our data showed a significant reduction of tumour volume (measured with digital caliper) in the drug-treated mice, when compared to the control group (Fig. 3A). Two-way ANOVA with time as repeated measure, revealed a time x treatment interaction ($F_{(1.363,\ 27.26)} = 29.81$, $p < 0.0001$). Following Tukey's multiple comparison test showed a main treatment effect at 4 ($p = 0.0102$), 8 ($p = 0.0003$), 12 ($p < 0.0001$), 16 ($p < 0.0001$), 20 ($p < 0.0001$) and 24 ($p = 0.0001$) days post-Bevacizumab treatment. This physical tumour mass reduction was overall confirmed by BLI, which tracks the functional tumour burden *in vivo* through photon flux (radiant efficiency), carried out in an experimental subset of the same animals (n = 5/treatment) (Fig. 3B). Two-way RM ANOVA, with time as repeated measure, showed a treatment x time interaction ($F_{(1.249,\ 9.994)} = 8.689$, $p = 0.0115$), with treatment effect at 12- and 24-days following drug administration (Tukey's multiple comparison test: day 12, $p = 0.0332$; day 24, $p = 0.0231$). At the time of animals' sacrifice, images were taken (Fig. 4A) and tissue from control mice was collected to be processed for histological analysis. Evaluation of slides stained with H&E revealed cords and islands of proliferating squamous cells surrounded by desmoplastic stroma (Fig. 4B). Concentric, rounded nests of keratinized tumour cells were also observed, consistent with keratin pearls (Fig. 4C). Cells with dark shrunken peripheral nuclei surrounded by a clear halo, namely koilocytes, suggestive of papillomavirus infection, were visible within the neoplastic nests, consistently with previous studies (Altamura et al., 2018) (Fig. 4C). Based on these histological features, a diagnosis of SCC was confirmed.

Discussion

Characterizing the molecular landscape of FOSCC is pivotal to identify new molecular targets and test new approaches based on biological drugs, such as mAbs, aiming at improving therapeutic protocols and thus prognosis for affected cats. In this work, we aimed at gaining new insights into VEGF-A/VEGFR-2 pathway in FOSCC, in terms of molecular pathology and possible therapeutic relevance as target of Bevacizumab. Expression of this ligand/receptor axis has been ascertained in canine and equine tumours as well as feline cutaneous SCC and mammary carcinoma, however it has not been fully investigated in FOSCC (Gudenschwager-Basso et al., 2022; Martano et al., 2018; Martano et al., 2016; Nascimento et al., 2021). Here we report expression of VEGF-A gene in SCCF1, SCCF2 and SCCF3, in agreement with previous studies, strengthening the reliability of these cell models (Harris et al., 2019; Nasry et al., 2025).. Importantly, expression of VEGF-A_{163}, the feline counterpart of the human most biologically active isoform VEGF-A_{165}, and that of native and activated VEGFR-2 was demonstrated for the first time in these FOSCC cell lines (Koga et al., 2002; Muellerleile et al., 2019). Concomitant expression of VEGF-A and VEGFR-2 in human and animal cancer is associated with activation of an autocrine signaling loop supporting tumour progression, therefore a similar scenario is conceivable in FOSCC pathogenesis (Adamcic et al., 2012; Millanta et al., 2006; Wang et al., 2024); previous work showed that SCCF1, SCCF2 and SCCF3 actively secrete VEGF-A in culture medium in serum-deprived conditions and, accordingly, we found persisting VEGFR-2 activation in serum-starved cells, confirming this hypothesis (Harris et al., 2019). Taken together, these results put the rationale for testing the anti-VEGF-A mAb Bevacizumab. In this regard, binding of Bevacizumab to feline VEGF-A has been previously ascertained, supporting the feasibility of such approach (Michishita et al., 2016; Muellerleile et al., 2019). Consistently, treatment with Bevacizumab decreased activation of VEGFR-2 and its downstream mediator AKT in all the cell lines, suggesting that the drug interrupted the autocrine loop. However, a decrease of activated ERK was found in SCCF1 but not

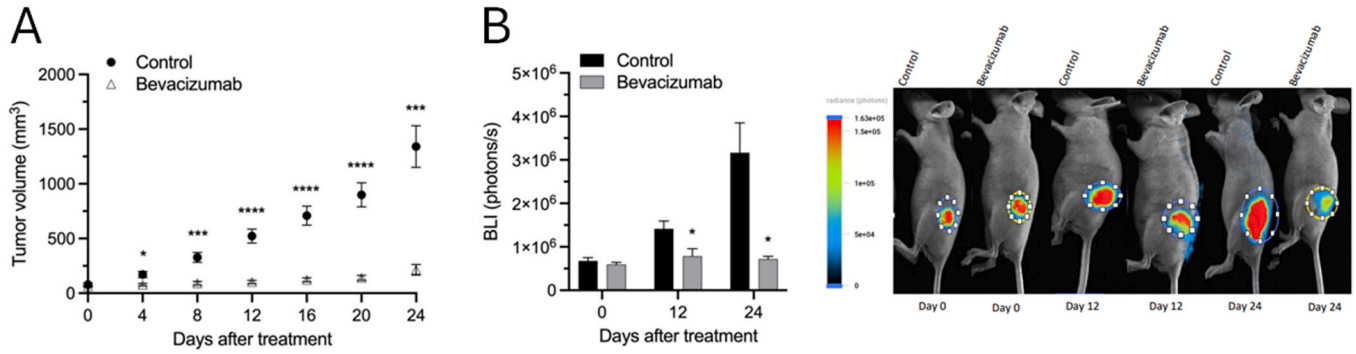

Fig. 3. Bevacizumab inhibits FOSCC growth in a xenograft mice model. (A) Tumour growth evaluation (expressed in mm^3) using a digital caliper, in female athymic mice (n = 11/treatment), subcutaneously inoculated with 4×10^6 SCCF3 Luc cells. **(B)** Bioluminescence imaging (BLI) was performed in a subset of the above indicated mice (n = 5/treatment) on days 0, 12 and 28, after tumour cell inoculation, to assess viable tumour cell burden over time. Statistical analysis was carried out using two-way RM ANOVA, with time as repeated measure, followed by Tukey's multiple comparison test, when required. * $p < 0.05$, *** $p = 0.0001$, **** $p < 0.0001$, compared to control group within each time point. All values are given as mean ± standard error of the mean.

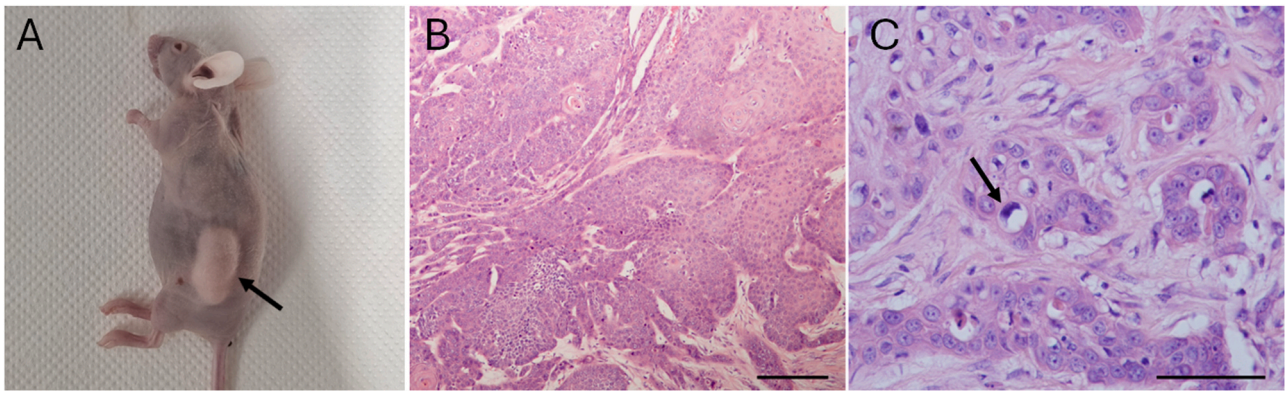

Fig. 4. Squamous cell carcinoma induced with xenograft of SCCF3 Luc cell line in nude mice. (A) Tumour mass grown from xenograft of SCCF3 Luc cells (black arrow) on the left flank in nude mice. (B) Histological analysis revealed cords and islands of proliferating squamous cells surrounded by desmoplastic stroma, consistent with a diagnosis of SCC. Haematoxylin and Eosin, 20x. Scale bar: 200 μm. **(C)** Concentric, rounded nests of keratinized tumour cells (keratin pearls). One koilocyte (cell with dark shrunken peripheral nucleus surrounded by a clear halo) is visible within a neoplastic nest (black arrow). Haematoxylin and Eosin, 40x. Scale bar: 200 μm.

SCCF3, whilst in SCCF2 an unpredicted increase could be even observed, despite being treated at higher doses. Previous research describes this ERK rebound in response to high doses of Bevacizumab as the result of compensatory activation of the MAPK cascade mediated by alternative tyrosine kinase receptors, suggesting a complex cross-talk between receptors and soluble factors that could bypass the inhibition of VEGF-A/VEGFR-2 signalling pathway (Liu et al., 2022). An uneven ERK response was also obtained in our feline models upon treatment with Cetuximab, suggesting that FOSCC cells might activate such an adaptive signalling in response to diverse anti-tumour stimuli (Altamura and Borzacchiello, 2022). The addition of MAPK inhibitors to Bevacizumab and Cetuximab has been shown to improve anti-tumour effects in human cancer, and this could be object of our future research (Rong et al., 2020; Wang et al., 2016; Zhai et al., 2023). Most importantly, the biochemical inhibition of VEGF-A/VEGFR-2 axis *in vitro* corresponded to anti-tumour effects *in vivo*, indeed we found that Bevacizumab counteracted tumour growth in a FOSCC xenograft mouse model. It is worthwhile noting that Bevacizumab does not bind to murine VEGF-A, therefore it is likely that the anti-tumour effect was due to inhibitory activity of the drug against feline VEGF-A secreted by FOSCC cells, consistently with *in vitro* data (Yu et al., 2008). Considering that in cultured cells Bevacizumab affected AKT, an anti-apoptotic mediator, but not ERK, which transduces proliferative signals, it is plausible that the observed inhibition of tumour growth might be related to induction of apoptosis rather than stoppage of cell proliferation (Ranieri et al., 2013). This hypothesis is in accordance with prior research showing that anti-tumour effect of Bevacizumab in feline mammary carcinoma xenograft models results in enhanced apoptosis along with diminished angiogenesis but does not affect proliferation index (Michishita et al., 2016). Whether Bevacizumab-mediated inhibition of angiogenesis contributes to stop tumour growth also in FOSCC will be investigated in future studies. Noteworthy, anti-tumour activity of Bevacizumab had been successfully assessed also in canine hemangiopericytoma and osteosarcoma xenograft models, confirming that this mAb might be of use for cancer treatment in pet oncology (Michishita et al., 2013; Scharf et al., 2013).

In this regard, therapeutic mAbs are entering the scene in veterinary medicine decades later than in human practice (Wang et al., 2025). Comparative oncology is the discipline studying animal models to gain advances in human medicine; however, thinking in a “One Health” perspective, we should consider also the opposite, making some efforts to walk the path traced by human disciplines in the attempt to bridge this gap (Altamura and Borzacchiello, 2023). We are walking this path since some years, endeavouring to transfer Cetuximab, a mAb employed in the treatment of HNSCC since 2006, in FOSCC therapy (Altamura and Borzacchiello, 2022; Altamura et al., 2024). With this study, we took a step forward, demonstrating that Bevacizumab, a therapeutic mAb in human oncology, exerts anti-tumour effects in preclinical models of FOSCC, paving the way for future translational studies aiming at employing this mAb, alone or in combination therapies, against this lethal tumour of cat.

CRediT authorship contribution statement

Gennaro Altamura: Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Francesco Napolitano:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Giuseppe Borzacchiello:** Writing – original draft, Supervision, Project administration, Conceptualization. **Carmen Avagliano:** Methodology, Investigation. **Alessandra Pelagalli:** Methodology, Investigation. **Domenico Sorrentino:** Writing – original draft, Visualization, Methodology, Investigation. **Caterina Squillacioti:** Writing – original draft, Methodology, Investigation.

Declaration of Generative AI and AI-assisted technologies in the writing process

No generative AI or AI-assisted technologies were used in the writing or preparation of this manuscript. The authors take full responsibility for the integrity and originality of the content.

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Declaration of Competing Interest

None.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2026.106648.

Data availability

All the data supporting the findings of this work are available from corresponding author upon reasonable request.

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Glossary

FOSCC: feline oral squamous cell carcinoma
HNSCC: head and neck squamous cell carcinoma
mAb: Monoclonal antibody
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor