Effects of epidermal growth factor receptor kinase inhibition on radiation response in canine osteosarcoma cells

by

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ABSTRACT

EFFECTS OF EPIDERMAL GROWTH FACTOR RECEPTOR KINASE INHIBITION ON RADIATION RESPONSE IN CANINE OSTEOSARCOMA CELLS

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Radiation therapy for canine osteosarcoma promotes transient improvement in analgesia. The addition of sensitizing agents may enhance tumour response to radiation therapy, improving patient outcomes. The epidermal growth factor receptor (EGFR) is expressed in canine osteosarcoma and correlated to prognosis. This research evaluated the effects of the EGFR inhibitor erlotinib on canine osteosarcoma cell radiation responses, target and downstream protein expression in vitro. The impact of this treatment on levels of vascular endothelial growth factor (VEGF) was also investigated.

Clonogenic survival and cell viability assays were performed after treatment with erlotinib as a single agent and in combination with radiation at doses of 2-10 Gy on three canine osteosarcoma cell lines. Target and downstream protein expression was assessed by Western blot after treatment with erlotinib as a single agent or in combination with 2 Gy radiation. Levels of VEGF in conditioned media were measured by enzyme-linked immunosorbent assay (ELISA).

Radiation at doses of 2-10 Gy demonstrated a dose dependent reduction in clonogenic survival in all cell lines. Erlotinib treatment reduced clonogenic survival in two and enhanced the impact of radiation in one of three cell lines. In cell viability assays, erlotinib exhibited single agent activity in one cell line at 10 μM dose, and in all three cell lines at 40 μM dose. Erlotinib at
40 μM demonstrated radiation enhancement effects at 2 and 4 Gy for all cell lines. Erlotinib did not alter total levels of EGFR, nor inhibit downstream protein kinase B (PKB/Akt) activation. On the contrary, erlotinib treatment increased phosphorylated Akt in two of three cell lines. Levels of VEGF in conditioned media increased after erlotinib treatment as a single agent and in combination with radiation in two of three cell lines, and decreased with erlotinib treatment in the third cell line.

Erlotinib treatment promoted modest enhancement of radiation effects in canine osteosarcoma cells, and possessed activity as a single agent, indicating a potential role for EGFR inhibition in the treatment of a subset of osteosarcoma patients. EGFR signalling and angiogenic responses to radiation and erlotinib are likely to be multifactorial and require further investigation.
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DECLARATION OF THE WORK PERFORMED

I declare that, with the exception of the items below, all work in this thesis was performed by me, Fernanda Mantovani.

With my guidance, direction and supervision, laboratory technician Jodi Morrison performed several replicates of cell survival assays, Western Blots and ELISA assays.

All data analysis, figure plotting and writing of all chapters in this thesis was performed by me, Fernanda Mantovani. Dr. Anthony Mutsaers made edits in three paragraphs of chapter 3, in preparation of this work for submission for publication.

The statistical analysis was performed with the help of Dr. Arata Matsuyama of the Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario.
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<tr>
<td>Akt</td>
<td>Serine/threonine kinase, also known as protein kinase B</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
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<td>NSCLC</td>
<td>Non-small-cell lung cancer</td>
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<td>OSA</td>
<td>Osteosarcoma</td>
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<tr>
<td>p-Akt</td>
<td>Phosphorylated Akt</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Radiation therapy</td>
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<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER I

Literature review

1.1) Canine osteosarcoma

Osteosarcoma (OSA) is the most common primary bone tumour affecting dogs, representing up to 85% of skeletal malignancies in this species [1]. The incidence of OSA in the United States is estimated to be more than 10,000 cases per year, however the true number of affected dogs is likely higher, because not all cases are confirmed and/or recorded [1]. The median age of affected dogs is 7 years, although there is also a small incidence peak at 1.5 to 2 years [1]. Osteosarcoma is observed more frequently in large and giant breed dogs, and one study showed an increased risk in Greyhounds, Rottweilers and Great Danes [2]. Increasing standard weight and height are risk factors for developing OSA, and dogs with a body weight over 40 kg accounted for 29% of affected dogs in a review of 1462 cases [1, 3].

Osteosarcoma most commonly affects the appendicular skeleton and only approximately 5% of tumours occur in bones of the axial skeleton [4]. The metaphyseal region of long bones is the most common primary site, and the thoracic limbs are two times more likely to be affected than the pelvic limbs [5]. Tumours of the oral cavity, affecting the mandible and maxilla, have been reported to account for approximately 50% of axial OSA cases [4]. In rare cases, OSA can also affect extra skeletal sites as a primary tumour location, such as the mammary gland, spleen, liver, kidney and skeletal muscles, and these forms of OSA are typically biologically more aggressive than their primary skeletal counterparts [6].
Canine OSA displays a locally aggressive and highly metastatic behaviour. Despite the fact that only 10 to 15% of dogs have clinically detectable metastasis at the time of diagnosis, approximately 90% of dogs eventually succumb to metastatic disease. Metastasis develops most commonly in the lungs, and also in bones, regional lymph node and other soft tissue sites. Local tumour progression leads to severe pain and lameness secondary to bone lysis, proliferation or both. Soft tissue swelling usually accompanies the primary lesion, and pathologic fracture of the affected bone can occur [1], especially in cases of lytic tumours affecting the femur and tibia [7]. Therefore treatment of OSA requires considerations for control of the primary tumour and delaying of metastatic disease.

Surgical removal of the primary tumour, either by amputation of the affected limb or by limb-sparing surgery, followed by adjuvant chemotherapy is considered the standard of care for canine OSA. When limb amputation is used as the sole treatment modality, median survival times of approximately 19 weeks have been reported, with 1- and 2-year survival rates of 11.5 and 2.0%, respectively [8]. In this study, 72.5% of affected dogs were euthanized due to documented consequences of distant metastasis, which occurred most commonly to the lungs (60.8%), the skeleton (5.2%), or both (4.6%). The addition of post-operative chemotherapy delays the onset of distant metastasis in dogs with OSA [9, 10].

The most commonly used chemotherapy protocols in the adjuvant treatment of OSA include single agent carboplatin, doxorubicin and cisplatin, or alternating carboplatin and doxorubicin protocols; however concurrent administration of doxorubicin and carboplatin, doxorubicin and cisplatin, and carboplatin and gemcitabine have also been reported [11-23]. The median survival times for affected dogs in these studies ranged from 235 to 365 days, and direct comparisons between different chemotherapy protocols have been performed in two studies [20,
In a retrospective investigation comparing toxicity and outcomes between various carboplatin and doxorubicin-based chemotherapy protocols after limb amputation, no significant differences in patient outcome were found [20]. In contrast, a prospective phase III clinical trial showed that dogs treated with six doses of single agent carboplatin had a statistically significantly longer time to metastasis compared to dogs treated with an alternating carboplatin and doxorubicin protocol [22]. Although a standard chemotherapy protocol has not been established for the treatment of dogs with OSA, survival times of dogs treated with surgery and adjuvant chemotherapy have remained static for the past two decades.

1.2) Radiation therapy for canine osteosarcoma

Despite providing the best success for treating the local tumour, surgery is not performed in all dogs with OSA. Limb amputation may be contraindicated in dogs with pre-existing orthopaedic or neurologic disease, or may not be elected by owners; surgical removal of the primary tumour may not be feasible in cases of OSA affecting the axial skeleton, and limb sparing surgeries are associated with a high chance of complications, including a 50% infection rate [1, 24], and are significantly more costly than limb amputation surgeries. Additionally, some dogs with OSA may not be candidates for limb spare surgery, such as in cases of tumour involving more than 50% of the affected bone, as those patients could be at increased risk of mechanical failure post-operatively [25]. Thus, there is increasing interest in treating the primary tumour by utilizing external beam radiation therapy (RT).

In veterinary oncology, the most commonly used RT modality is external beam RT, also called teletherapy, in which photons or electron beams are produced from a radiation unit [26]. Radiation therapy has mainly been applied in palliative settings to provide pain relief and
improve quality of life for canine OSA patients. Most reports in the veterinary literature describe radiation protocols consisting of two to four treatments (fractions), delivering total doses of 16 to 32 Gray (Gy) [27-30]. Gray is the unit utilized to quantify radiation, and 1Gy is equivalent to 1 joule/kilogram. Although pain control is achieved in approximately 70-90% of treated dogs, responses seen with palliative RT protocols are transient, with clinical improvement lasting approximately 2 to 4 months [26]. Treatment failure is associated with recurrent primary tumour growth and secondary pain, as the effects of RT on inhibition of OSA growth are incomplete and usually short-lived [26].

Stereotactic radiosurgery has also been investigated as a curative intent treatment modality for canine OSA [31]. This modality of RT consists of the delivery of high doses of radiation, typically delivered in one to three fractions, utilizing very precise radiation beams in a pattern that conforms to the shape of the target, while using a steep dose gradient between the tumour and surrounding normal tissue [31]. Stereotactic radiosurgery requires extreme accuracy to minimize damage to the surrounding healthy tissue, which requires a set up with specific radiation equipment, treatment planning programs and immobilization devices that allow for the radiation beams to be delivered with extreme precision. As technological advances have made it possible to deliver precise and conformal radiation to patients, and such technology is becoming more available in veterinary centres, stereotactic radiosurgery has been increasingly used in veterinary oncology; however, there is currently only one published report on the outcome of dogs with OSA treated in stereotactic radiosurgery [31]. In this study, 11 dogs were treated, 6 of which also received carboplatin chemotherapy, and limb function was considered good to excellent in all dogs for 3 months after treatment, and continued to be good in 7 dogs after that period. The median overall survival was 363 days, and the main complications were pathological
fracture, which occurred in 4 dogs, and open skin wounds in the RT field in 2 dogs [31]. Despite the promising results seen with stereotactic radiosurgery, this modality is not available at all veterinary radiation oncology centres, and dogs with extensively lytic tumours may be at higher risk of pathological fracture post-treatment, therefore limiting the use of this RT technique for only a subset of dogs with OSA.

1.3) Cellular effects of radiation therapy

The interaction of ionizing radiation with cells promotes both direct and indirect effects. Energy absorption can induce direct damage of molecules; however, most of the energy deposited within cells is absorbed by water, generating free radicals. These free radicals are highly reactive molecules, as they contain an unpaired electron on their outer shell, and can cause damage to deoxyribonucleic acid (DNA), including single and/or double strand breaks, alteration or loss of DNA bases, and formation of crosslinks, either between DNA strands or between DNA and chromosomal proteins [32, 33]. Double strand DNA breaks cause chromosomal abnormalities that, during cell division, either result in severe chromosomal aberrations or cell death by mitotic catastrophe. Free radicals induced by ionizing radiation can also interact with proteins in cell membranes, causing apoptosis in certain cell types [32, 33].

Mammalian cells have specialized mechanisms of response to DNA damage, including repairing damaged DNA, halting cell cycle progression and inducing apoptosis [33]. There are two different pathways involved in the repair of double strand DNA breaks depending on the phase of the cell cycle at the time that DNA damage occurs. The first one is homologous recombination repair, which relies on the presence of an undamaged DNA strand to serve as a template and is, therefore, error-free. This pathway occurs primarily in the S/G2 phase of the cell
The other pathway is nonhomologous end-joining, which relies in a modification of the damaged ends of DNA that allows them to be rejoined together. This mechanism is prone to errors that can cause mutagenic lesions, and occurs in the G1 phase of the cell cycle, when there is not a sister chromatid to act as DNA template [33]. Repair of DNA damage is an important strategy for cell survival post-radiation. If damaged DNA is not successfully repaired, either cell death or chromosomal aberrations may occur upon cell division [32]. With the exception of a few cell types, such as hematopoietic and lymphoid cells, which undergo apoptosis shortly after radiation exposure, most cell death secondary to irradiation takes place upon mitosis, as cells die attempting to divide because of damaged chromosomes [32, 33]. Rapidly proliferating cells have a high rate of cell division, and therefore will be more sensitive to radiation effects, or at least manifest the consequences of radiation damage sooner than slower dividing cell populations. However, cells that are proficient in DNA repair will be more resistant to radiation induced cytotoxicity [32].

The cell cycle checkpoints are involved in halting cell cycle progression in response to DNA damage or defects in replication (G1 checkpoint), as well as ensuring the mitotic spindle is intact and formed correctly prior to cell division (G2 checkpoint) [32]. In mammalian cells, RT results in delays in the cell cycle progression both in G1 and G2 [34]. One of the main regulators of the G1 checkpoint is the protein p53, which normally is found at low cellular concentrations due to its short half-life; however the cellular levels of p53 increase in response to DNA damage, including damage resulting from exposure to ionizing radiation [32, 34, 35]. The protein p53 induces the transcription of a broad variety of DNA repair proteins, and promotes downstream signalling that ultimately produces the protein p21, which has an important role in maintaining G1 arrest [32, 35]. Therefore p53 has an important role in halting the cell cycle, promoting DNA
repair and maintaining G1 arrest until DNA damage has been successfully repaired, and cells containing mutant, and therefore abnormally functional, p53 protein have a radiosensitive phenotype [32]. Additionally, p53 also has roles in the induction of apoptosis when DNA damage is too extensive for successful repair [35].

Cellular repair between radiation doses is the main reason why larger total doses of radiation can be tolerated when RT is delivered in multiple fractions [32]. The RT-induced delays in cell cycle can also cause synchronization of a population of cells, as cells in different phases of the cell cycle have different sensitivity to RT, with cells in M/G2 being the most radiosensitive, cells in G1 being intermediately radiosensitive, and cells in S phase being the least radiosensitive [32, 33]. There is an established correlation between the dose required for tumour control and the mean surviving fraction at 2 Gy for a given cell type, which is a useful indicator of tumour cell intrinsic radiosensitivity and clinical response to RT [36]. Pre-clinical work conducted in vitro using cell lines has indicated that canine OSA is a moderately radioresistant tumour, with a high mean surviving fraction after treatment with 2 Gy [37]. This moderate radioresistance of canine OSA was also seen clinically when curative RT protocols delivering doses of 2.7 to 3 Gy per fraction and chemotherapy did not provide outcomes equivalent to standard of care treatment [38]. Given the moderate resistance of OSA to radiation therapy, and lack of durable analgesia achieved in patients, increasing the sensitivity of OSA cells to ionizing radiation by utilizing sensitizing agents could enhance RT effects, possibly improving patient outcomes.

Radiation therapy also plays an important role in the treatment of cancer pain. The mechanisms that induce pain secondary to tumour infiltration include the release of chemical mediators within the tumour microenvironment, increased pressure within the bone, stretching of
the periosteum and microfractures [39], leading to both ongoing and movement-evoked pain [40]. The effects of RT in reducing bone pain in patients with OSA are proposed to result from acute disruption of inflammatory cells, decreased progression of tumour-induced osteolysis and reduction in tumour size; however the exact molecular mechanisms of radiation-induced pain relief are still unknown [26, 40]. Inflammatory cells, such as lymphocytes, which are very radiosensitive may die of radiation induced apoptosis within 24 hours of RT, inhibiting the release of chemical pain mediators and promoting quick analgesic effects [26, 39]. Recently the nociceptive ligands involved in OSA pain have been investigated [41]. In this study, canine OSA cells and naturally occurring OSA tumour samples expressed nerve growth factor, endothelin-1 and prostaglandin E2, and decreases in plasma levels of endothelin-1 and prostaglandin E2 were seen after dogs with OSA received palliative treatment with 10 Gy RT delivered in two fractions on consecutive days in combination with intravenous zoledronate and oral pain medications [41].

1.4) Epidermal growth factor receptor

Advanced studying in the field of molecular biology have allowed for the discovery of pathways that play a role in the pathogenesis and progression of cancer, which can be utilized as therapeutic targets. Kinases are enzymes that perform phosphorylation, which is the addition of phosphate groups derived from high energy molecules, such as adenosine triphosphate (ATP), to other target molecules. Tyrosine amino acids are phosphorylated by tyrosine kinases, and receptor tyrosine kinases (RTK) are a type of transmembrane protein with an intrinsic, ligand-controlled tyrosine kinase activity, and are involved in cellular signal transduction [42, 43].
All RTKs have a similar molecular structure, composed of an extracellular domain with ligand-binding sites, a transmembrane section, and a cytoplasmic protein kinase domain containing a regulatory carboxyl terminal segment [42, 44]. Ligand attachment to RTKs induces receptor dimerization, which changes the architecture of the receptor and allows for binding of ATP and subsequent tyrosine autophosphorylation, which then leads to the recruitment and phosphorylation of several intracellular substrates and autophosphorylation of tyrosine residues [42, 45]. This cascade of events ultimately results in the activation of downstream pathways that regulate cellular proliferation, migration, metabolism, differentiation and survival [42, 45]. Whereas in normal cells the activity of a RTK is tightly controlled, mutation or structural alterations in these molecules have been shown to be involved in the development and progression of many cancers [42].

The epidermal growth factor receptor (EGFR) is part of a family of RTKs comprised of the EGFR itself (also known as ERBB1 or HER1), ERBB2 or HER2, ERBB3 and ERBB4 [42, 44]. Upon ligand binding, receptor dimerization occurs in either the form of homodimers with another EGFR molecule, or as heterodimers with ERBB2/HER2. Ligands of EGFR include growth factors that bind specifically to EGFR: epidermal growth factor (EGF), transforming growth factor-α, and amphiregulin; and ligands that have dual specificity and bind to both EGFR and ERBB4: betacellulin, heparin-binding EGF and epiregulin [46]. Epidermal growth factor receptor signaling increases cell proliferation, as well as a range of processes that are essential for tumour progression, including cell motility, cell adhesion, cell survival, tumour invasion and angiogenesis [42, 47]. Overexpression of EGFR as a result of gene amplification, as well as constitutive activation of EGFR due to the presence of EGF ligands in the tumour cells or
surrounding stromal cells, have been found in numerous human cancers, including breast, lung and head and neck carcinomas [44, 46].

In veterinary oncology, EGFR expression has been identified in various epithelial malignancies, including canine lung, nasal, mammary, transitional cell carcinoma and medullary thyroid carcinoma, and feline squamous cell carcinoma (SCC) [43, 48-53]. Additionally, higher expression levels of EGFR have been associated with more aggressive cancer behaviour [43, 48, 49, 51, 52, 54]. Targeting of EGFR could play a role in the treatment of many veterinary cancer patients, and warrants investigation.

1.5) Epidermal growth factor receptor and osteosarcoma

As outlined in the above section, overexpression and constitutive activation of EGFR has been documented in many epithelial malignancies. However, the role of aberrant activation of EGFR in the pathogenesis of mesenchymal tumours, such as OSA, is less well defined. Expression of EGFR has been documented in 57 to 90% of human OSA biopsy samples and in 50 to 75% of human OSA cell lines [55, 56]. Possible associations with levels of EGFR expression and patient outcomes remain unclear, mainly due to the small sample size of these studies.

The expression of EGFR in canine OSA and its association with clinicopathological parameters and prognosis has been investigated [57]. This study evaluated frozen OSA tissues and microarrays, a panel of OSA cell lines, and normal bone. Expression of EGFR was scored on immunohistochemistry, and classified low if 10-50% of cells were positive, and high if over 50% of cells were positive. Positive cells for EGFR were present in 34.7% of tissue samples and in all
OSA cell lines. Although high expression of EGFR was noted in only 13.3% of primary OSA tissue samples, the survival times for these dogs was shorter in comparison to dogs with low or negative expression of EGFR. *In vitro* targeting of EGFR with RTK inhibitors has been reported in the veterinary literature, with successful inhibition of cell proliferation and growth of canine mammary [58] and OSA cell lines [59]. Findings of these studies are encouraging for the potential of EGFR as a therapeutic target for dogs with OSA.

**1.6) Epidermal growth factor receptor targeting in cancer treatment**

Epidermal growth factor receptor is overexpressed in many tumours commonly treated in human and veterinary oncology, and increased EGFR expression correlates with a poorer clinical outcome in many cases. It has also been demonstrated that increased number of receptors is often associated with increased production of ligands by the same tumour cells, resulting in autocrine receptor activation [44, 46]. For these reasons EGFR is considered a good target for cancer therapy.

Two main strategies have been utilized to impede EGFR signaling pathways: monoclonal antibodies that bind to the extracellular domain, and tyrosine kinase inhibitors that compete with ATP in the intracellular tyrosine kinase domain [46]. Treatment with EGFR inhibitors produces cytostatic effects *in vitro* by blocking cell-cycle progression and proliferation; however, their effects *in vivo* are greater, possibly as a result of effects on angiogenesis, invasion and metastasis [60]. Therapeutic approaches targeting EGFR signaling pathways are already in place in human oncology. Cetuximab and panitumumab are monoclonal antibodies against EGFR approved for treatment of metastatic colorectal cancer. Small molecule tyrosine kinase inhibitors that target EGFR include erlotinib, gefitinib, lapatinib and vandetanib [47]. Cetuximab, gefitinib and
erlotinib are approved for treatment of advanced colorectal cancer and non-small cell lung cancer (NSLC) [47]. Erlotinib is approved for treatment of metastatic NSLC and pancreatic cancer, and is currently being tested for colorectal cancer. Gefitinib is approved in Europe for treatment of advanced or metastatic NSLC, and is currently being studied in patients with colorectal cancer [47]. Lapatinib, a dual EGFR and HER2 inhibitor, is used in women with advanced stage or metastatic breast cancer [61], and vandetanib is being evaluated in clinical trials for non-small cell lung cancer and investigated for medullary thyroid carcinoma [62, 63].

In veterinary oncology, very few studies evaluating the use of EGFR as a therapeutic target have been performed. Kennedy and colleagues investigated the effects of vandetanib on canine mammary tumour cell lines, showing a dose dependent inhibition of EGFR phosphorylation, resulting in inhibition of EGF-stimulated proliferation and migration [58]. Gefintinib combined with crizotinib, a tyrosine kinase inhibitor that targets MET, showed an additive effect of inhibition of proliferation of canine OSA cells [59]. Cetuximab, a mouse-human chimeric anti human EGFR monoclonal antibody, has been shown to bind to canine mammary carcinoma cells in vitro, leading to inhibition of tumour cell proliferation [64]. There are currently no reports of the use of EGFR inhibitors in clinical settings in veterinary oncology, and additional investigations are required to further examine the effects and safety profile of these drugs, and their clinical applications in companion animals with spontaneous cancers.

1.7) Epidermal growth factor signaling and the PI3K-Akt pathway

The effect of EGFR tyrosine kinase activation and subsequent autophosphorylation of tyrosine residues initiates a cascade of intracellular signaling pathways. The EGFR ligand and dimer partner determine which sites are autophosphorylated and hence which signaling proteins
are subsequently engaged [65]. Intracellular signaling pathways downstream of EGFR include the phosphatidylinositol-3-kinase (PI3K) signaling cascade, which results in the activation of the serine/threonine kinase Protein kinase B (PKB/Akt). Upon stimulation of EGFR, PI3K is activated and generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn acts as a second messenger for activation of Akt. Activation of Akt leads to phosphorylation and thereby activation of numerous downstream cytoplasmic and nuclear substrates involved in processes such as cell cycle progression, apoptosis suppression, and glucose uptake and metabolism, ultimately resulting in enhanced cell survival, proliferation, and inhibition of apoptosis [65-67]. The PI3K-Akt pathway can be inappropriately activated in tumours overexpressing EGFR, which has been shown to be associated with increased radioresistance in human tumour cell lines [60].

Radiation treatment may result in enhancement of the PI3K-Akt signaling pathway in cancer cells as a mechanism of cellular stress response. When human carcinoma and glioblastoma cells were exposed to radiation in vitro, EGFR was activated via a ligand-independent mechanism, with subsequent activation of Akt, promoting increased cell survival and proliferation [66, 68]. In these studies, increased levels of phosphorylated-Akt (p-Akt) were found within 4 hours of RT, and inhibition of Akt enhanced radiosensitivity of tumour cells [66, 68]. It is possible that similar EGFR signaling pathway activation and secondary increases in levels of p-Akt could be seen following RT of canine OSA cells. Furthermore, the PI3K/Akt pathway could potentially serve as a surrogate biomarker for inhibition of upstream receptor targets like EGFR after treatment with targeted agents.
1.8) Radiosensitization of tumour cells targeting the epidermal growth factor receptor

The combining of RT with cytotoxic chemotherapy and/or targeted cancer therapeutics has been widely investigated in human oncology, with the goal of improving the effectiveness of radiation (radiosensitization) [60]. The main biological factors of tumours that affect the response to RT are the ability of surviving cells to repopulate within the time frame of the RT protocol, the extent of hypoxia in the tumour microenvironment, and the intrinsic radioresistance of tumour cells [69]. In addition, RT can induce pro-survival signaling pathways involved in cell cycle arrest and subsequent DNA repair, as well as suppression of apoptosis, which protect cancer cells from the cytotoxic effects of RT and lead to radioresistance [70].

Strategies to overcome or modulate these factors have been developed to increase the radiation-induced killing of tumour cells, such as the use of concurrent chemotherapy and RT. Unfortunately there is a significant increase in toxicity when RT and chemotherapy are used concurrently, including the development of leukopenia and mucositis [71]. The use of therapies targeting a specific pathway that is more active in cancer cells rather than in all rapidly proliferating cells present the potential to enhance RT induced tumour cell kill without increasing toxicity to the normal tissue [60, 70].

Radiosensitization of cancer cells by targeting the EGFR pathway is an attractive approach for multiple reasons. Tumours expressing high levels of EGFR have decreased sensitivity to RT [60, 72], and exposure of cancer cells to RT may activate EGFR signaling independent of ligand binding (a protective response to radiation-induced cellular stress that contributes to radioresistance) [73, 74]. Therefore, neutralizing this tumour response to radiation
by inhibiting EGFR signaling could maintain tumour sensitivity. Additionally, EGFR inhibitors commonly produce a cytostatic effect with arrest in the G1 phase of the cell cycle, which can prevent tumour cell repopulation between fractions of a RT protocol [75, 76].

The implementation of an EGFR inhibitor in combination with RT in human oncology has lead to some positive results. When the monoclonal antibody cetuximab was used in combination with RT in patients with locally advanced head and neck cancer, significant improvements in local control and overall survival were seen [77]. However, when cetuximab was combined with RT and cisplatin in a randomized phase III trial, increased toxicity and no improvement in survival was achieved in the cetuximab-containing treatment group [78]. It is still unclear if cetuximab can replace chemotherapy as a radiation sensitizer in a subset of patients with head and neck cancer, and similar toxicities were seen in patients receiving cetuximab or chemotherapy in combination with RT [79]. The addition of cetuximab to neoadjuvant chemotherapy before radiation in patients with high-risk rectal cancer significantly improved radiologic response and overall survival [80].

In human oncology, the use of erlotinib as a radiosensitizer has been successful in pre-clinical work with bladder cancer cells containing wild type EGFR [81], and with nasopharyngeal carcinoma cells tested both in vitro and in a mouse xenograft model [82]. One study with human NSCLC and prostate cancer cells showed treatment with erlotinib in combination with RT induced accumulation of cells in G1 phase with a reduction of cells in S phase, enhanced radiation-induced apoptosis, inhibited RT induced activation of EGFR, attenuated RT-induced cell repair and modulated radiosensitivity [83]. In clinical studies, erlotinib in combination with RT was well tolerated in elderly patients with esophageal cancer [84], and showed evidence of clinical efficacy with no increase in toxicity in combination with
low-dose cisplatin and RT for head and neck SCC [85], and was feasible in combination with capecitabine for patients with locally advanced pancreatic cancer [86]. A phase II clinical trial evaluating the combination of erlotinib with stereotactic RT in patients with metastatic NSCLC resulted in survival greater than historical values for patients treated solely with systemic agents [87]. These studies indicate that erlotinib can be safely and successfully used as a radiosensitizer, and similar responses could potentially be seen in veterinary oncology, including the treatment of dogs with OSA.

1.9) Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a fundamental regulator of physiological and pathological growth of blood vessels. It is an endothelial-cell-specific mitogen that increases vascular permeability, which results in the formation of an extravascular fibrin gel used as substrate for endothelial and tumour cell growth. Vascular endothelial growth factor also induces the expression of anti-apoptotic proteins in endothelial cells, resulting in cell survival [88]. The documented effects of VEGF in vitro include promoting growth of vascular endothelial cells derived from blood vessels, and inducing endothelial cells to invade collagen gels and form capillary-like structures [89]. The angiogenic effects of VEGF have also been observed in multiple in vivo models [90-92]. Vascular endothelial growth factor is produced and secreted by several cell types including platelets, lymphocytes, neutrophils, macrophages, smooth muscle cells and fibroblasts [88]. Cellular VEGF expression is stimulated by a variety of factors including hypoxia, inflammatory cytokines, growth factors, hormones and oncogenic mutations [89].
Tumours influence the surrounding host stroma by inducing angiogenesis to supply their oxygen and nutrient needs. A wide variety of tumours express VEGF, including canine mast cell tumour [93] and mammary gland cancer [94], to cite just a few, and increased VEGF production has been associated with a more aggressive tumour phenotype. Increased serum and/or plasma levels of VEGF have been identified in dogs with lymphoma, OSA, mammary tumours and hemangiosarcoma [95-98], indicating a role of VEGF in canine tumour growth and progression. In addition to tumour cells, tumour-associated stromal cells also secrete VEGF, in response to chemotactic signals released by tumour cells [99].

The expression of VEGF and its correlation with outcome has been documented in canine OSA. Detectable VEGF have been observed in canine OSA cells [100], and dogs with OSA that had higher pre-treatment serum VEGF levels had a decreased disease free interval after treatment with limb amputation and adjuvant chemotherapy [98]. The effects of treatment with anti-angiogenic agents, RTK inhibitors and RT on the expression and production of VEGF at the cellular and systemic levels has not been fully established. After treating canine OSA cells with masitinib, a RTK inhibitor which targets c-Kit and platelet-derived growth factor, VEGF levels in conditioned media increased relative to cell viability [100]. When canine OSA xenografts were treated with tepoxalin, a dual cyclooxygenase and lipoxygenase inhibitor, an increase in VEGF levels was seen, which was not accompanied by an increase in tumour growth. In this study, levels of systemic VEGF in mice implanted with xenografts and healthy dogs did not increase with tepoxalin treatment, illustrating the complexity of in vitro and in vivo correlations between angiogenesis markers [101].

One important mechanism of tumour kill by RT is the effect on the vascular supply, depriving the surviving tumour cells of nutrients and oxygen. With respect to endothelial cells,
VEGF provides a protective effect on radiation-induced damage, which may limit the effects of RT on the local vasculature [102]. Dose-dependent increases in VEGF levels after RT have been documented in human glioblastoma cells and in lung cancer mouse xenografts [103, 104], and are proposed to be associated with radioresistance. In the veterinary literature, the effects of RT on VEGF levels have been investigated in melanoma and mast cell tumours. Radiation therapy up-regulated VEGF production in a melanoma cell line in a dose-dependent manner [105], but no changes in VEGF levels post-radiation were seen in a mast cell tumour cell line [106]. The effects of RT on levels of VEGF expression by canine OSA cell lines in vitro has not been reported, and identifying the angiogenic responses to RT could help elucidate the role of combining RT with anti-angiogenic therapy in the clinic, including in dogs with OSA.

Additionally, the EGFR and VEGF pathways are correlated, as these share parallel and reciprocal downstream signaling mechanisms, and exert direct and indirect effects on tumour cells that contribute to cancer progression [107]. The epidermal growth factor, which as listed in previous sections is an important ligand for EGFR, also drives VEGF expression, and an overactive VEGF pathway plays a role in tumour resistance to treatment with EGFR inhibitors [107, 108]. In a model of murine renal cell carcinoma the interaction between EGFR, VEGF and angiogenesis have also been shown because treatment with gefitinib, a selective EGFR RTK inhibitor, resulted in decreased cell proliferation, decreased microvascular density and decreased VEGF levels [109]. Therefore, in addition to direct effects on cancer cells, treatment with EGFR inhibitors, including erlotinib, could possibly exert anti-angiogenic effects on canine OSA, further benefiting affected patients.
1.10) Rationale, Hypothesis and Objectives

Osteosarcoma is a common cancer affecting dogs and displays a locally aggressive and highly metastatic behaviour. Radiation therapy plays an important role in the treatment of the local tumour, and new strategies to improve the effectiveness of tumour cell kill from RT could improve the outcomes of dogs with OSA. Targeting EGFR is an established strategy for radiosensitization in human oncology but has not been investigated in canine OSA, a known EGFR-expressing cancer. In addition, one of the mechanisms of action for both radiation and EGFR inhibition is inhibition of angiogenesis, which may be assessed \textit{in vitro} by measuring cancer cell-produced VEGF levels.

This study hypothesized erlotinib, a tyrosine kinase inhibitor targeting EGFR, would enhance the effects of radiation therapy against canine OSA cells \textit{in vitro}.

The objectives of the study were:

1) To determine the effects of treatment with erlotinib as a single agent or in combination with radiation therapy on clonogenic survival and viability of canine OSA cells.

2) To determine the levels of EGFR, and total and phosphorylated Akt expression in canine OSA cells after treatment with erlotinib as a single agent or in combination with radiation therapy.

3) To investigate the impact of treatment with erlotinib as a single agent or in combination with radiation therapy on levels of VEGF secreted into conditioned media by canine OSA cells.
1.11) References


CHAPTER II

Introduction to cell culture techniques, cell viability, clonogenic survival and Western blot assays: pilot studies.

Cell culture models are widely utilized in pre-clinical cancer research. Cancer cells are able to grow well *in vitro* as they do not respond to signals for contact inhibition when cells become confluent on a Petri dish; therefore, cancer cells become immortalized [1]. Cell culture models allow for investigation of an anti-cancer agent’s ability to induce cytotoxic effects or to inhibit cell proliferation, and are an essential first step in the evaluation of a new anti-cancer compound, combination treatment, or application of a known anti-cancer agent to treat a specific cancer type in a novel setting [2]. Limitations of cell culture in monolayers include the lack of cancer-stromal cell interaction and tumour microenvironment, therefore limiting the ability to predict the effectiveness of the studied drug or radiation protocol in solid tumours [2].

2.1) Cell viability assays

2.1.1) Introduction

Cell viability tests assess the number of metabolically active cells in culture, and reflect the effects of treatment in reduction of cell proliferation or induction of rapid cell kill and apoptosis. Widely used assays include tetrazolium reduction, resazurin reduction, and protease activity, which all measure some aspect of general metabolism or an enzymatic activity as a marker of viable cells [3]. These assays require incubation of a reagent with viable cells for the
metabolic conversion of a substrate to a coloured or fluorescent product. The amount of substrate can be quantified with fluorescence or absorbance plate readers to record data, and these assays can be used in multiple tissue culture well formats, in which cells have been treated with a range of doses of drug [2, 3]. As only viable cells are able to convert substrate to product, these assays provide an evaluation of cellular growth for a short period, typically 1 to 5 days [4].

Resazurin is a cell permeable redox indicator. Metabolically active and therefore viable cells are able to reduce resazurin into the resorufin product. Resorufin is pink and fluorescent, and can be quantified using a microplate fluorometer equipped with a 560 nm excitation / 590 nm emission filter set. As the production of resorufin relies on the presence of viable cells in a Petri dish, the amount of measured resorufin is therefore proportional to the number of viable cells. The major advantages of the resazurin reduction assay are that it is relatively inexpensive, it is more sensitive than the tetrazolium assay, and can be multiplexed with other assays that give information on the mechanism leading to cytotoxicity [5]. One important limitation of the resazurin assay is that cell viability is a surrogate rather than absolute measurement of cell proliferation/growth, and in scenarios where cell metabolism continues even though cell growth has ceased, there would be a disconnect between cell viability assays and functional proliferation and clonogenic survival assays. Additionally, metabolically active cells with a low proliferation rate, such as cancer stem cells and autophagic cells, could still be detected on cell viability assay, although these cell populations may be constant across treatment groups, as those are typically not killed by anti-cancer treatments that target rapidly proliferating cell populations [4].

In the introductory part of this project, with the goal of familiarization with cell culture techniques and cell viability assays, the rezasurin assay was utilized to assess the cytotoxic effects of multiple doses of the chemotherapy drug doxorubicin on canine osteosarcoma cells.
2.1.2) Materials and Methods

The canine OSA cell lines Tonka, Dharma and D17 were utilized in this pilot study. Cells were grown in Dulbecco’s modified Eagle’s media (HyClone DMEM - Fisher Scientific- Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (Life Technologies, Burlington, ON, Canada) and 1% penicillin/streptomycin (BioWhittaker, Mississauga, ON, Canada). All cell cultures were maintained at 37°C and 5% CO2 in a humidified incubator. Cells were seeded into 96-well plates at a density of 1,000 cells/well, and incubated for 24 hours. Wells were treated with serial dilutions of doxorubicin, for final concentrations of 50, 10, 2, 0.4, 0.08, 0.016 and 0.0032 µM, and incubated for 24 hours. To assess cell viability, Resazurin Cell Viability Kit (Sigma-Aldrich, Oakville, ON, Canada) was used at a concentration of 5.0 mg/ml. 100 µl of resazurin solution was pipetted into each well, and the plate was observed for a change in colour every hour for a total of 12 hours. Absorbance readings were obtained from a Synergy 2 spectrophotometer (BioTek, Winooski, VT, USA), at an excitation wavelength of 570 nm and emission wavelength of 600 nm.

2.1.3) Results and Conclusion

No visually appreciable change in colour was observed in any of the wells in a 12 hour interval after addition of resazurin solution. Readings from triplicate wells were used to calculate results, and the reported results represent the 6 hour reading time point. The experiment was performed once.

The absence of colour change and lack of change in cell viability overtime, including lack of increased cell viability in control wells, are most consistent with a low cell density and
absence of viable cells. If the absence of viable cells had only been observed in treated groups with no differences among doxorubicin concentrations, it would represent pronounced cytotoxic effects at all doses utilized in the experiment. Alternatively, there could have been a problem with the drug’s activity or a miscalculation in the doses. However, as the control wells did not show an increase in cell viability over time, it indicates an overall lack of viable cells in the plate.

The cell density was later adjusted for future cell viability assays, as the 1,000 cell/well appeared to be too low for a short-term assay.

![Graph showing cell viability](image)

**Figure 2.1.1.** Effects of multiple concentrations of doxorubicin on cell viability of three canine osteosarcoma cell lines. There was no consistent reduction in cell viability, and viability of control wells did not increase over time.
2.2) Clonogenic survival assays

2.2.1) Introduction

An important activity of an anti-cancer therapy is to induce loss of indefinite proliferation of cancer cells. Cells that do not retain proliferative ability may undergo a few cell divisions or remain metabolically active, but lose their ability to generate colonies of cells that continue unlimited cell divisions [4]. If a single cell undergoes at least 6 cell divisions, it will most likely generate a colony containing more than 50 cells, which is usually capable of continued cell proliferation. Therefore cell colonies ≥50 cells are regarded as having arisen from a cell that survived treatment [4]. The number of cells plated can be divided by the number of colonies formed to generate the plating efficiency, and the ratio of the plating efficiency from treated cells to control cells is defined as the surviving fraction [2]. The surviving fraction is plotted on a logarithmic scale against doses of a drug or radiation, to produce a survival curve. Exponential decreases in cell survival are rarely seen with increasing doses of chemotherapy, but may be observed for radiation [2].

In the introductory part of this project, clonogenic survival assays were utilized to assess preliminary effects of treatment with erlotinib at 10 µM as a single agent, radiation therapy at 2Gy, and the combination of erlotinib 10 µM and 2 Gy radiation on D17 osteosarcoma cells.

2.2.2) Materials and Methods

Cells were seeded into 6 well plates at a density of 500 cells/well with 3 mls of Dulbecco’s modified Eagle’s media (Hyclone DMEM - Fisher Scientific - Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (Life Technologies, Burlington, ON, Canada) and
1% penicillin/streptomycin (BioWhittaker, Mississauga, ON, Canada). Plates were maintained in a humidified incubator at 37°C and 5% CO2 for 24 hours. Erlotinib (SelleckChem, Houston, TX, USA) at 10 µM was added to treatment group wells, and after 4-6 hours, a dose of 2 Gy of radiation was delivered to the plates, at ambient temperature and pressure, at a rate of 400 monitor units/min utilizing a 6-MV linear accelerator (Clinac IX System, Varian Medical Systems, Inc., Palo Alto, CA, USA). Control plates were kept outside the radiation vault during treatments. Plates were replaced in the incubator, and colony formation was monitored daily and the experiment stopped after 10 to 14 days, before the control colonies became confluent. Cells were stained with 0.5% crystal violet in 20% methanol. Colonies were visualized by light microscopy and counted. A colony was defined as an aggregate of ≥ 50 cells. This experiment was performed one time only, and the mean and standard deviation of 3 wells per each treatment group was utilized to calculate results.

2.2.3) Results and Conclusion

Treatment with 10 µM erlotinib in combination with 2 Gy radiation promoted the highest reduction in number of colonies compared to control group. This indicated that the proposed techniques would be feasible to evaluate the effects of erlotinib treatment and multiple doses of radiation in canine osteosarcoma cells. Colonies were easy to identify and count under light microscopy. An incubation period with erlotinib of 4-6 hours was adequate to provide measurable effects on clonogenic survival.
Figure 2.2.1. Preliminary evaluation of effects of treatment with erlotinib at 10 µM as a single agent, radiation therapy at 2 Gy, and the combination of erlotinib and radiation (2 Gy) on clonogenic survival of D17 canine osteosarcoma cells. Values are normalized to percentage of control group colonies. Error bars represent standard deviation of mean number of colonies in each group, which is 0.5 for the Erlotinib plus radiation combination treatment group and therefore not shown on graph.

2.3) Western blot assays

2.3.1) Introduction

Western blot assays are widely used for the detection of specific proteins in cell homogenate or extract. The transfer of biological samples from a gel to a membrane followed by their detection on the membrane’s surface is referred to as "blotting". Western blot assays utilize antibodies specific to the target protein, selectively detecting only the
protein of interest at concentrations as low as 0.1 ng, providing both qualitative and semiquantitative data when compared to a control protein used to assess equal lane loading [6]. There are important limitations and challenges associated with Western blot assays. The assays are laborious, with multiple steps that require technical expertise, and are relatively expensive due to the cost of antibodies [7]. Although protein detection by antibody is a very specific reaction, it is highly dependent on the antibody utilized, and false negative results could occur if an antibody directed against a target protein of a different species is utilized (for example, anti-human antibodies used to detect canine proteins), especially if the antibody has not been previously validated for use in the studied species.

The initial step in a Western blot assay is to perform polyacrylamide gel electrophoresis to separate the macromolecules, which are then transferred onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Proteins are most commonly transferred utilizing the electroelution or electrophoretic method, due to its speed and transfer efficiency. The membrane is then blocked to prevent any nonspecific binding of antibodies, and the blocked membrane is probed with a primary antibody that recognizes a specific protein or epitope on a group of proteins. Indirect detection is generally utilized in Western blot assays, as the primary antibody that recognizes the target protein not directly detectable. Therefore, tagged secondary antibodies or other detection reagents are used to ultimately detect the target antigen [6, 7].

The antibody probes that are bound to the protein of interest must be detected, which can be done utilizing colourimetric or chemiluminescent detection. For colourimetric detection, the Western blot is incubated with a substrate that reacts with the reporter enzyme that is bound to
the secondary antibody, which converts the soluble dye into an insoluble form of a different colour that precipitates next to the enzyme and thereby stains the membrane. Chemiluminescent detection methods depend on incubation of the Western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. A densitometry reading is then obtained, which is used for qualitative and semiquantitative information on the target protein [6, 7].

2.3.2) Material and Methods

D17 canine osteosarcoma cells were utilized for this experiment. Cells were grown in Dulbecco’s modified Eagle’s media (Hyclone DMEM - Fisher Scientific- Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (Life Technologies, Burlington, ON, Canada) and 1% penicillin/streptomycin (BioWhittaker, Mississauga, ON, Canada), and maintained at 37°C and 5% CO2 in a humidified incubator. Cells were seeded into six-well plates at a density of 150,000 cells/well and settled for 24 hours. Erlotinib at 10 µM was added to half of the wells of both the radiation and control plates. After a 6 hour incubation period, one plate was administered a 2 Gy radiation dose, while another plate was kept outside the radiation vault (control plate). Radiation was delivered utilizing a 6-MV linear accelerator (Clinac IX System, Varian Medical Systems, Inc., Palo Alto, CA, USA). Cells were lysed in ice cold buffer (Cell Signaling technology, Whitby, ON, Canada) containing aprotinin, phenylmethanesulfonyl fluoride and a phosphatase inhibitor cocktail, and collected 2, 24 and 48 hours post radiation, and placed immediately on ice. Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to a polyvinyl difluoride membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA). Membranes were hybridized to primary antibody against β-
actin, EGFR (Cell Signaling Technology, Whitby, ON, Canada) and horseradish peroxidase (HRP) conjugated secondary goat anti-rabbit antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA), then visualized using the Bio-Rad Chemi-Doc system (Universal Hood III).

2.3.3) Results and Conclusions

The expression of EGFR was confirmed in the canine osteosarcoma cell line D17. Treatment with erlotinib as a single agent promoted an increase in EGFR levels at 24 and 48 hours. The conditions of this pilot experiment were adequate to detect measurable protein levels, and were further utilized during additional phases of this research.
Figure 2.3.1. Western blot analysis of epidermal growth factor receptor (EGFR) levels in D17 osteosarcoma cell lysate after treatment with erlotinib at 10 µM as a single agent, radiation therapy at dose of 2 Gy, or the combination of erlotinib (10 µM) and 2 Gy radiation. The vertical y axis represents densitometry readings. Graphed results were normalized to the control β-actin.
2.4) References


CHAPTER III

Effects of epidermal growth factor receptor kinase inhibition on radiation response in canine osteosarcoma cells

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3.1 Abstract

Background

Radiation therapy is a palliative treatment modality for canine osteosarcoma, with transient improvement in analgesia observed in many cases. However there is room for improvement in outcome for these patients. It is possible that the addition of sensitizing agents may increase tumour response to radiation therapy and prolong quality of life. Epidermal growth factor receptor (EGFR) expression has been documented in canine osteosarcoma and higher EGFR levels have been correlated to a worse prognosis. However, effects of EGFR inhibition on radiation responsiveness in canine osteosarcoma have not been previously characterized. This study examined the effects of the small molecule EGFR inhibitor erlotinib on canine osteosarcoma cells’ radiation responses, target and downstream protein expression in vitro. Additionally, to assess the potential impact of treatment on tumour angiogenesis, vascular endothelial growth factor (VEGF) levels in conditioned media were measured.

Results

Erlotinib as a single agent reduced clonogenic survival in two canine osteosarcoma cell lines and enhanced the impact of radiation in one of three cell lines investigated. In cell viability assays, erlotinib enhanced radiation effects and demonstrated single agent effects. Erlotinib did not alter total levels of EGFR, nor inhibit downstream protein kinase B (PKB/Akt) activation. On the contrary, erlotinib treatment increased phosphorylated Akt in these osteosarcoma cell lines. VEGF levels in conditioned media increased after erlotinib treatment as a single agent and in combination with radiation in two of three cell lines investigated. However, VEGF levels decreased with erlotinib treatment in the third cell line.
Conclusions

Erlotinib treatment promoted modest enhancement of radiation effects in one of the canine osteosarcoma cell lines, and possessed activity as a single agent in two of the cell lines, indicating a potential role for EGFR inhibition in the treatment of a subset of osteosarcoma patients. The relative radioresistance of osteosarcoma cells does not appear to be related to EGFR signalling exclusively. Angiogenic responses to radiation and kinase inhibitors are similarly likely to be multifactorial and require further investigation.

Keywords
Osteosarcoma, dog, canine, erlotinib, epidermal growth factor receptor (EGFR), radiation, vascular endothelial growth factor (VEGF), radiosensitization

3.2 Background

Osteosarcoma (OSA) is the most common primary bone tumour of the domestic dog, occurring predominantly in large breeds, and accounting for up to 85% of skeletal tumours in this species [1]. Local tumour growth causes severe pain and lameness secondary to bone lyses, proliferation or both, and eventual metastasis from OSA to the lungs and other locations occurs in the vast majority of cases [1]. Surgical removal of the primary tumour, either by amputation of the affected limb or by limb-sparing surgery, followed by adjuvant chemotherapy is considered the standard of care for canine OSA. However, surgery may be contraindicated in dogs with preexisting orthopedic or neurologic disease, may not be elected by owners, or may not be feasible in cases of tumours affecting the axial skeleton. Thus, there is increasing interest in treating the primary tumour by utilizing external beam radiation therapy (RT) for dogs with
OSA. Radiation therapy has mainly been applied in palliative settings to provide analgesia and improve quality of life for canine OSA patients. Most reports in the veterinary literature describe radiation protocols consisting of two to four treatments (fractions), delivering total doses of 16 to 32 Gray (Gy) [2]. Although pain control is achieved in approximately 70-90% of treated dogs, responses seen with palliative RT protocols are transient, with clinical improvement lasting approximately 2 to 4 months [2]. Treatment failure is associated with recurrent primary tumour growth and therefore novel strategies to improve the response to RT for canine OSA may translate into better clinical outcomes for these patients. Pre-clinical work conducted in vitro using cell lines has indicated that canine OSA is a moderately radioresistant tumour, with a high mean surviving fraction after treatment with 2 Gy [3]. Increasing the sensitivity of OSA cells to ionizing radiation could enhance the effects of RT, possibly improving patient outcomes.

Advances in molecular biology have resulted in the identification of several pathways involved in the pathogenesis and progression of cancer, which can be utilized as therapeutic targets. The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase (RTK) involved in signaling for cell growth, proliferation, invasion and survival [4]. Over-expression and constitutive activation of EGFR have been found in numerous human cancers, including breast, lung and head and neck carcinomas [5]. In veterinary oncology, EGFR expression has been identified in various epithelial malignancies, including canine lung, nasal, mammary and transitional cell carcinoma, and feline squamous cell carcinoma (SCC) [6-11]. Additionally, higher expression levels of EGFR have been associated with more aggressive cancer behavior [6-11]. The role of aberrant activation of EGFR in the pathogenesis of mesenchymal tumours, such as OSA, is less well defined. Expression of EGFR has been documented in human [12, 13] and canine OSA [14], and correlated with a worse prognosis,
indicating that EGFR may play a role in OSA tumour biology and therefore EGFR pathway inhibition could represent a viable treatment option for OSA. In vitro targeting of EGFR with RTK inhibitors has been reported in the veterinary literature, with successful inhibition of cell proliferation and growth of canine mammary carcinoma and OSA cell lines [15, 16], further supporting EGFR inhibition as a possible treatment approach for canine OSA.

The combining of RT with cytotoxic chemotherapy and/or more targeted cancer therapeutics has been widely investigated in human oncology, with the goal of improving the effectiveness of radiation (radiosensitization) [4]. Targeting the EGFR pathway is an attractive approach for radiosensitization for multiple reasons. EGFR inhibitors commonly produce a cytostatic effect with arrest in the G1 phase of the cell cycle, which can prevent tumour cell repopulation post-radiation [17, 18]. Additionally, exposure of tumour cells to ionizing radiation can activate EGFR independently from ligands, contributing to tumour radioresistance [4, 19, 20]. Therefore, neutralizing this tumour response to radiation by inhibiting EGFR signaling could maintain tumour sensitivity. Erlotinib is a selective inhibitor of EGFR tyrosine kinase, which blocks cell cycle progression at the G1 phase and induces apoptosis of select human carcinoma cells in vitro [21]. Erlotinib has been used in the treatment of several human malignancies, and is approved for the treatment of non-small-cell lung cancer (NSCLC) and advanced pancreatic cancer in the United States. In human oncology, the use of erlotinib as a radiosensitizer has been successful in pre-clinical work [22-24], and has shown promising results in phase I/II clinical trials for head and neck SCC and NSCLC [25-27].

The effects of EGFR activation are exerted via subsequent activation of multiple downstream intracellular signaling pathways, including the phosphatidylinositol-3-kinase (PI3K) signaling cascade that culminates with activation of the serine/threonine kinase Protein kinase B
(PKB/Akt). Upon stimulation of EGFR, PI3K is activated and generates phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn acts as a second messenger for activation of Akt. Upon activation, Akt phosphorylates numerous downstream cytoplasmic and nuclear substrates, ultimately resulting in enhanced cell survival, proliferation, and inhibition of apoptosis [28, 29]. Radiation treatment may lead to enhancement of this signaling pathway in cancer cells as a response to treatment. Exposure of human carcinoma and glioblastoma cells to radiation in vitro activated Akt, and promoted increased cell survival and proliferation [28-30], through activation of EGFR via a ligand-independent mechanism. In these studies, increased levels of phosphorylated-Akt (p-Akt) were found within 4 hours of RT, and inhibition of Akt enhanced radiosensitivity of tumour cells [28-30]. It is possible that similar EGFR activation and secondary increases in p-Akt levels could be seen following RT of canine OSA. Furthermore, evaluating the PI3K/Akt pathway could potentially serve as a surrogate biomarker for inhibition of upstream receptor targets like EGFR after treatment with erlotinib or other agents.

This study investigated the effects of erlotinib alone and in combination with RT on canine OSA cell lines. Therapeutic effects were evaluated by clonogenic survival, cell viability, and the expression of target and downstream proteins. Additionally, one of the mechanisms of action for both radiation and EGFR inhibition has been shown to be inhibition of angiogenesis; therefore we investigated the impact of treatment on the levels of the potent angiogenesis factor vascular endothelial growth factor (VEGF) secreted by OSA cells into conditioned media.
3.3) Methods

3.3.1) Cell culture

Canine osteosarcoma cell lines D17, Abrams and Dharma were used. D17 cells were obtained from Sigma-Aldrich/European Collection of Cell Cultures (ECACC). Abrams cells were a generous gift from Mike Huelsmeyer at the University of Wisconsin. Both D17 and Abrams cell lines have been utilized in several published studies and have been characterized as canine OSA cells based on morphology and xenograft analysis [31]. Dharma cells were isolated and adapted to culture from a clinical case by Dr. Anthony Mutsaers, and validated as OSA by histopathologic evaluation of tumours produced from successful xenograft outgrowth following implantation in immunocompromised (nude) mice. All cells were grown in Dulbecco’s modified Eagle’s media (HyClone DMEM - Fisher Scientific- Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (Life Technologies, Burlington, ON, Canada) and 1% penicillin/streptomycin (BioWhittaker, Mississauga, ON, Canada). All cell cultures were maintained at 37°C and 5% CO2 in a humidified incubator.

3.3.2) Radiation therapy

Cell culture plates were irradiated at ambient temperature and pressure, at a rate of 400 monitor units/min utilizing a 6-MV linear accelerator (Clinac IX System, Varian Medical Systems, Inc., Palo Alto, CA, USA). Cell culture dishes were placed between two solid water-equivalent plates, with thickness of 4.5 cm on top and 5 cm on the bottom. The dose distribution for this set up was verified by a medical physicist. Control cell culture plates were transported to the radiation therapy area but kept outside the radiation vault during treatments.
3.3.3) Clonogenic survival

Cells were seeded into six-well plates (D17 and Abrams at 500 cells/well, and Dharma at 1,500 cells/well) with 3 ml of media. After 24 hours, the media of all wells was replaced and erlotinib (SelleckChem, Houston, TX, USA) at 10 µM was added to treatment group wells. Erlotinib was diluted in dimethyl sulfoxide (DMSO) resulting in a final concentration of 0.04% DMSO in each well. After incubation for 4 to 6 hours, doses of 0, 2, 4, 6, 8 and 10 Gy of radiation were administered to individual plates. Colony formation was monitored daily and the experiment stopped after 10 to 14 days, before the control colonies became confluent. Cells were stained with 0.5% crystal violet in 20% methanol for 30 minutes, then washed gently twice with tap water [32]. Colonies were visualized by light microscopy and counted. A colony was defined as an aggregate of ≥ 50 cells. The cell surviving fraction, normalized for plating efficiency, was determined for each radiation dose. All experiments were repeated three times.

3.3.4) Cell viability

To assess cell viability, Resazurin Cell Viability Kit (Sigma-Aldrich, Oakville, ON, Canada) was used at a concentration of 5.0 mg/ml. Cells were seeded into 96-well plates (D17 and Abrams at 500 cells/well, and Dharma at 2,000 cells/well), and settled for 24 hours at 37°C and 5% CO2 [33]. Erlotinib was administered at 10 µM and 40 µM, plates were incubated for 4 to 6 hours, and doses of 0, 2, 4, 6, 8 and 10 Gy of radiation were delivered to individual plates. After 72 hours, 100 µl of Resazurin solution was pipetted into each well. As the solution changed in color, absorbance readings were obtained from a Synergy 2 spectrophotometer (BioTek, Winooski, VT, USA), at an excitation wavelength of 570 nm and emission wavelength of 600 nm. Relative viable cell number was assessed in sextuplicate for each erlotinib
concentration and corresponding control group, and each experiment was repeated three times. Absorbance values were corrected for media only readings in sextuplicate wells.

3.3.5) **Protein detection**

Cells were seeded into six-well plates (D17 and Abrams at 150,000 cells/well, and Dharma at 200,000 cells/well) and settled for 24 hours at 37°C and 5% CO2. The media of all wells was replaced to divide groups into erlotinib at 10 µM or control, followed by incubation for 4 to 6 hours. Plates were irradiated with a 2 Gy dose or kept outside the radiation vault in the radiation control room during treatment. Cells were lysed in ice cold buffer (Cell Signaling technology, Whitby, ON, Canada) containing aprotinin, phenylmethanesulfonyl fluoride and a phosphatase inhibitor cocktail, and collected 0.25, 0.5, 1, 2, 24 and 48 hours post radiation, and placed immediately on ice. Cell lysis buffer additives were obtained from Sigma-Aldrich (Oakville, ON, Canada). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to a polyvinyl difluoride membrane (Roche Diagnostics Corporation, Indianapolis, IN, USA). Membranes were hybridized to an appropriate primary antibody and horseradish peroxidase (HRP) conjugated secondary antibody, then visualized using the Bio-Rad Chemi-Doc system (Universal Hood III). Primary antibodies against β-actin, EGFR, Akt and p-Akt were purchased from Cell Signaling Technology (Whitby, ON, Canada). The secondary antibodies, HRP-conjugated goat anti-rabbit IgG were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).
3.3.6) VEGF levels

Conditioned media was collected and pooled from sextuplicate wells treated with erlotinib at 10 µM, with or without radiation treatment at 2 Gy and 8 Gy after 72 hours. Levels of VEGF were quantified using the Quantikine Canine VEGF ELISA Kit (R&D Systems, Minneapolis, MN, USA), following the manufacturer’s instructions [33]. The optical density of the standard solutions was plotted against their corresponding concentrations to generate a standard curve and allow determination of sample VEGF concentrations. Absorbance was read at 450 nm and corrected by subtracting readings at 540 nm, as per manufacturer recommendation.

3.4) Statistical analysis

Statistical analyses were performed with Graph-Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). For clonogenic survival and cell viability assays, a two-way analysis of variance (ANOVA) with Sidak method for multiple comparisons was used to determine whether erlotinib treatment had an effect in clonogenic survival and cell viability compared to radiation only treatment groups. For VEGF levels, a one-way ANOVA was used to determine whether treatment with erlotinib and/or radiation had an effect on VEGF concentrations compared to control groups. To account for changes in cell number that may influence VEGF levels, readings were normalized to cell viability of respective wells, as measured by Resazurin assay. Overall significance was set at p<0.05.
3.5) Results

3.5.1) Effects of erlotinib and radiation on clonogenic survival.

Erlotinib showed single agent activity against the canine OSA cell lines through reduction in clonogenic survival in 2 of 3 cell lines: p < 0.0001 for Dharma and p = 0.0003 for D17 (Figure 3.1). Radiation administered at doses ranging from 2 to 10 Gy demonstrated a dose dependent reduction in clonogenic survival, as expected, in all 3 OSA cell lines examined (Figure 3.2). Treatment with erlotinib four to six hours prior to radiation therapy resulted in a significant reduction in clonogenic survival of Dharma OSA cells for the lower radiation doses of 2 Gy (p < 0.0001), 4 Gy (p < 0.0001) and 6 Gy (p = 0.0127). This effect was lost at the higher radiation doses that resulted in a lower survival fraction from radiation treatment alone. In this cell line the shape of the survival curve for the erlotinib group had a more narrow shoulder compared to the control curve (Figure 3.2), indicating the potential for reduced sublethal damage repair in these erlotinib treated cells. Enhancement of radiation effects was not observed in D17 (p = 0.39) and Abrams (p = 0.71) OSA cells.

3.5.2) Effects of erlotinib and radiation on cell viability

Cell viability assays were assessed 72 hours post-radiation (Figure 3.3). Radiation administered at doses ranging from 2 to 10 Gy demonstrated dose dependent reductions in cell viability for 2 of 3 OSA cell lines. The viability of Dharma cells was less impacted by radiation but interestingly, these cells were more sensitive to single agent erlotinib on cell viability assays, with statistically significant reductions in cell viability for all erlotinib treated groups (p < 0.0001), as shown in Figure 3.3. Given the lack of response seen in 2 cell lines with erlotinib at 10 µM, a higher dose of 40 µM was tested. The viability of all 3 cell lines was reduced by this
higher, but clinically/pharmacologically less relevant concentration of erlotinib. Addition of erlotinib at 40 µM resulted in decreased cell viability compared to radiation alone for all cell lines, however these effects were not statistically significant for Abrams cells at radiation doses above 4 Gy (p = 0.14). Treatment with erlotinib at the 10 µM dose further decreased viability in irradiated Dharma cells (p ≤ 0.0002), but failed to provide enhancement of radiation effects for Abrams (p = 0.25) and D17 cells (p = 0.38).

3.5.3) Expression of target proteins

Western blot analyses detected endogenous expression of EGFR, total Akt and p-Akt in all three OSA cell lines investigated. Treatment with erlotinib, with or without radiation, increased levels of p-Akt in Dharma and D17 cells at 0.25, 0.5, 1, 2 and 24 hours after radiation treatment (Figure 3.4). Levels of p-Akt showed minimal variation among treatment groups in Abrams cells. Total Akt and EGFR were detected in all cell lines at all time points and treatment combinations, with no consistent variations seen among treatment groups.

3.5.4) Effects of erlotinib and radiation on VEGF levels

Secreted VEGF was detected in the conditioned media from all three canine OSA cell lines investigated. Changes in VEGF levels compared to control occurred more consistently after combination treatment with radiation doses of 2 and 8 Gy (Figure 3.5). Interestingly, conditioned media from Dharma and Abrams cells showed increases in VEGF levels, whereas D17 cells showed decreases. Exposure to radiation at 8 Gy provided a significant reduction in VEGF levels for D17 cells (p > 0.09), but not other statistical significant changes.
3.6) Discussion

The interaction of ionizing radiation with cells promotes both direct and indirect effects. Energy absorption can induce direct damage of molecules; however, most of the energy deposited within cells is absorbed by water, generating free radicals. These are highly reactive molecules that can cause breakage of deoxyribonucleic acid (DNA) strands. If damaged DNA is not successfully repaired, either cell death or chromosomal aberrations may occur upon cell division [34]. With the exception of a few cell types, such as lymphocytes, that undergo apoptosis shortly after radiation exposure, most cell death secondary to irradiation takes place by mitotic catastrophe [34]. Rapidly proliferating cells have a high rate of cell division, and will therefore be more sensitive to radiation effects, or at least manifest the consequences of radiation damage sooner than slower dividing cell populations. However, cells that are proficient in DNA repair will be more resistant to radiation cytotoxicity. The rate of cell death and repair can influence assays utilized to evaluate the effects of RT in vitro, such as the clonogenic survival and cell viability assays utilized in this study.

After irradiation, cells may continue to be metabolically active (which is detectable in viability assays), but they may lose the capacity to undergo normal cell division and maintain continued reproductive ability [34]. Clonogenic survival assays after RT assess a cell’s ability to survive treatment, preserve cell division and repopulate the tumour, and therefore these assays provide an important in vitro assessment of potential therapeutic success. Radiation dose-response cell survival curves based on colony formation assays represent the total cumulative clonogenic outgrowth. The shoulders of these curves illustrate the capability of cells to repair sublethal DNA damage, and a wider shoulder indicates more efficient repair and subsequent repopulation, keeping clonogenic survival high. Clonogenic survival curves in this current
study were in agreement with previously reported RT dose-response curves for canine OSA cells, displaying a wide shoulder and moderate radioresistance [3]. Treatment with erlotinib provided statistically significant reductions in clonogenic survival of Dharma cells at doses of 2, 4 and 6 Gy compared to control groups (Figure 3.2). The radiosensitization effects of erlotinib are proposed to be, at least in part, secondary to cell cycle arrest in the G1 phase [4, 17, 18]. Cells in G1 phase are less radiosensitive than cells in G2 or M phases of the cell cycle, which makes radiosensitization by EGFR inhibitors appear counterintuitive. However, arrest in G1 also provides a cytostatic effect that prevents tumour cell repopulation between fractions of RT, still potentially enhancing the efficacy of a radiation protocol [4, 17, 18, 34]. It is possible that the sequence and timing of treatment with a targeted EGFR inhibitor may play a role in radiosensitization. In this study, cells were pre-treated with erlotinib 4 hours prior to radiation, and drug treatment after radiation was not investigated. Figure 3.2 also illustrates a narrowing of the shoulder in the cell survival curve for Dharma cells, indicating that decreased repopulation might have contributed to enhancement of radiation effects seen on this cell line. Colony formation assays in the present study showed no radiosensitizing effects of erlotinib on Abrams or D17 cells.

Cell viability assays, such as the Resazurin assay, rely on bioreduction of the reagent dye by metabolically active cells, providing an indirect determination of cell viability. Such assays may not reflect the later death following cell divisions that is reflected in clonogenic survival assay results. Nevertheless, cell viability was assessed in the present study to investigate possible radiation enhancing effects of erlotinib by multiple mechanisms (Figure 3.3). Abrams cells showed marked radiation dose-dependent reduction of cell viability, but moderate radioresistance on clonogenic survival assays. This discrepancy could be explained by efficient
repopulation. Abrams cells are very fast growing with a doubling time of approximately 17 hours. Therefore at 72 hours post-radiation, multiple cell divisions would likely have occurred, with consequent mitotic deaths, and corresponding low cell viability on Resazurin assays. Any surviving clones would have then undergone repopulation, resulting in the cell survival curves generated after several days of growth and shown in Figure 3.2. It could be concluded that erlotinib failed to prevent repopulation of Abrams cells, at least at the 10 µM dose. These findings were in contrast with Dharma cells, which showed less pronounced dose-dependent effects of radiation on cell viability assays, yet radiation demonstrated consistent suppression of clonogenic survival. Dharma cells have a doubling time of 34 hours. Thus, after only 72 hours the cytotoxic effects of RT may have been undetectable, as a significant proportion of cells had not yet undergone mitosis. D17 cells showed moderate radiation dose-response sensitivity on cell viability assays, which was more pronounced on clonogenic survival assays. Interestingly, the doubling time of D17 cells is 23 hours, which is longer than Abrams cells but shorter than Dharma cells.

Erlotinib treatment promoted cytotoxic effects as a single agent at 10 µM for Dharma and D17 cells, and at 40 µM for all three cell lines investigated. Additionally, enhancement of radiation effects was seen at the 40 µM dose for all cell lines on cell viability assays. In addition to dosing, the order of treatment and period of exposure can influence the effects of combination therapy. In this study erlotinib was administered to cells 4 to 6 hours prior to RT, and remained in the media until the end of experiments, in an effort to mimic how RTK inhibitors are used clinically. It is possible that a more prolonged period of erlotinib exposure prior to RT could have promoted enhancement of RT effects on D17 and Abrams cells. Nevertheless, the cytotoxic and radiation enhancing effects of erlotinib demonstrated in this current study support in vivo
evaluation of EGFR inhibition as a possible treatment strategy for a subset of canine OSA cases. As the erlotinib-induced enhancement of RT effects on cell viability were more pronounced at RT doses of 2 and 4 Gy, it can be speculated that EGFR inhibition might be more effective in potentiating the effects of hyperfractionated curative protocols as opposed to the currently used palliative hypofractionated RT protocols. Further investigations of the ideal dosing, timing of drug exposure and RT protocol, utilizing additional OSA cells, xenograft models and other EGFR inhibitors is recommended to improve our understanding of potential radiosensitization effects of EGFR targeting in canine OSA. Given that not all patients are likely to benefit from this therapy, evaluation of potential biomarkers of treatment response, including and beyond EGFR expression and pathway activation for individual tumours could be investigated further.

Protein analysis by Western blot confirmed EGFR expression in all three cell lines, with no variation in levels among treatment groups consistently throughout the time points examined. In contrast to antibody therapeutics such as cetuximab that can impact receptor trafficking, the small molecule kinase inhibitor erlotinib may not be expected to cause decreased total EGFR with signaling inhibition. The protein Akt was evaluated as a potential downstream indicator of EGFR kinase signaling inhibition. Activation of Akt post-radiation has been documented in human carcinoma and glioblastoma cells in vitro [28-30]. In the current study, increased levels of p-Akt post RT were not observed. It is possible that RT treatment does not activate the EGFR pathways in canine OSA cells as occurs with human carcinoma and glioblastoma cells [28-30]. Interestingly however, increased levels of p-Akt were observed after erlotinib was used as a single agent or in combination with RT in D17 and Dharma cells. Increased levels of p-Akt may contribute to cell survival, and this was an unexpected finding with erlotinib treatment. These findings in the context of EGFR inhibitor use suggest that the EGFR pathway may not be
exclusively responsible for the radioresistance of canine OSA, and illustrate that signaling responses after molecular targeting agents may be multifaceted. Other signaling cascades downstream of EGFR not investigated herein, such as the mitogen-activated protein kinases (MAPK/erk) pathway, could also be involved in the cytotoxic effects of erlotinib. Further evaluation of signaling events post RT and EGFR inhibition for canine OSA cells is warranted, as such studies could shed more light on the potential mechanisms involved in this treatment and improve targeted therapeutic strategies for this cancer.

The amount of VEGF secreted by OSA cells constitutively and after treatment with RT, erlotinib and combinations was quantified in this study. Increased serum VEGF levels in dogs with OSA has been correlated with decreased disease free intervals [35], and constitutive VEGF levels have previously been observed in canine OSA cells [33]. Dose-dependent increases in VEGF levels after RT have been documented in human glioblastoma cells and in lung cancer mouse xenografts [36, 37], and have been proposed to be associated with radioresistance. In the veterinary literature, RT up-regulated VEGF production in a melanoma cell line in a dose-dependent manner [38], but no changes in VEGF levels post-radiation were seen in a mast cell tumour cell line [39]. There are also correlations between the EGFR and VEGF pathways, as these share parallel and reciprocal downstream signaling mechanisms, and exert direct and indirect effects on tumour cells that contribute to cancer progression [40]. Additionally, epidermal growth factor, an important ligand for EGFR, also drives VEGF expression, and an overactive VEGF pathway plays a role in tumour resistance to treatment with EGFR inhibitors [40,41]. Treatment with gefitinib, a selective EGFR RTK, resulted in decreased cell proliferation and decreased microvascular density and VEGF levels in murine renal cell carcinoma [42].
In the present study, VEGF production was not up-regulated after RT, and statistically significant decreased levels were seen by D17 cells after 8 Gy of radiation. Treatment with RTK inhibitors can modulate VEGF levels in an off-target manner. Increased VEGF levels have been found in vitro after canine OSA cells were treated with masitinib, a RTK inhibitor targeting c-Kit and platelet-derived growth factor receptor [33]. In our study, statistically significant changes in VEGF levels occurred more consistently after combination therapy. Additionally, D17 cells showed decreases whereas Dharma and Abrams cells had increases in VEGF production. This variability in VEGF levels post tyrosine kinase inhibitor treatment and RT illustrate the complexity of responses of individual cancers to cytotoxic stimuli, and the need for further investigation of angiogenic responses to anti-cancer therapeutics.

3.7 Conclusions

Erlotinib treatment promoted modest enhancement of radiation effects in canine OSA cells, and showed activity as a single agent, indicating a possible role of EGFR inhibition in the treatment of a subset of OSA patients. Radioresistance of OSA cells does not appear to depend exclusively on EGFR signaling. Expanding research into signaling cascade alterations and angiogenic responses to combinations of RT with RTK inhibitors are worthy of further investigation.
3.8 List of Abbreviations

Akt: Serine/threonine kinase, also known as protein kinase B
DNA: Deoxyribonucleic acid
EGFR: Epidermal growth factor receptor
MAPK: mitogen-activated protein kinase, also known as erk
NSCLC: Non-small-cell lung cancer
OSA: Osteosarcoma
p-Akt: Phosphorylated Akt
PI3K: Phosphatidylinositol-3-kinase
PIP3: Phosphatidylinositol-3,4,5-trisphosphate
RT: Radiation therapy
RTK: Receptor tyrosine kinase
SCC: Squamous cell carcinoma
VEGF: Vascular endothelial growth factor

3.9 Competing interests

The authors declare that they have no competing interests.

3.10 Author’s contributions

FM carried out clonogenic and cell viability assays, carried out the experiments for protein analysis, analyzed the data and drafted the manuscript. JM carried out Western blots and ELISA analyses, conducted replicates of cell viability experiments and helped with data analysis.
AJM conceived of the study and participated in its design and coordination, and revised the manuscript. All authors read and approved the final manuscript.

3.11 Acknowledgements

The authors would like to thank the radiation therapists from the Ontario Veterinary College, Laura Furness, Maria Helena Hartono and Kim Stewart for their keen contribution with irradiation of cells throughout the study period, radiation oncologist Valerie Poirier, as well as medical physicist Andre Fleck from the Grand River Cancer Centre for verification of dose distribution. The authors also thank Dr. Arata Matsuyama for his input regarding statistical analysis.

3.12 References


3.13 Figures

**Figure 3.1. Effects of single agent erlotinib on survival of control colonies.** Canine OSA cells treated with erlotinib at 10 µM for 4-6 hours (erlotinib). Experiments were repeated three times and average of results is shown, expressed as percentage of control, set to 100%. Erlotinib showed single agent activity through reduction in clonogenic survival in 2 out of 3 cell lines. * p < 0.05 indicates statistical significant reduction in clonogenic survival compared to control, and error bars represent standard deviation of mean number of colonies in each group.
Figure 3.2. Clonogenic survival curves. Canine OSA cells were treated with radiation only (control) or in combination with erlotinib at 10 µM (erlotinib) given 4-6 hours before RT. Experiments were repeated three times. Averages of results are shown and error bars represent standard deviation for each group. Survival fractions are plotted on a log-scale. Erlotinib treatment resulted in statistically significant reduction in cell survival of Dharma cells for radiation doses of 0, 2, 4 and 6 Gy, and statistically significant reduction in cell survival for D17 cells at 0 Gy, but did not promote enhancement of radiation effects for D17 or Abrams cell lines. * p < 0.05 indicates statistically significant reduction in clonogenic survival compared to control at the corresponding radiation dose.
Figure 3.3. Cell viability assays 72 hours post-radiation. Cells were treated with either radiation only (control), or radiation plus erlotinib at 10 µM or 40 µM. Experiments were repeated three times. The averages of results are shown and error bars represent standard deviation for each group. Addition of erlotinib resulted in statistically significant decreases in cell viability for Dharma cells at 10 µM, and for all cell lines at 40 µM dose. Enhancement of radiation effects were less pronounced at 10 µM, as seen in Dharma cells. * p<0.05 indicates statistically significant reduction in percentage of viable cells compared to control group at the corresponding radiation dose.
Figure 3.4. Western blot analysis of EGFR and downstream proteins. EGFR, total Akt and p-Akt were detected in all OSA cell lines investigated. Higher levels of p-Akt were seen after treatment with erlotinib, with or without radiation, in Dharma and D17 cells at 0.25, 0.5, 1, 2 and 24 hours post RT.
Figure 3.5. **VEGF in conditioned media 72 hours post-radiation.** VEGF levels are expressed as a ratio of change from control. * p < 0.05 indicates statistical significant change and error bars represent standard deviation in each group. Changes in VEGF levels were variable among cell lines, but significant changes occurred most consistently with higher dose of erlotinib (40 µM) and with combination RT plus erlotinib treatment.
Table 3.1. Median VEGF concentration in conditioned media 72 hours post-radiation (pg/mL)

<table>
<thead>
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<th></th>
<th>Abrams</th>
<th>Dharma</th>
<th>D17</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>57.8 ± 36.4</td>
<td>476.7 ± 177.2</td>
<td>143.7 ± 60.1</td>
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<tr>
<td>Erlotinib</td>
<td>144.1 ± 63.4</td>
<td>413.9 ± 204.6</td>
<td>157.6 ± 91.4</td>
</tr>
<tr>
<td>2Gy</td>
<td>34.8 ± 20.4</td>
<td>465.8 ± 181.1</td>
<td>139.2 ± 57.1</td>
</tr>
<tr>
<td>8Gy</td>
<td>21.1 ± 7.7</td>
<td>447.3 ± 162.9</td>
<td>135.5 ± 37.8</td>
</tr>
<tr>
<td>2Gy+Erlotinib</td>
<td>130.4 ± 55.6</td>
<td>490.9 ± 225.3</td>
<td>148.9 ± 73.3</td>
</tr>
<tr>
<td>8Gy+Erlotinib</td>
<td>52.8 ± 15.9</td>
<td>398.8 ± 92</td>
<td>163.4 ± 54.9</td>
</tr>
</tbody>
</table>

Table 3.2. Median VEGF concentration 72 hours post-radiation normalized to cell viability (pg/mL) * indicates significant change from control (p<0.05)

<table>
<thead>
<tr>
<th></th>
<th>Abrams</th>
<th>Dharma</th>
<th>D17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.57</td>
<td>4.76</td>
<td>0.76</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>1.22*</td>
<td>7.66</td>
<td>0.75</td>
</tr>
<tr>
<td>2Gy</td>
<td>0.37</td>
<td>5.22</td>
<td>0.61</td>
</tr>
<tr>
<td>8Gy</td>
<td>0.44</td>
<td>5.67</td>
<td>0.49*</td>
</tr>
<tr>
<td>2Gy+Erlotinib</td>
<td>1.32*</td>
<td>9.96</td>
<td>0.56</td>
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<td>8Gy+Erlotinib</td>
<td>1.14*</td>
<td>9.32*</td>
<td>0.38*</td>
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CHAPTER IV

Discussion and conclusions

Canine OSA is a locally aggressive and highly metastatic tumour, which affects at least tens of thousands of dogs yearly in the United States [1], and many more in Canada and worldwide. Therefore novel treatment strategies for canine OSA would benefit many patients. Radiation therapy has become more available in many veterinary centres in North America, and is an important modality in the treatment of dogs with OSA. Radiation therapy has most commonly been used in palliative settings for dogs with OSA, and unfortunately most dogs treated with palliative RT succumb to progressive local pain and lameness within 2-4 months [2]. One of the likely reasons why the effects of RT in dogs with OSA are short-lived is the intrinsic radioresistance of canine OSA cells [3]; therefore, trying to enhance the effects of RT on OSA by utilizing sensitizing drugs could improve the outcome of these patients. However, radiosensitization strategies for canine OSA have not been previously reported. Epidermal growth factor receptor targeting has been utilized as a radiosensitization strategy in human oncology [4-10]. Because EGFR expression has been documented in canine OSA and correlated with a worse outcome [11], EGFR expression has been associated with radioresistance in many tumours [12], and drugs targeting EGFR are available for clinical and pre-clinical use, this pathway was the chosen target of the present study, which investigated the effects of the addition of erlotinib, a selective EGFR RTK inhibitor, on the radiation response of canine OSA cells.

Clonogenic survival assays were utilized in the first part of this study. When this assay is utilized after treatment with radiation, a cell’s ability to survive treatment, preserve cell division
and repopulate the tumour is assessed [13]. Treatment with radiation at doses ranging from 2 to 10 Gy promoted a dose dependent reduction in clonogenic survival, as expected, in all three OSA cell lines examined. Treatment with erlotinib provided enhancement of radiation effects in Dharma cells, which was not observed in D17 and Abrams OSA cells. Statistically significant reductions in cell survival of Dharma cells at radiation doses of 2, 4 and 6 Gy were seen in erlotinib treated cells compared to control groups. Interestingly, this effect was not observed at the higher radiation doses that resulted in a lower survival fraction from radiation treatment alone. As canine OSA cells are more sensitive to higher RT doses, it can be speculated that only a very small fraction of the cells would survive and then repopulate the tumour at RT doses of 8 and 10 Gy, making the effects of erlotinib treatment on radiation dose-response less pronounced. It is also possible that the total number of surviving colonies present in wells after 8 and 10 Gy RT doses was too small for differences between control and erlotinib treated groups to be observed, underestimating the effects of erlotinib at the higher RT doses. As tumour cell repopulation between fractions is an important cause of tumour recurrence following RT, it is possible that EGFR inhibition with erlotinib may be more effective in potentiating the effects of curative intent RT protocols for canine OSA, in which doses of 2 to 4 Gy are typically utilized daily for 2 to 4 weeks, as erlotinib exerts cytostatic effects of arrest in the G1 phase of the cell cycle [14].

Other factors could have impacted the modest radiation enhancement results of erlotinib treatment in the present study. The period of drug exposure and the order of treatment can influence the effects of combination therapy. In this study erlotinib was administered to cells 4 to 6 hours prior to RT, and remained in the media until the end of experiments. The timing of erlotinib treatment pre-RT was determined based on the availability of the radiation therapy unit,
typically in the late afternoon after all the patients in the veterinary hospital were treated, and the media was not changed in an effort to mimic how RTK inhibitors are used clinically, with oral daily to every other day dosing utilized most commonly. It is possible that a more prolonged period of erlotinib exposure prior to RT would have promoted more pronounced enhancement of RT effects in the present study. Further investigation of timing and order of erlotinib pre-radiation could help further define the radiosensitizing effects of erlotinib in canine OSA.

After irradiation, cells may continue to be metabolically active, but they may lose the capacity to undergo normal cell division and reproductive ability [13]. Therefore, cell viability assays, such as the resazurin assay utilized in this study, may not accurately reflect the later death following cell divisions that is reflected in clonogenic survival assay results [13]. The differential cytotoxicity properties measured by each assay could explain the discrepancy in results found between clonogenic survival and resazurin assays in this study. Radiation dose-dependent reductions in cell viability were observed for D17 and Abrams cells; however the viability of Dharma cells was less impacted by RT at the clinically-relevant doses studied. Cell death after irradiation typically takes place upon cell division, and with rapidly growing cell lines, this usually occurs within the 72 hours time point of the resazurin assay used in the present study. Therefore, it is possible that a significant proportion of Dharma cells might have not yet undergone mitosis. This is in contrast with D17 and Abrams cells, which have a faster growth rate than Dharma cells and demonstrated a more pronounced radiation dose-dependent reduction in cell viability at 72 hours. The effects of erlotinib on cell viability were also variable among the three cell lines. The 10 µM dose of erlotinib utilized in this study was chosen based on preliminary data on cell viability and apoptosis assays from preliminary experiments in canine and murine OSA cells performed by other researchers at our laboratory. In this study, when the
10 µM dose of erlotinib was utilized, no reduction in cell viability was seen for D17 and Abrams OSA cells. Given this lack of response seen in the two cell lines, a higher dose of 40 µM was also tested. Interestingly, Dharma cells were more sensitive to single agent erlotinib, with statistically significant reductions in cell viability for all erlotinib treated groups for both 10 and 40 µM concentrations. It is possible that this more pronounced sensitivity to erlotinib resulted from cytostatic effects of arrest in G1 phase of the cell cycle [14] and associated reduced metabolic activity, as the resazurin assay relies on bioreduction of the reagent dye by metabolically active cells. It is also possible that Dharma cells were more dependent on the EGFR pathway for survival and proliferation, and therefore intrinsically more sensitive to EGFR inhibition by erlotinib. One of the limitations of this study is that all the assays were performed in vitro, with a relatively small number of OSA cell lines, limiting the extrapolation of results to a general population of patients affected by OSA.

Although the evaluation of the cytotoxic effects of erlotinib was not a primary objective of this study, the activity of erlotinib alone demonstrated in both the clonogenic survival and the cell viability assays are encouraging for a possible role of EGFR inhibition in the treatment of canine OSA. The use of an EGFR inhibitor in the treatment of canine OSA has only been reported in vitro with gefitinib, a selective EGFR RTK inhibitor [15], which promoted a dose-dependent reduction in cell proliferation in one OSA cell line (OSA8). Additionally, evaluating the effects of combination treatment with erlotinib and chemotherapy in canine OSA should be considered, as RTK inhibitors can enhance the effects of chemotherapy agents. Masitinib, a RTK inhibitor targeting c-Kit and platelet-derived growth factor receptor, when used in combination with gemcitabine in vitro generated synergistic growth inhibition of canine OSA cells Abrams and D17 [16], and it is possible that erlotinib treatment could provide similar effects. Further in
*vitro* investigations utilizing various dosages, timing and order of exposure, and combination with chemotherapy agents commonly utilized in the treatment of canine OSA, such as doxorubicin and carboplatin, would provide valuable information on the effects of erlotinib against canine OSA.

When erlotinib was used at 40 µM dose in the present study, a reduction in viability was seen for all three OSA cell lines examined. This higher concentration of erlotinib is unfortunately less relevant clinically and pharmacologically. The standard dose of erlotinib used in human oncology is 150 mg administered once daily. Pharmacokinetic evaluation of serum levels after erlotinib administration in patients with NSCLC showed a median serum level of 3.9 µM after 7 days of therapy, and patients that experienced skin rash as a side effect had higher median serum erlotinib levels of 6.8 µM [17]. Therefore erlotinib concentrations in the 40 µM range would be ten times above these reported serum levels post-erlotinib treatment. It is difficult to predict how the serum levels in dogs treated with erlotinib would relate to the reported post-erlotinib treatment serum levels in humans. The clinical use of erlotinib has not been reported in tumour bearing dogs, and studies evaluating the safety, bioavailability, and correlations with serum levels and target inhibition would be necessary to determine the role of erlotinib in veterinary oncology. It is also possible that the addition of erlotinib, or other sensitizing agents targeting EGFR, could induce a higher rate of severe radiation side effects, such as mucositis and cutaneous moist desquamation, secondary to increased radiosensitivity of the normal tissue in the RT field. Another strategy that could be considered is combining erlotinib with stereotactic radiosurgery, in which very precise radiation beams are delivered in a pattern that conforms to the shape of the target. As this modality of RT allows for significantly sparing of the normal tissue surrounding the tumour, radiation side effects could be minimized despite the addition of a
sensitizing agent. Studies evaluating the safety of radiosensitizing agents in veterinary patients are an important step to determine the role of this treatment strategy in patients undergoing RT.

An important limitation of this study is that only one drug targeting EGFR was investigated. There are other commercially available small molecule RTK inhibitors targeting EGFR, such as gefitinib and vandetanib, as well as monoclonal antibodies targeting EGFR, which can be considered as radiosensitizing agents for canine OSA. Cetuximab, a mouse-human chimeric anti human EGFR monoclonal antibody, has been shown to bind to canine mammary carcinoma cells in vitro, leading to inhibition of tumour cell proliferation [18], and it is possible that cetuximab would also bind to canine OSA cells. In human OSA cells expressing EGFR, treatment with cetuximab augmented the cytolytic function of natural killer cells by antibody-dependent cellular toxicity [19], indicating antibody targeting might be a viable strategy for EGFR inhibition in OSA. As previously discussed, treatment with RT induces constitutive activation of EGFR pathway in certain cancer cells [20, 21]. Therefore inhibition of EGFR could counteract these effects and increase the cytotoxic effects of RT. It is possible that treatment with erlotinib after rather than prior to RT could provide a different effect in counteracting this ligand-independent constitutive activation of EGFR, possibly providing more pronounced radiation enhancement effects. However, it is also possible that the EGFR pathway is not activated after RT in canine OSA cells, limiting the radiosensitizing properties of erlotinib in this tumour. Further studies exploring different drug exposure times and order of treatment utilizing additional cell lines and xenograft models are indicated to further assess the radiosensitization properties of erlotinib in canine OSA. Nevertheless, the radiation enhancement effects found in the present study are encouraging for a possible role of combination treatment with erlotinib and RT for canine OSA. This combination treatment could also be explored in other companion
animal tumours with known overexpression of EGFR, such as feline squamous cell carcinoma and canine nasal carcinoma [22, 23]. In addition to EGFR, other targets could be considered and investigated for radiosensitization strategies in canine OSA, including VEGF. Toceranib, a small molecule RTK inhibitor that targets vascular endothelial growth factor receptor 2, among other RTKs, is a veterinary specific drug that has reported biological activity against canine OSA [24], and has been administered to dogs receiving concurrent palliative RT for mast cell tumours [25]. The effects of toceranib as a radiosensitizing agent have not been reported, and could be evaluated in vitro and in clinical settings for canine OSA.

Expression of EGFR was confirmed by Western Blot analysis in all canine OSA cells examined in this study, in agreement with previous reports of EGFR expression in canine OSA [11, 15]. No consistent variation in EGFR levels was observed among treatment groups at 0.25, 0.5, 1, 24 and 48 hours post RT. This finding was not surprising as signaling inhibition of EGFR pathway with erlotinib may not be expected to cause decreased total EGFR. Instead, changes in downstream protein activation and levels would be expected to occur with RTK inhibition. The protein Akt was evaluated as a potential downstream indicator of EGFR kinase signaling inhibition. Interestingly, in the present study increased levels of p-Akt were observed after erlotinib was used as a single agent or in combination with RT in D17 and Dharma cells. Activation of the PI3K-Akt pathway and secondary increase in levels of p-Akt may contribute to cell survival, and this was an unexpected finding with erlotinib treatment. It is possible that the levels of p-Akt changed over time and perhaps they were not increased at the 72 hour and 10-14 day time points evaluated in the cell viability and clonogenic assays, respectively, when cytotoxic effects of erlotinib and RT were documented. The earlier time-points for protein analysis were elected based on previous work showing increased levels of p-Akt post-radiation
in human epithelial and glioblastoma cells [26, 27]. In the current study, increased levels of p-Akt post RT were not observed. It is possible that RT treatment does not activate the EGFR pathway in canine OSA cells as occurs with human carcinoma and glioblastoma cells, indicating that the EGFR pathway may not be responsible for the inherent radioresistance of canine OSA. Another interesting finding was that levels of p-Akt had the least appreciable variation in all treatment groups compared to control for Abrams cells, which were the cells most resistant to erlotinib and RT. Other signaling cascades downstream of EGFR not investigated in the present study, such as the mitogen-activated protein kinase (MAPK/erk) pathway, could also be involved in the cytotoxic effects of erlotinib. The lack of decrease in p-Akt levels post-erlotinib treatment illustrates that signaling responses after molecular targeting agents may be multifaceted and complex.

The final objective of the study was to quantify the amount of VEGF secreted by OSA cells constitutively and after treatment with erlotinib, RT and combined erlotinib with RT. Increases in VEGF levels were seen for Dharma and Abrams OSA cells in the erlotinib, 8 Gy radiation, and combination erlotinib and 8 Gy radiation treatment groups. Treatment with RTK inhibitors can modulate VEGF levels in an off-target manner, and increases in VEGF levels in conditioned media have been reported after OSA cells were treated with masitinib [28]. A similar effect was seen after erlotinib treatment in two of three cell lines examined in the present study, including a statistically significant increase in VEGF levels in Abrams cells compared to the control group. Relationships between the EGFR and VEGF pathways have been demonstrated, as activation of EGFR induces VEGF expression in cell culture models [20], and these pathways share parallel and reciprocal downstream signaling mechanisms, and exert direct and indirect effects on tumour cells that contribute to cancer progression [21]. Correlations
between the changes in VEGF levels and the cytotoxic effects of erlotinib were not observed in the present study. Levels of VEGF displayed a statistically significantly increase in Abrams cells, which were most resistant to erlotinib treatment, whereas Dharma cells, which were the most sensitive to erlotinib treatment, had increased VEGF that was not statistically significant. D17 cells had decreases in VEGF levels despite no reduction in cell viability assay at the respective time-point. Therefore more definitive conclusions regarding treatment with EGFR blockade and consequent cytotoxic and angiogenic responses in canine OSA require further investigation. The effects of RT on VEGF secretion by canine OSA have not been previously reported. Levels of VEGF after RT have been evaluated in canine melanoma and canine mast cell tumour cells, with a dose dependent increase in VEGF production demonstrated in the melanoma cell line [29], but no changes in VEGF levels were seen in the mast cell tumour cell line [30]. In the present study, treatment with RT at the 8 Gy dose promoted increases in VEGF levels in Abrams and Dharma cells, but promoted a statistically significant decrease in VEGF levels in D17 cells. As these previous studies utilized only one cell line [29, 30], and variable results were observed among OSA cell lines in the present study, definitive conclusions on the effects of RT on VEGF production cannot be made at this time. The effects on angiogenesis after RT treatment also results from cytotoxic effects on endothelial cells within the tumour microenvironment and in the normal tissues in the radiation field. Therefore the cell culture model has limitations for not representing such interactions between RT and the tumour environment. The variability in VEGF levels post tyrosine kinase inhibitor treatment and RT found in the present study illustrates the complexity of responses of individual cancers to cytotoxic stimuli, and the need for further investigation of angiogenic responses to anti-cancer therapeutics.
In conclusion, treatment with erlotinib demonstrated single agent activity and modest radiation enhancement effects on canine OSA cells, indicating a possible role of EGFR inhibition in the treatment of canine OSA. This study has also provided off-target effect data to support the further investigation of RTK inhibitors as a radiosensitization strategy for canine OSA, as modulation of VEGF levels can result from RTK treatment. In addition, it does not appear that RT treatment alone activates the EGFR pathway in canine OSA cells, and therefore radioresistance of OSA cells may not depend exclusively on EGFR signaling. Future studies evaluating multiple signaling cascade alterations and angiogenic responses, including modeling the tumour microenvironment, following treatment combinations of RT with RTK inhibitors are worthy of further investigation.

References.


CHAPTER V
APPENDICES

5.1 Figures

**Figure 5.1.** Photograph of the radiation therapy set up. Cell culture dishes were placed between two solid water-equivalent plates, with thickness of 4.5 cm on top and 5 cm on the bottom. The dose distribution for this set up was medical physicist verified. Control cell culture plates were transported to the radiation therapy area but kept outside the radiation vault during treatments. A 6-MV linear accelerator (Clinac IX System, Varian Medical Systems, Inc., Palo Alto, CA, USA) was utilized to deliver radiation for all experiments.
Figure 5.2. Photograph representative of clonogenic survival experiments. D17 canine osteosarcoma cells treated with 2 Gy radiation or the combination of 2 Gy radiation and erlotinib at 10 µM. Cells were stained with 0.5% crystal violet in 20% methanol, and colonies were visually inspected counted under light microscopy. A colony was defined as an aggregate of ≥ 50 cells.
Figure 5.3. Effect of radiation therapy and erlotinib (10 µM), on clonogenic survival of canine osteosarcoma cells. * p < 0.05 indicates statistical significant reduction in clonogenic survival compared to control at the corresponding radiation dose. Erlotinib treatment enhanced the effects of radiation on Dharma cells for radiation doses of 2, 4 and 6 Gy, and showed single agent effects in reduction of colony numbers for Dharma and D17 cells.
Figure 5.4. Western blot analysis of EGFR, total and phosphor-AKT expression in Abrams OSA cells in control groups and after treatment with erlotinib, RT and combination therapy. Time points listed represent hours post-RT.
Figure 5.5. Western blot analysis of EGFR, total and phosphor-Akt expression in Dharma OSA cells in control groups and after treatment with erlotinib, RT and combination therapy. Time points listed represent hours post-RT.
**Figure 5.6.** Western blot analysis of EGFR, total and phosphor-Akt expression in D17 OSA cells in control groups and after treatment with erlotinib, RT and combination therapy. Time points listed represent hours post-RT.
Figure 5.7. Cell viability assays 24 hours post-radiation. Cells were treated with either radiation only (“control”), or radiation plus erlotinib at 10 µM or 40 µM. Experiments were repeated three times and the averages of results are shown. A radiation dose-response decrease in cell viability was not observed at 24 hours for any of the cell lines examined. This time-point was therefore not utilized for statistical analysis or evaluation of effects of erlotinib in combination with RT in canine osteosarcoma.
Figure 5.8. Cell viability assays 48 hours post-radiation. Cells were treated with either radiation only (“control”), or radiation plus erlotinib at 10 µM or 40 µM. Experiments were repeated three times and the averages of results are shown. Abrams cells showed a radiation dose-response decrease in cell viability, which was not observed in the other two cell lines at 48 hours. This time-point was therefore not utilized for statistical analysis or evaluation of effects of erlotinib in combination with RT in canine osteosarcoma.
Figure 5.9. Doubling time of Abrams, Dharma and D17 osteosarcoma cell lines. The three cell lines examined have different growth rate and doubling time. Abrams cells have the shortest doubling time, while Dharma cells have the longest doubling time.
Figure 5.10. Photographs of canine osteosarcoma cell colonies. At day 7 of clonogenic survival experiments, cells start to form colonies and are visualized by light microscopy.