Topical 5-Fluorouracil – A Novel Targeted Therapy for the Keratocystic Odontogenic Tumour

by

Nicholas John Ledderhof

A thesis submitted in conformity with the requirements for the degree of Masters of Science in Oral and Maxillofacial Surgery and Anaesthesia

Discipline of Oral and Maxillofacial Surgery
Faculty of Dentistry
University of Toronto

© Copyright by Nicholas John Ledderhof 2016
Abstract

5-Fluorouracil (5-FU) is used to treat basal cell carcinomas (BCCs), which are similar in etiopathogenesis to Keratocystic Odontogenic Tumours (KOTs). We hypothesized that KOTs are molecularly targeted by 5-FU, and 5-FU-treated KOT cases have lower recurrence rates and less morbidity compared to those treated with Modified Carnoy’s (MC). An ambispective study of recurrence rates and morbidity in patients with KOTs treated with 5-FU or MC was completed. There were no KOT recurrences with 5-FU; whereas, there were 4 recurrences with MC (p=0.190). There was also a lower incidence of inferior alveolar nerve paresthesia with 5-FU treatment (p=0.039). Immunohistochemistry revealed up-regulation of thymidine phosphorylase (TP, p<0.0001) and dihydropyrimidine dehydrogenase (DPD, p<0.0001), and no change in thymidylate synthetase (TS, p>0.05) in inflamed KOTs. Our findings suggest that topical 5-FU is a novel therapy for KOTs and the therapeutic efficacy of 5-FU is likely via molecular interactions with TP, DPD and TS in KOTs.
Acknowledgments

This work was supported by a Canadian Association of Oral and Maxillofacial Surgeons Research Support Grant and the Bertha Rosenstadt Endowment Fund. My sincerest gratitude goes to my supervisor Dr. David K Lam and my thesis committee members: Drs. Marco Caminiti and Grace Bradley for their guidance and support over the course of the project. I would also like to thank the surgeons, pathologists and staff at Mount Sinai Hospital, the Crescent Oral Surgery group and the Princess Margaret Hospital. Finally, thank you to my family and friends for their love and support over the entire course of my education!
# Table of Contents

Abstract .......................................................................................................................... ii

Acknowledgements........................................................................................................ iii

Table of Contents.......................................................................................................... iv

List of Tables................................................................................................................ vii

List of Figures................................................................................................................ viii

List of Abbreviations..................................................................................................... ix

List of Appendices......................................................................................................... xi

Chapter 1. Introduction................................................................................................... 1

1.1 Background............................................................................................................... 1

1.2 Clinical Features..................................................................................................... 1

1.3 Radiographic Features........................................................................................... 2

1.4 Histopathology....................................................................................................... 2

1.5 Etiopathogenesis.................................................................................................... 3

1.6 KOT Proliferation Marker...................................................................................... 6

1.7 Patched Tumour Supressor and Nevoid Basal Cell Carcinoma Syndrome.......... 7

1.8 Treatment Options................................................................................................. 8

1.9 Prognosis and Recurrences..................................................................................... 9

1.10 Inferior Alveolar Nerve Injury............................................................................. 11

1.11 5-Fluorouracil..................................................................................................... 11
Chapter 2. Statement of Objectives and Hypotheses
2.1 Objectives
2.2 Hypotheses

Chapter 3. Topical 5-Fluorouracil – A Novel Targeted therapy for the Keratocystic Odontogenic Tumour
3.1 Abstract
3.2 Introduction
3.3 Materials and Methods
3.4 Results
3.5 Discussion
3.6 Conclusions
3.7 Disclosures and Acknowledgements
3.8 References

Chapter 4. Discussion
4.1 Topical 5-Fluorouracil
4.2 KOT Recurrences and Post-Operative Nerve Injury
4.3 KOT Proliferation
4.4 Expression of TS, TP and DPD
4.5 Inflammation in KOTs
4.6 Study Limitations and Future Directions
List of Tables

Table 3.1 Dilution and incubation times for primary antibodies.................................................24

Table 3.2 Demographics of Keratocystic Odontogenic Tumour cases treated using Modified Carnoy’s (MC) or 5-Fluorouracil (5-FU)........................................................................................................26

Table 3.3 Recurrences in Keratocystic Odontogenic Tumour (KOT) treated with Modified Carnoy’s (MC) or 5-Fluorouracil (5-FU).........................................................................................................27

Table 3.4 Inferior alveolar nerve injury data for mandibular cases treated with Modified Carnoy’s (MC) or 5-Fluorouracil (5-FU) (* = p < 0.05)..................................................................29

Table 3.5 Immunohistochemical staining scores for predictors of 5-Fluorouracil response in Keratocystic Odontogenic Tumours (KOTs)...................................................................................31

Table 3.6 Immunohistochemical staining scores for predictors of 5-Fluorouracil response according to the presence of inflammation in Keratocystic Odontogenic Tumours (KOTs).……32
List of Figures

Figure 1.1 Representative histology of a Keratocystic Odontogenic Tumour (KOT)..................3

Figure 1.2 Sonic Hedgehog (SHH) pathway.................................................................5

Figure 1.3 5-Fluorouracil (5-FU) Schematic...............................................................13

Figure 3.1 5-Fluorouracil application technique for the Keratocystic Odontogenic Tumour (KOT)..................................................................................................................22

Figure 3.2 Representative example of Keratocystic Odontogenic Tumour (KOT) treated with 5-fluorouracil..........................................................28

Figure 3.3 Representative immunohistochemistry of non-inflamed and inflamed areas of Keratocystic Odontogenic Tumours (KOTs) including p53, Ki-67, Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP), and Dihydropyrimidine Dehydrogenase (DPD) at 200x magnification........................................................30
List of Abbreviations

5-FU  5-Fluorouracil
BCC  Basal Cell Carcinoma
BMP  Bone Morphogenetic Protein
CS   Carnoy’s Solution
CT   Computed Tomography
DPD  Dihydropyrimidine Dehydrogenase
DAB  3, 3’ – Diaminobenzidine Tetrahydrochloride
dUMP Deoxyuridine Monophosphate
dTMP Deoxythymidine Monophosphate
FdUMP Fluorodeoxyuridine Monophosphate
FdUTP Fluorodeoxyuridine Triphosphate
FGF  Fibroblast Growth Factor
FUTP Fluorodine Triphosphate
Gli1 Glioma Associated Oncogene 1
HGF  Human Growth Factor
IAN  Inferior Alveolar Nerve
Il-1a Interleukin-1a
KOT  Keratocystic Odontogenic Tumour
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI</td>
<td>Labeling Index</td>
</tr>
<tr>
<td>MC</td>
<td>Modified Carnoy’s Solution</td>
</tr>
<tr>
<td>NBCCS</td>
<td>Nevoid Basal Cell Carcinoma Syndrome</td>
</tr>
<tr>
<td>OKC</td>
<td>Odontogenic Keratocyst</td>
</tr>
<tr>
<td>OPRT</td>
<td>Orotate Phosphoribosyltransferase</td>
</tr>
<tr>
<td>PRPP</td>
<td>Phosphoribosyl pyrophosphate</td>
</tr>
<tr>
<td>PTCH</td>
<td>Patched</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>SMO</td>
<td>Smoothened</td>
</tr>
<tr>
<td>TGF beta</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TNF alpha</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>TP</td>
<td>Thymidine Phosphorylase</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate Synthetase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Tdt-Mediated dUTP-biotin nick end labeling</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
List of Appendices

Appendix A. Manuscript submitted to the Journal of Oral and Maxillofacial Surgery………..62
Chapter 1
Introduction

1 Keratocystic Odontogenic Tumour

1.1 Background

Keratocystic Odontogenic Tumours (KOTs) are locally destructive, benign odontogenic tumours with no clear consensus on optimal treatment modality. Pindborg and Hansen originally described the odontogenic keratocyst (OKC) in 1963. In 2005, the lesion was reclassified as a tumour by the World Health Organization (WHO) due to its neoplastic features (Barnes et al., 2005). Historically, treatment of KOTs involved simple enucleation; however, this was suboptimal due to high recurrence rates.

KOTs can cause significant destruction in the maxilla and mandible as well as extend to other areas of the head and neck when not diagnosed early or effectively treated. Tumour invasion into the orbit (Chuong et al., 1982), temporal fossa (Metkees et al., 2015) and skull base (Jackson et al., 1993) have been reported, demonstrating the destructive effects of KOTs due to local-regional spread in the head and neck.

1.2 Clinical Features

Individuals who present with KOTs are usually 10-40 years of age (Meara et al., 1996) with an average age of 38-40 years (Woolgar et al., 1987). There is a male predilection of 1.6:1 (Chirapathomsakul et al., 2006) with the mandible more frequently affected than the maxilla at a ratio of 2:1, and most lesions present in the posterior mandibular body and ramus (Eryilmaz et al., 2009). Large areas of the maxilla or mandible can be afflicted with a KOT without noticeable signs or symptoms (Cardes and Slootweg, 2006). Stoelinga (2001) found that 37.8% of patients were asymptomatic, 20.7% had swelling and 41.5% had some degree of inflammation in a study of 82 patients.
1.3 Radiographic Features

KOTs may present as a solitary, well-defined, corticated unilocular or multilocular radiolucency, often associated with an impacted tooth, with or without osseous septations. Lesions in the mandible and maxilla tend to grow along the long axis of the bone causing little expansion; however, lesions of the maxilla may grow into the maxillary sinus (Neville et al, 2002). KOTs present with a ratio of 2.5:1 unilocular to multilocular radiolucencies (Chirapathomsakul et al, 2006); a multilocular pattern is 1.7 times more likely to be a KOT, based on a study of 130 patients comparing KOTs with dentigerous cysts (Buckley et al, 2012). Plain films are most suited for provisional diagnosis and surveillance; however, computed tomography (CT) scans best delineate tumour extent within bone, cortical perforation, small loculations and cortical thinning (van Rensburg et al, 2003). Differential diagnoses for well-defined, corticated, radiolucent lesions in the mandible and maxilla may include but are not limited to: dentigerous cysts, KOTs, ameloblastomas, central giant cell granulomas and odontogenic myxomas. Due to a broad differential diagnosis for this type of lesion, the shape and pattern of an osseous lesion of the maxilla or mandible are not pathognomonic for a KOT.

1.4 Histopathology

Histopathology of the neoplastic KOT is identifiable by a characteristic 5-8 cell thick stratified squamous epithelial lining with a palisading basal layer of columnar cells and a parakeratinized, corrugated surface layer (Gonzalez-Alva et al, 2008) (see Figure 1.1). Inside the cystic lumen of the neoplasm, one often finds keratinaceous material, often described as “cheesey” in consistency which is postulated to arise from apoptosis of the outer layer of the epithelium. This is different from the basal epithelial layer where anti-apoptotic Bcl-2 has been shown to be active (Kichi et al, 2005). These findings contribute to the higher tendency for recurrence and aggressive nature of the KOT (Rangiani and Motahhary, 2009). Diagnosis is based on histopathology, radiographic features and clinical findings. Importantly, the presence of inflammation can dramatically change the histopathological appearance of the KOT, imparting a diagnostic challenge for the evaluating pathologist (see Figure 1.1).
Figure 1.1 Representative histopathology of Keratocystic Odontogenic Tumours (KOT).
(A) A representative section of a non-inflamed KOT with a characteristic palisaded basal cell layer, a corrugated parakeratotic superficial layer and a uniform epithelial lining that is 5-8 cells in thickness. (B) A representative section of an inflamed KOT demonstrating loss of the characteristic diagnostic features, an inflammatory infiltrate and hyperemia marked by increased vascularity.

1.5 Etiopathogenesis

KOTs develop from cell rests of the dental lamina without expansion due to internal oncotic pressures. KOT growth may be related to factors associated with the epithelium or activity in the cyst wall (Neville et al, 2002).

Several signaling pathways are known to be involved in the complex process of odontogenesis. Sonic hedgehog (SHH), bone morphogenetic protein (BMP), Wnt, human growth factor (HGF) and fibroblast growth factor (FGF) are a small subset of factors involved (Tucker and Sharpe, 1999; Jernvall and Thesleff, 2000). As with any tissue, inactivation of tumour suppressor genes can result in tumour proliferation (Knudson, 1971 and Knudson, 1993). Odontogenic neoplasms express a variety of signaling molecules including: SHH, patch (PTCH), smoothened (SMO) and glioma associated oncogene-1 (Gli1) (Barreto et al, 2002) suggesting an important role for the
SHH signaling pathway.

SHH is an important regulatory pathway controlling cell fate in a variety of tissues including teeth, which is accomplished largely via PTCH (Colborne et al, 2004) (see Figure 1.2). Evidence suggests that PTCH forms a transmembrane receptor complex with SMO creating a receptor site for ligands of SHH allowing for the activation of Gli1 (Zhang et al, 2005). Gli1 in turn upregulates transcription factors and genes responsible for cellular proliferation (Ingham and McMahon, 2001). Therefore, SHH activation is postulated to cause tumourgenesis (Barreto et al, 2002), which is further evidenced by SMO gene alterations suggesting a role in the development of syndromic and non-syndromic KOTs (Rui et al, 2014).
**Figure 1.2 Sonic Hedgehog (SHH) pathway.** Without SHH protein, patch (PTCH) causes inhibition of smoothened (SMO) resulting in inactivity of downstream signaling within the SHH pathway. Binding of SHH to PTCH receptor results in activation transcription and genes associated with cell proliferation. 5-FU may disrupt cellular proliferation through DNA and RNA damage. Adapted from Bhargava et al, 2012.

The PTCH gene encodes a SHH receptor, known as PTCH1 which is found on cell membranes (Caro and Low, 2010) and downregulates SMO, a crucial transmembrane protein. Resulting from binding of SHH to PTCH1, SMO is activated encouraging Gli1 stimulation and cellular proliferation (de Zwann and Haass, 2010). Gli1 induces PTCH1 and itself resulting in a positive feedback loop with induction of other factors including: Bcl-2, bone morphogenetic protein.
(BMP) and transforming growth factor beta (TGF-beta). Tumourgenesis happens when PTCH1 becomes inactive resulting in the inability to suppress SMO (de Zwaan and Haass, 2010).

KOTs have germline mutations and loss of heterozygosity at the PTCH gene locus 9q22.3 (Ohki et al, 2004). Patients with Nevoid Basal Cell Carcinoma Syndrome (NBCCS), characterized by multiple BCCs and KOTs, are postulated to have one mutation in the genetic make up (Lench et al, 1996) which only requires one more hit to result in tumour growth according to Knudson’s 2 hit theory (Knudson, 1971). Thus KOTs develop secondary to cells that already possess allelic loss of heterozygosity (Barreto et al, 2000). Non-syndromic KOTs happen after 2 hits secondary to failure of PTCH function whereby dysregulation of p53 and other genetic changes cause the potential for tumour proliferation (Lo Muzio et al, 1999).

1.6 KOT Proliferation Markers

Due to the complicated mechanism by which p53 transmits apoptotic signals, one function may be as an apoptotic protein and the other may be as a marker of cellular proliferation (Schmitt and Lowe, 1999; Shear, 2002). Higher expression of p53 in the intermediate layers of KOT epithelium compared to dentigerous and radicular cysts (Ogden et al, 1992; Kimi et al, 2000) may be due to an increased proliferative capacity of the KOT (Slootweg, 1995).

Ki-67 is a protein associated with cellular proliferation (Mendes et al, 2010). Evidence from previous studies confirms that p53 (Slootweg, 1995; Lombardi et al, 1995 and Li et al, 1996) and Ki-67 (Slootweg, 1995 and Kimi et al, 2000) are present in higher concentrations in the lining epithelium of KOTs than other odontogenic cysts. It is expressed highly in the suprabasal layers of KOT epithelium, suggesting that this layer exhibits the highest ability to proliferate during the development of KOTs (Kichi et al, 2005). Ki-67 and p53 protein expression are correlated with each other in KOTs; over-expression of p53 and Ki-67 may suggest increasing aggressiveness and may be useful as prognostic markers in odontogenic pathoses (Gadbail et al, 2012).

Antiapoptotic mechanisms exist within the basal layers of the proliferating KOT (Kimi et al, 2000 and Kim et al, 2003). However, apoptotic cells have been demonstrated in the superficial epithelial layer via Tdt-mediated dUTP-biotin nick end labeling (TUNEL). Bcl-2 is a proto-oncogene that functions to suppress apoptosis without promoting proliferation (Chylicki et al, 2000) and has been found in KOTs, dentigerous cysts and ameloblastomas (Lo Muzio et al,
Bcl-2 cells are identified in the basal epithelium suggesting no apoptosis in this layer; whereas, TUNEL positive cells are found exclusively in the surface layer of KOT epithelium (Kichi et al., 2005). This may explain the high concentrations of cystic keratinization and a consistent 5-8 cell layer thickness of tumour epithelium in KOTs.

1.7 Patched (PTCH) Tumour Suppressor and Nevoid Basal Cell Carcinoma Syndrome (NBCCS)

Initially identified in the 1960s, NBCCS, also known as, Gorlin Goltz syndrome was based on a triad of findings including: multiple basal cell carcinomas (BCCs), multiple maxillary and/or mandibular keratocysts and bifid ribs; however, multiple other features are also present. These include but are not limited to: calcification of the falx cerebri, kyphoscoliosis, frontal bossing, ovarian fibromas, cardiac fibromas and medulloblastomas. NBCCS is a rare disorder with no racial nor gender bias, an autosomal dominant inheritance pattern and affects approximately 1/57,000 – 1/256,000 individuals (Endo et al., 2012, Lo Muzio, 2008 and Epstein, 2008). Inheritance of this condition is due to germline mutations in the SHH pathway, most commonly compromising the PTCH gene (Johnson et al., 1996 and Epstein, 2008).

Molecular studies focusing on the PTCH tumour suppressor gene pathway yields a targeted approach for treating BCCs. Activation of SMO or downregulation of PTCH results in increased actions of SHH and reacts with cyclopamine (Taipale et al., 2000). Cyclopamine arrests the growth of KOT cells through the inhibition of SMO in the SHH pathway (Ren et al., 2012). More recently, it was demonstrated that SMO gene alterations likely play an important role in KOT and NBCCS development (Rui et al., 2014). This suggests that SHH signaling pathway antagonism may be an efficient way to molecularly target KOTs through SMO inhibition and suppression of SHH transcription factors (Zhang et al., 2006). A recent case report has shown efficacy of an orally administered SHH inhibitor, vismodegib in decreasing the number and morbidity of multiple BCCs and KOTs in patients with NBCCS [Booms et al., 2015].

Interestingly, PTCH1 mutation was found in approximately 85% of NBCCS and previous studies showed less than 30% mutation in sporadic KOTs; however, one study showed sporadic KOTs with 84% PTCH1 mutation that they attributed to masking properties of connective tissue capsules that they dissected off of the KOT epithelium and therefore demonstrated similar rates of PTCH1 mutations in non syndromic and syndromic patients (Qu et al., 2015).
Treatment of BCCs includes: excision, photodynamic therapy, laser therapy, topical 5-FU, topical 5% imiquimod and vismodegib (Lam et al, 2013). Using topical 5% 5-FU for superficial BCCs twice daily over 12 weeks has a cure rate of 90% (Gross et al, 2007).

1.8 Treatment Options for KOTs

No consensus exists on the ideal treatment for KOTs. Enucleation as a sole treatment option is commonly used (Myoung et al, 2001). However, the thin, friable epithelial lining predisposes leaving remnants of the lesion behind. This is especially true in multilocular lesions when one may have a difficult time accessing the tissue remnants. Incomplete excision of the lesion and the perilesional connective tissue entirely is likely the most important factor causing tumour recurrence (Forssell, 1988).

Various adjunctive agents and techniques have been used in the treatment of these lesions. The most commonly used adjunctive treatment techniques for KOTs include peripheral ostectomy (i.e. using a handpiece to burr down the inner aspect of the osseous cavity in hopes of eliminating remnants) and/or some form of Carnoy’s Solution (CS) following enucleation in an effort to further decrease recurrence rates. Removal of chloroform from CS was due to concerns regarding carcinogenicity (Gosau et al, 2010 and Pitak-Arnnop et al, 2010). New reports of decreased efficacy of Modified Carnoy’s Solution (MC) by Dashow and colleagues (2015) suggests a different protocol for MC may be necessary to achieve similar results to CS. It is not ideal to use a substance that fixes surrounding tissue when it comes in contact with neurovascular tissue or sinus mucosa for fear of causing irreversible damage or necrosis (Stoelinga, 2003). Cryosurgery using multiple applications of liquid nitrogen to the enucleated cavity is another useful adjunct in the management of KOTs (Schmidt and Pogrel, 2001).

Marsupialization involves an osteotomy, completing a partial cystectomy and maintaining communication to the oral cavity so that the patient is able to thoroughly irrigate the lesion on a daily basis. Removal of the remaining lesion is completed weeks or months later when the lesion has decompressed in hopes of decreasing morbidity through less nerve and tooth involvement and greater bone strength. This increases the overall ease of lesion excision. Decreasing the oncotic pressure via decompression allows the tumour to shrink creating a smaller lesion and lowers the risk of damage to surrounding structures. Marsupialization and later cystectomy are treatment
options for children and medically compromised individuals that can help alleviate the need for general anesthesia, retains teeth and avoids damage to vital structures (Pogrel and Jordan, 2004).

Resection (composite, segmental or marginal) is the most invasive but most successful surgical measure used for KOTs. Most studies cite that resection produces the fewest recurrences, but this is poorly documented (Blanas et al, 2000; Warburton et al, 2015). However, this treatment modality may not be entirely devoid of recurrences, with reports of recurrent KOTs developing in bone graft reconstruction following resection (Warburton et al, 2015).

Frerich and colleagues (1994) defined critical exposure time of CS to the inferior alveolar nerve as 3-minutes in rabbits without causing irreparable damage to the endoneurium in their landmark paper. They described a critical exposure time of 3-minutes when applying CS. The optimal application time for MC has not been elucidated; therefore it is difficult to compare reports on recurrence rates and morbidity of KOTs treated with this adjunctive agent.

1.9 Prognosis and Recurrences

A recurrence rate of 17-56% was found when enucleation alone was used to treat KOTs (Blanas et al, 2000). In a recent study, Dashow and colleagues (2015) show that the recurrence rate (10%) of enucleation with the addition of CS is significantly less than the recurrence rate (35%) of enucleation along with Modified Carnoy’s Solution (MC) without chloroform. Enucleation with the addition of peripheral ostectomy is another method to eliminate KOTs. Two recurrences in 11 patients resulted in a recurrence rate of 18.18% with this method of treatment (Kaczmarzyk et al, 2012). Enucleation, peripheral ostectomy and CS application showed no recurrences in the same systematic review (Kaczmarzyk et al, 2012).

Marsupialization allows for decompression of the KOT by inserting a drain after biopsy is completed, increasing the overall ease of lesion excision. Without access to antibiotics, Partsch advocated for marsupialization because primary closure often lead to infection (Pogrel and Jordan, 2004). The proliferation marker bcl-2 was significantly downregulated following marsupialization (Pogrel and Jordan, 2004) along with decreased expression of IL-1a, which in combination is associated with KOT decompression (Pitak-Arnnop et al, 2010). A 24-40% recurrence rate is noted with marsupialization (Blanas et al, 2000; Kaczmarzyk et al, 2012).
Cryosurgery is a useful adjunct in the management of KOTs (Schmidt and Pogrel, 2001). In a study of 26 patients with a 3.5 year follow up of KOTs treated with enucleation followed by two, 1-minute applications of liquid nitrogen spray with a 5 minute thaw between sprays resulted in 3 recurrences for a rate of 11.5% (Schmidt and Pogrel, 2001). However, 1 patient had a pathological fracture, so it is postulated that immediate bone grafting may be required with this technique (Pogrel, 2005). The main advantage of using cryosurgery is to preserve the osseous architecture required for osteoconduction of new bone (Schmidt and Pogrel, 2001).

In a large study, there were no recurrences in 52 patients treated with resection followed from 3-11 years (Zhao et al, 2002). Warburton and colleagues (2015) proposed guidelines in which resection should be used. They advocate for resection following multiple failed conservative treatment attempts, when there is extraosseous spread into the pterygoid musculature and malignant transformation.

With rates of recurrences of 17-56% (Blanas et al, 2000), conservative options are less than ideal for management of the KOT. Conversely, resection offers an excellent cure rate but the associated morbidity including neurosensory deficits, donor site morbidity, increased hospital stay and facial defects are less than desirable and may not be worth it to many patients who may opt for more conservative treatment even if it means multiple surgeries. Kaczmarzyk and colleagues (2012) described significant limitations in the ability to study outcomes of surgeries for KOT extirpation including: poor follow up times, not distinguishing the parakeratinizing true KOT from orthokeratinizing cysts and small sample sizes which makes a nearly impossible task to prepare an adequate systematic review and draw conclusions applicable to the community of surgeons providing care for these patients.

KOT size does not correlate to higher recurrence rates (Kuroyanagi et al, 2009 and Madras and Lapointe, 2008). A more important gauge of tumour recurrence and aggressiveness is postulated to relate to the condition of the cortical plates. Perforated and thinned cortices are indicators of a higher rate of recurrence and should be treated as such with some advocating more aggressive treatment approaches (Pitak-Arnnop et al, 2010). Maxillary KOTs are diagnosed earlier due to the proximity of the maxillary sinus and thinner, more cancellous bone (Chow, 1998); whereas lesions of the posterior mandible may increase in size to a greater extent prior to developing signs and symptoms (Myoung et al, 2001).
Recurrences most likely happen within the first 2 years after the first surgery and therefore, a 5 year follow-up period is suggested by most surgeons (Stoelinga, 2001). Radiographic evidence of recurrence has been documented to occur an average of 2.2 years following the initial surgery (Apajalahti et al, 2011). There have been 19 documented cases of malignant transformation in KOTs (Lukandu and Micha, 2015 and Warburton et al, 2015). Carcinoma ex KOTs are said to have a prevalence of 0.12% (Timosca et al, 1995) but malignant transformation is difficult to estimate because lesions may arise de novo or from a previous KOT.

Maxillary and mandibular KOTs are found in approximately 90% of individuals with NBCCS in their first 2 decades of life. KOTs are thought to continue to develop into the 3rd decade of life in areas of maxillary/mandibular odontogenesis (Lo Muzio et al, 1999). Syndromic patients often develop multiple KOTs and people without NBCCS often will only develop one lesion, not including recurrences (Lo Muzio et al, 1999).

1.10 Inferior alveolar nerve injury

The inferior alveolar nerve (IAN) provides sensation to the lower lip, chin, gingiva and teeth and is at significant risk for injury during surgical removal of KOTs in the mandible. The slow growing nature of KOTs tends to displace the IAN inferiorly towards the inferior mandibular cortex and often times the IAN is exposed along the inferior surface of the KOT during removal. This can make removal of KOTs challenging because of the increased chance of permanent damage to the IAN. Rates of temporary post-operative neuropathy have been documented as 18.2 - 30.1% (Ribeiro Junior et al, 2012 and Leung et al, 2016); with corresponding rates of permanent paresthesia or anesthesia documented as 0 – 16.0% (Ribeiro Junior et al, 2012 and Leung et al, 2016). Patients averaged 4.6 months to neurosensory recovery in one reported study (Leung et al, 2016). Perforation of the bony cavity and application of CS may induce further injury to the nerve; however, when following the 3-minute protocol defined by Frerich and colleagues (1994), no axonal degeneration is thought to occur.

1.11 5-Fluorouracil (5-FU)

Given the similar etiopathogenesis of KOTs and BCCs, 5-FU may be a promising novel treatment option for KOTs. Antimetabolite drugs are a specific class of chemotherapeutic drug used to treat cancers of the head and neck, bowel, breast and skin (Labianca et al, 1995).
Fluoropyrimidines mimic uracil and cause misincorporation of fluoronucleotides into DNA and RNA resulting in the inhibition of thymidylate synthetase (TS) (Rutman et al., 1954). In particular, 5-FU incorporates into the cellular DNA and RNA causing inhibition of the normal biosynthetic process. 5-FU is generally used in conjunction with other chemotherapeutic agents to improve outcomes of medical therapy in malignancies (Johnston and Kaye, 2001).

Fluorine for hydrogen substitution at the C-5 position of uracil allows its analogue 5-FU to enter the cell replacing uracil (Wohlhueter et al., 1980) (see Figure 1.3). Once 5-FU enters cellular metabolism, it is broken down into metabolites including: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorodine triphosphate (FUTP) resulting in the inhibition of TS and RNA synthesis (Diasio and Harris, 1989). Without TS, DNA replication is impossible and the methylation of normal metabolites deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using cofactor 5,10-methylenetetrahydrofolate does not occur (Nazki et al., 2014 and Wang et al., 2004). FdUMP competitively binds and blocks dUMP inhibiting normal DNA synthesis (Sommer and Santi, 1974 and Santi et al., 1974).

5-FU has been shown in hepatocellular carcinoma cell lines to down-regulate the effects of SHH at the mRNA and protein levels (Wang et al., 2008). 5-FU induced apoptosis and inhibited the motility of SHH-activated HCC cells showing a role in treatment. However the mechanism of action and why 5-FU is efficacious remains incompletely understood.
Figure 1.3 5-Flourouracil (5-FU) is converted to active metabolites fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) through thymidine phosphorylase (TP) and orotate phosphoribosyltransferase (OPRT) with cofactor phosphoribosyl pyrophosphate (PRPP). Active metabolite FdUMP results in the inhibition of thymidylate synthetase (TS), which is required for DNA replication and repair. Dihydropyrimidine dehydrogenase (DPD) inactivates and breaks down 5-FU into its excretory metabolites. Adapted from Longley et al, 2003.

1.12 Independent Predictive Markers for Sensitivity to 5-FU: Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP), and Dihydropyrimidine Dehydrogenase (DPD)

Salonga and colleagues (2000) showed that TS, TP and DPD are predictive of 5-FU efficacy independently and measurement of these markers predicts tumour responsiveness to 5-FU treatment with malignant tumours. Expression of TS is crucial to 5-FU susceptibility. Increases in TS mRNA have been shown as a marker of resistance to 5-FU (Lu et al, 2013 and Ijichi et al, 2014). Immunohistochemical and RT-PCR analysis suggest that low tumour expression of TS improved clinical outcomes in 5-FU-based chemotherapeutic regimens (Lenz et al, 1998).
Studies gauging TS expression by cell culture and western blot analysis demonstrate increasing resistance to treatment with 5-FU with high expression of TS (Ijichi et al, 2014). Downregulation of TS via various medications has been shown to increase the efficacy of 5-FU with colorectal cancer cell lines (Yang et al, 2015).

Thymidine phosphorylase (TP) also known as platelet-derived endothelial cell growth factor exhibits a dual role in metabolic processing of pyrimidine metabolites and, secondly, promotes angiogenesis (Bronkaers et al, 2009). TP reversibly converts 5-FU to fluoroxyuridine (FUDR) which is then converted to the 5-FU metabolite FdUMP which is the primary metabolite responsible for the cytotoxicity of the medication binding and inhibiting TS (Kim et al, 2013). High expression of TP in vitro suggests more responsiveness to 5-FU because of increased FdUMP (Evrard et al, 1999).

Dihydropyrimidine dehydrogenase (DPD) is a catabolic enzyme in uracil and thymidine catabolism and is responsible for the breakdown of 5-FU into its metabolites (Heggie et al, 1987). Overexpression suggests resistance to 5-FU because DPD is used to break down 5-FU into its excretory inactive metabolites (Salonga et al, 2000), therefore low intramural expression of DPD tends to suggest an improved response to the medication. Studies have demonstrated tumours with high DPD expression had significantly shorter disease-free survival time compared to patients with low DPD expression (Ciaparrone et al, 2006). Other authors suggest that intrahepatic upregulation of DPD may play an important role in acquired resistance to 5-FU (Li et al, 2013).
Chapter 2
Statement of Objectives and Hypotheses

2 Statement of Objectives and Hypotheses

2.1 Objectives

1) Determine the incidence of inferior alveolar nerve injury and recurrence rate of Keratocystic Odontogenic Tumours (KOTs) treated with enucleation, peripheral ostectomy and topical application of Modified Carnoy’s solution (MC).

2) Determine the incidence of inferior alveolar nerve injury and recurrence rate of KOTs treated with enucleation, peripheral ostectomy and application of topical 5\% 5-Fluorouracil (5-FU).

3) To evaluate the expression of molecular markers [thymidylate synthetase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD)] that may predict response to 5-FU.

2.2 Hypotheses

1) KOTs treated with enucleation, peripheral ostectomy and 5-FU have a lower recurrence rate than treatment with conventional enucleation, peripheral ostectomy and application of MC solution.

2) Rates of inferior alveolar nerve injury are lower with 5-FU compared to MC.

3) The therapeutic efficacy of 5-FU is supported by immunohistochemical expression of TS, TP and DPD in KOTs.
Chapter 3

3 Topical 5-Fluorouracil – A Novel Targeted Therapy for the Keratocystic Odontogenic Tumour

Submitted for publication in the Journal of Oral and Maxillofacial Surgery

Nicholas J. Ledderhof DDS, a Marco F. Caminiti DDS, MEd, FRCD(C), b Grace Bradley DDS, MSc, FRCD(C), c and David K. Lam MD, DDS, PhD, FRCD(C). d

a Chief Resident, Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

b Assistant Professor, Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

c Professor and Head, Oral Pathology & Oral Medicine, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

d Assistant Professor and Head, Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

Corresponding Author: Nicholas Ledderhof

Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Room 143, Toronto, Ontario, Canada M5G 1G6

Telephone: 416-979-4922 Ext 4329

Fax: (416) 979-4936

Email: nick.ledderhof@mail.utoronto.ca
3.1 Abstract

Purpose

The antimetabolite drug, 5-fluorouracil (5-FU), is used in the treatment of various cancers including basal cell carcinomas (BCCs). We hypothesized that Keratocystic Odontogenic Tumors (KOTs) respond to 5-FU treatment due to their similarities in etiopathogenesis to BCCs. We: (1) tested the efficacy of KOT adjunctive treatment with 5-FU compared to conventional treatment with Modified Carnoy’s solution (MC), and (2) assessed KOTs for expression of markers that may predict response to 5-FU treatment.

Methods

We conducted an ambispective cohort study of the treatment efficacy of KOTs treated with topical application of either 5% 5-FU or MC following enucleation and peripheral ostectomy, at the University of Toronto from 2006-2014. Outcome variables included KOT recurrence, time to recurrence, and peripheral nerve injury. KOT specimens in these patients were immunostained with p53, Ki-67, thymidylate synthetase (TS), thymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) antibodies. Semi-quantitative staining scores were calculated for all immunohistochemistry sections examined. Descriptive statistics were computed and the p value was set at 0.05.

Results

5-FU was an effective adjunctive treatment for KOTs with no adverse side effects. There were no KOT recurrences in the 5-FU group (n=11); whereas there were 4 recurrences in the MC group (n=21, p=0.190). There was a significantly lower incidence of inferior alveolar nerve paresthesia with 5-FU treatment (p=0.039). Immunohistochemical staining revealed up-regulation of TP (p<0.0001) and DPD (p<0.0001), and no change in TS (p>0.05) in inflamed KOTs.
Conclusions

5-FU effectively treats KOTs with less post-operative morbidity than conventional treatment with MC. The therapeutic efficacy of 5-FU is likely via molecular interactions with TP, DPD and TS in KOTs. Low TS and up-regulated TP in inflamed KOTs suggests increased 5-FU efficacy in inflamed KOTs. Topical 5-FU is a novel therapy for KOTs and provides a targeted molecular approach to treatment.
3.2 Introduction

Keratocystic odontogenic tumors (KOTs) are benign lesions occurring in the maxilla and/or mandible with potential for significant morbidity. Reports of bone erosion,\(^1\) orbital invasion,\(^2\) skull base extension,\(^3\) and temporal fossa violation\(^4\) demonstrate the aggressive nature of this lesion. Previously known as Odontogenic Keratocysts (OKCs), KOTs were subsequently reclassified as a tumor by the World Health Organization (WHO) to better represent the neoplastic nature of this lesion.\(^5\)

Historically, treatment of the KOT involved simple enucleation; however, this was suboptimal due to a high recurrence rate. Other treatment options include marsupialization, curettage, peripheral ostectomy, adjunctive solution application, removal of overlying mucosa, or resection, either alone or in combination.\(^6, 7\) and \(^8\) Enucleation alone resulted in recurrence rates as high as 56%, whereas resection resulted in recurrence rates closer to 0%.\(^8\) and \(^9\) Adjunctive application of a chemical fixative called Carnoy’s solution (absolute alcohol, glacial acetic acid, chloroform, ferric chloride) was shown to decrease the rate of recurrence after enucleation. However, chloroform was removed from Carnoy’s solution due to its carcinogenicity, giving rise to Modified Carnoy’s solution (MC).\(^10\) and \(^11\) A recent study demonstrated that the use of MC was associated with significantly higher recurrence rates than with the original Carnoy’s solution (CS).\(^12\) Rates of peripheral nerve injury after the application of CS are estimated at 18.2%\(^13\) after direct application of the solution onto the nerve with the 3-minute protocol defined by Frerich and colleagues.\(^14\) Liquid nitrogen following enucleation of the lesion resulted in recurrence rates of 11.5%,\(^15\) which is similar to recurrence rates with CS.\(^8\) and \(^13\)

A targeted approach to KOT treatment has been proposed, based on current understanding of the molecular genetics of KOTs.\(^16\) and \(^17\) Molecular studies focusing on the PTCH tumor suppressor gene pathway yields a targeted treatment approach for basal cell carcinomas (BCCs). It is known that KOTs develop via PTCH gene mutations, similar to basal cell carcinomas (BCCs).\(^18\) Mutations in PTCH1 causes smoothened (SMO) activation and Sonic Hedgehog (SHH) signaling resulting in neoplastic growth.\(^19\) More recently Rui and colleagues\(^20\) demonstrated SMO gene alterations likely play an important role in KOT development. This finding suggests that SHH signaling pathway antagonism may be an efficient way to molecularly target KOTs
through SMO inhibition and suppression of SHH transcription factors.\textsuperscript{21} A recent study has shown that orally administered Vismodegib, a SHH inhibitor, may help to decrease the number and morbidity of multiple BCCs and KOTs in patients with Nevoid Basal Cell Carcinoma Syndrome (NBCCS).\textsuperscript{22}

The antimetabolite drug, 5-fluorouracil (5-FU) was shown to induce apoptosis by inhibiting SHH in hepatocellular carcinoma cells.\textsuperscript{23} 5-FU has a variety of applications in treatment of malignant disease including topical application to treat superficial BCCs.\textsuperscript{24} Salonga and colleagues\textsuperscript{25} showed that thymidylate synthetase (TS), dihydropyrimidine dehydrogenase (DPD), and thymidine phosphorylase (TP) are independent predictive measures of tumor responsiveness to 5-FU treatment. Increases in TS mRNA have been shown as a marker of resistance to 5-FU.\textsuperscript{26,27,28,29} Downregulation of TS results in increased efficacy of 5-FU in colorectal cancer cell lines.\textsuperscript{31} DPD is an enzyme involved in uracil and thymidine catabolism and is responsible for the breakdown of 5-FU into its excretory metabolites.\textsuperscript{32} Low expression of DPD suggests an improved response to 5-FU treatment since DPD is used to break down 5-FU.\textsuperscript{25} Conversely, increased expression of TP suggests improved responsiveness to 5-FU because of increased FdUMP, an active metabolite of 5-FU.\textsuperscript{33}

Due to similarities in the molecular etiopathogenesis of BCCs and KOTs, KOTs may similarly respond favorably to treatment with topical application of 5-FU. \textit{We hypothesized that: (1) 5-FU is efficacious in the treatment of KOTs; (2) KOTs treated with 5-FU have a similar or lower recurrence rate than treatment with conventional application of MC; (3) rates of trigeminal nerve injury are decreased when using 5-FU compared to MC; and (4) KOTs express molecular markers that predict responsiveness to 5-FU.}
3.3 Methods

Study Population and Design

An ambispective study of patients treated with topical application of 5-FU, or MC, following enucleation and peripheral ostectomy of KOT at the University of Toronto and Mount Sinai Hospital (Toronto, Canada) from 2006-2014 was performed. Ethics approval was obtained from the Mount Sinai Hospital (protocol #: 15-0011-E) and the University of Toronto (protocol #: 31638) Research Ethics Boards. Patient records were located via a retrograde search of operating room case lists, and by searching cyst enucleation codes for procedures performed in the clinic as set out by the Ontario Dental Association 2014 fee guide, for all attending Oral and Maxillofacial Surgeons at Mount Sinai Hospital from 2006-2014. Key words to identify and locate charts included: cyst, enucleation, Carnoy’s solution, 5-Fluorouracil, KOT, OKC, Keratocyst, Keratocystic Odontogenic Tumor, and Odontogenic Keratocyst. Operative notes, pathology reports and associated clinical records were reviewed.

Inclusion criteria for patients in the study were (1) biopsy-proven KOT/OKC, (2) complete history and clinical examination prior to definitive surgical intervention, and (3) completed surgical intervention for the KOT. Exclusion criteria were (1) having a diagnosed psychiatric condition, (2) multiple KOTs or diagnosed Gorlin-Goltz Syndrome, (3) recurrent KOT, (4) prior trigeminal nerve injury or existing paresthesia, and (5) patients with a diagnosis of orthokeratinizing odontogenic cyst or odontogenic keratocyst – orthokeratinized variant.

Clinical Examination and Oral Biopsy

A comprehensive history and examination was performed on all patients to rule out a history of medical conditions or disorders that may alter their trigeminal sensory perception. Oral biopsy specimens of all patients meeting the inclusion criteria were evaluated by the Mount Sinai Hospital (Toronto, Canada) or the University of Toronto Oral Pathology Biopsy Service to confirm the diagnosis of KOT. Demographic information was collected for each patient including age, sex, lesion location, radiographic appearance and tumor size.
**Topical application of 5-FU**

Following enucleation and peripheral ostectomy of the KOT lesion, sterile quarter-inch ribbon gauze was coated with 5% 5-FU (Efudex®, Valeant Inc., Laval, Quebec, Canada) and packed into the surgical wound. The wound was then closed per usual manner, leaving a small distal end (approx. 1 cm) of gauze exposed to allow gauze removal at 24 hours post-operatively (Figure 3.1).

**Figure 3.1 5-Fluorouracil application technique for the Keratocystic Odontogenic Tumor (KOT).** (A) Panorex and computed tomographic (CT) imaging are used as necessary in the pre-operative work up and evaluation which includes incisional biopsy. The patient with a biopsy-confirmed KOT is brought to OR and undergoes enucleation and peripheral ostectomy in the standard fashion. B) Topical 5% 5-Fluorouracil cream (Efudex®) is applied generously to a ¼ inch ribbon gauze, and (C) packed into the entire wound covering all surfaces. The wound is closed in a standard fashion leaving approximately 1 cm of ribbon gauze out of the wound. The entire ribbon gauze is removed 24 hours post-operatively. No further lavage or rinsing of the surgical site is performed in the post-operative phase.
**Topical application of MC**

Following intraoperative enucleation and peripheral ostectomy of the KOT lesion, the surrounding soft tissues were protected with multiple sterile petroleum jelly-coated neuro patties. MC solution-saturated neuro patties were then carefully placed in the surgical wound so that every discernable surface of the lesional cavity was exposed to MC for 3 minutes followed by thorough normal saline irrigation. All instruments exposed to MC were then removed from the operative field, and the surgical team re-gowned and gloved in order to prevent possible injury to healthy tissues by the caustic MC solution during wound closure.

**Immunohistochemistry**

Immunohistochemical staining was performed to evaluate the expression of markers that may predict response to 5-FU. Proliferative activity and DNA damage response were assessed by staining for Ki-67 (mouse monoclonal against MIB-1, M7240, Dako North America Inc., Carpintaria, California, USA) and p53 (DO-1 mouse monoclonal M7001, Dako North American Inc.) respectively. Responsiveness to 5-FU was assessed by staining for TS (mouse monoclonal M3614, Dako North America Inc.), TP (mouse monoclonal ab3151, Abcam, Toronto, Ontario, Canada) and DPD (rabbit monoclonal ab134922, Abcam). In 14 of the 32 study cases, paraffin blocks with sufficient tissue were available for immunohistochemical analyses, using a similar methodology that we and others used previously. Four µm thick sections of formalin-fixed, paraffin embedded tissues were placed on charged slides (VWR Superfrost Plus, Cat. No.48311-703), dried at 60°C for 1 hour, deparaffinized and rehydrated through graded alcohols. Immunohistochemical staining was performed according to the manufacturer’s guidelines using the BenchMark XT automated slide stainer (Ventana Medical System) with standard antigen retrieval (CC1,Tris/Borate/EDTA pH8.0, #950-124). The dilution and incubation time for each primary antibody are shown in Table 3.1. Positive controls were selected according to information published in the Human Protein Atlas (http://www.proteinatlas.org) and included tonsil (TS, TP, p53), spleen (DPD) and colon (Ki-67/MIB-1). A Ventana Ultraview Universal DAB Detection Kit (#760-500), containing a cocktail of enzyme-labeled secondary antibodies that locate the bound primary antibody, was used. The complex was then visualized with hydrogen peroxide substrate and 3, 3'- diaminobenzidine tetrahydrochloride (DAB) chromogen, which produced a dark brown reaction product. The slides were counterstained with Gill
modified hematoxylin, dehydrated in graded alcohol, cleared in xylene, and coverslipped in Permount.

Table 3.1 Dilution and incubation times for primary antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Host</th>
<th>Product #</th>
<th>Dilution</th>
<th>Incubation time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>Dako</td>
<td>mouse</td>
<td>M7240</td>
<td>1:100</td>
<td>60</td>
</tr>
<tr>
<td>p53</td>
<td>Dako</td>
<td>mouse</td>
<td>M7001</td>
<td>1:250</td>
<td>32</td>
</tr>
<tr>
<td>Thymidylate Synthetase</td>
<td>Dako</td>
<td>mouse</td>
<td>M3614</td>
<td>1:50</td>
<td>60</td>
</tr>
<tr>
<td>Thymidine Phosphorylase</td>
<td>Abcam</td>
<td>mouse</td>
<td>ab3151</td>
<td>1:1000</td>
<td>60</td>
</tr>
<tr>
<td>DPD</td>
<td>Abcam</td>
<td>rabbit</td>
<td>ab134922</td>
<td>1:2000</td>
<td>60</td>
</tr>
</tbody>
</table>

Ki-67, p53, TS, TP and DPD labeling indices (LI) were calculated as percentages of positive cells among at least 500 epithelial cells in 5 randomly selected fields, comparing them to their respective positive controls. A Leica DM2500 microscope equipped with DFC320 camera and application suite 4.4.0 (build:454) software was used to obtain photomicrographs at 200X. A semi-quantitative scoring system was used initially. 0-10% staining was considered negative (-), 11-50% was considered positive (+) and 51-100% was considered strongly positive (++) for comparing non-inflamed and inflamed fields of KOTs, a simplified scoring system was used where positive and strongly positive staining were scored as 1 and negative stains were assigned as 0. All LI were analyzed independently by 2 blinded reviewers (NL and DL) and results compared. Any differences were resolved by direct comparison together at the microscope. The percentage of immunoreactive positive cells from LI were summarized as mean percentage.

Data analysis

Data are reported as mean ± SE; Fishers and Chi squared exact tests were used as appropriate (p<0.05 considered to reflect statistical significance) using SPSS version 22.0 software for analysis.
3.4 Results

**Patient Demographics**

A total of 32 KOTs were reviewed, with 41% in women and 59% in men. The mean age at diagnosis was 42 years, 2 months ± 2.9 years. Mandibular lesions accounted for 27/32 KOTs with the remaining 5/32 found in the maxilla. A total of 21 KOTs were treated with enucleation, peripheral ostectomy and topical application of MC, and 11 KOTs were treated by enucleation, peripheral ostectomy and topical application of 5% 5-FU cream (Table 3.2). There were no significant differences in patient demographics between the two treatment groups (*p > 0.05*).
Table 3.2 Demographics of Keratocystic Odontogenic Tumor cases treated using Modified Carnoy’s (MC) or 5-Fluorouracil (5-FU).

<table>
<thead>
<tr>
<th></th>
<th>MC</th>
<th>5-FU</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Cases</td>
<td>21</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>Age (+/- SE years)</td>
<td>42y 3m (3.7)</td>
<td>42y 1m (4.8)</td>
<td>42y 2m (2.9)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandibular body</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>mandibular ramus</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>mandibular condyle</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anterior mandible + body</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mandibular body + ramus</td>
<td>12</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Mandibular body + ramus</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>+ coronoid process</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior Maxilla</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Maxillary premolar + molar</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Anterior maxilla +</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>maxillary premolar + molar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiographic Appearance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilocular</td>
<td>12</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Multilocular</td>
<td>7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Mean lesion size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(width x height mm)</td>
<td>34.8 x 44.5</td>
<td>28.4 x 30.1</td>
<td>31.6 x 37.3</td>
</tr>
<tr>
<td>(+/- SE width x height)</td>
<td>(3.9 x 4.8)</td>
<td>(4.1 x 6.1)</td>
<td>(2.9 x 3.9)</td>
</tr>
</tbody>
</table>

KOT Recurrences

KOTs measured a mean of 34.8 x 44.5 ± (3.9 x 4.8) mm in the MC group and 28.4 x 30.1 ± (4.1 x 6.1) mm in the 5-FU group (p>0.05). There were 6 patients treated with MC that had cortical perforations, of which, 2 developed recurrences. In the 5-FU group, 3 cases that had cortical perforations were successfully treated without recurrence. In the MC group (n=21), there were 4 (19.0%) recurrences with a mean recurrence time of 26.3 ± 1.8 months and a mean follow up
time of $41.3 \pm 3.8$ months. In contrast, there were no recurrences in the 5-FU group ($n=11$) with a mean follow up time of $35.0 \pm 8.5$ months ($p=0.19$, Table 3.3). All 5-FU-treated cases demonstrated normal bony healing (Figure 3.2).

Table 3.3 Recurrences in Keratocystic Odontogenic Tumor (KOT) treated with Modified Carnoy’s (MC) or 5-Fluorouracil (5-FU).

<table>
<thead>
<tr>
<th></th>
<th>MC  (n=21)</th>
<th>5-FU (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrences</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mean time to recurrence in months (±SE)</td>
<td>26.25 (1.8)</td>
<td>N/A</td>
</tr>
<tr>
<td>Follow up time in months (±SE)</td>
<td>41.3 (3.8)</td>
<td>35.0 (8.5)</td>
</tr>
</tbody>
</table>
Figure 3.2 Representative example of Keratocystic Odontogenic Tumor (KOT) treated with 5-fluorouracil. (A) Pre-operative panorex with a biopsy-confirmed KOT involving the right mandibular body, ramus and coronoid process. (B) 2-year post-operative panorex demonstrating well-healed, tumor-free right mandible treated with enucleation, peripheral ostectomy and topical application of 5% 5-fluorouracil cream (Efudex ®).

**Patient Morbidity**

There were no adverse local or systemic events in response to 5-FU or MC application. In 14/18 mandibular cases (77.8%) treated with MC, post-operative inferior alveolar nerve paresthesia was noted with a mean recovery time of 29.0±10.6 weeks. Four of these cases (22.2%) resulted in permanent paresthesia. In contrast, only 3 cases (33.3%) of 5-FU-treated patients had transient paresthesia ($p=0.039$, Table 3.4) that resolved in a mean time of 42.0±10.0 weeks.
Table 3.4 Inferior alveolar nerve injury data for mandibular cases treated with Modified Carnoy’s (MC) or 5-Fluorouracil (5-FU) (* = p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>MC</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cases</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Post-operative nerve injury cases</td>
<td>14</td>
<td>3*</td>
</tr>
<tr>
<td>Average neurosensory recovery time in weeks (±SE)</td>
<td>29.0 (10.6)</td>
<td>42.0 (10.0)</td>
</tr>
<tr>
<td>Permanent nerve injury cases</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

**Immunohistochemistry**

Ki-67 and p53 immunopositivity was demonstrated in the basal and suprabasal nuclei of KOT epithelium with LI of 18.64% and 16.56%, respectively (Figure 3.3).
Figure 3.3 Representative immunohistochemical staining of non-inflamed and inflamed areas of Keratocystic Odontogenic Tumors (KOTs) for p53, Ki-67, Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP), and Dihydropyrimidine Dehydrogenase (DPD) at 200x magnification. p53 and Ki-67 staining was observed in the nuclei of basal and suprabasal epithelial cells. Minimal to no staining for TS was seen in either non-inflamed or inflamed areas, TP showed positive nuclear and cytoplasmic staining in the epithelial lining of inflamed areas and DPD showed positive cytoplasmic staining in the epithelial lining of inflamed areas.
TS and DPD staining was observed in the cytoplasm; whereas TP demonstrated both nuclear and cytoplasmic staining (Figure 3.3). Sum scores calculated for TS, TP and DPD demonstrated mainly negative TS immunostaining, while positive TP and DPD staining was seen in 8 of 14 cases (Table 3.5).

**Table 3.5 Immunohistochemical staining scores for predictors of 5-Fluorouracil response in Keratocystic Odontogenic Tumors (KOTs).** Sum scores calculated by counting total positive cells among 500 cells in 5 randomly selected fields (200x magnification) for Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP), and Dihydropyrimidine Dehydrogenase (DPD): negative (-) = 0-10%, positive (+) = 11-50%, and highly positive (++) = 51-100%.

<table>
<thead>
<tr>
<th>Case</th>
<th>TS</th>
<th>TP</th>
<th>DPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Of the 14 cases evaluated, 10/14 (71.4%) showed inflammation in the cyst lining in at least 1 area of all sections examined. Inflamed areas of KOT showed significantly higher expression of
TP ($p<0.0001$) and DPD ($p<0.0001$) in the epithelial lining compared with non-inflamed areas (Figure 3.3). TS expression was low in both non-inflamed and inflamed areas (Figure 3.2). There were no differences in the LI for Ki-67 or p53 between non-inflamed and inflamed fields ($p>0.05$).

All inflamed TP (27/27) and DPD (24/24) stained fields were positive; whereas TS (16/26) stained minimally in the inflamed fields. This is in contrast to the non-inflamed fields where minimal positive staining was observed for all 3 markers [TS (18/44), TP (7/43) and DPD (6/41)] (Table 3.6, Figure 3.3).

**Table 3.6 Immunohistochemical staining scores for predictors of 5-Fluorouracil response according to the presence of inflammation in Keratocystic Odontogenic Tumors (KOTs).**

Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP) and Dihydropyrimidine Dehydrogenase (DPD) staining was assessed in a total of 5 fields (200x magnification) for each case, counting 100 cells/field. Non-inflamed and inflamed fields were separated for each case. Positive and highly positive staining were both scored as 1 and negative staining was scored as 0. The proportion of positively stained fields was indicated for non-inflamed and inflamed fields (** = $p < 0.001$).

<table>
<thead>
<tr>
<th>Case</th>
<th>TS</th>
<th></th>
<th>TP</th>
<th></th>
<th>DPD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non Inflamed</td>
<td>Inflamed</td>
<td>Non Inflamed</td>
<td>Inflamed</td>
<td>Non Inflamed</td>
<td>Inflamed</td>
</tr>
<tr>
<td>1</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>2</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>3</td>
<td>1/5</td>
<td>-</td>
<td>1/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2/3</td>
<td>2/2</td>
<td>0/2</td>
<td>3/3</td>
<td>1/2</td>
<td>3/3</td>
</tr>
<tr>
<td>5</td>
<td>4/5</td>
<td>-</td>
<td>2/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0/3</td>
<td>0/2</td>
<td>0/3</td>
<td>2/2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>0/5</td>
<td>-</td>
<td>1/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>2/2</td>
<td>1/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>9</td>
<td>1/2</td>
<td>2/3</td>
<td>1/2</td>
<td>3/3</td>
<td>3/3</td>
<td>2/2</td>
</tr>
<tr>
<td>10</td>
<td>3/4</td>
<td>1/1</td>
<td>1/4</td>
<td>1/1</td>
<td>0/4</td>
<td>1/1</td>
</tr>
<tr>
<td>11</td>
<td>1/2</td>
<td>2/3</td>
<td>1/2</td>
<td>3/3</td>
<td>1/2</td>
<td>3/3</td>
</tr>
<tr>
<td>12</td>
<td>0/2</td>
<td>2/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>13</td>
<td>1/2</td>
<td>0/3</td>
<td>0/2</td>
<td>3/3</td>
<td>1/2</td>
<td>3/3</td>
</tr>
<tr>
<td>14</td>
<td>3/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>18/44</strong></td>
<td><strong>16/26</strong></td>
<td><strong>7/43</strong></td>
<td><strong>27/27</strong>*</td>
<td><strong>6/41</strong></td>
<td><strong>24/24</strong>*</td>
</tr>
</tbody>
</table>
3.5 Discussion

We demonstrate for the first time that 5-FU is an effective and novel, targeted treatment for KOTs. Topical application of 5-FU, following enucleation and peripheral ostectomy, effectively treats KOTs resulting in normal bony healing with no adverse local or systemic effects. In addition, 5-FU is more readily available, technically easier, and results in less morbidity than conventional treatment with MC.

We examined 14 representative study cases by immunohistochemical staining for markers that may predict responsiveness to 5-FU treatment. All KOTs in our study demonstrated a moderate proliferation index, which is in agreement with previous studies and suggests that KOTs are amenable to treatment by inhibition of DNA synthesis with an antimetabolite agent such as 5-FU. The overall low expression of TS in KOTs is suggestive of susceptibility to 5-FU treatment. Studies in cancer cell lines have shown an inverse relationship between TS expression and efficacy of 5-FU treatment.

The expression of TP and DPD was markedly altered by the presence of inflammation. Areas of inflammation in the cyst wall were seen in the majority of the cases (10/14) in our study and was likely induced by prior incisional biopsy. There was no change in the low expression of TS in inflamed KOT linings but there was increased expression of both TP and DPD. TP may be up-regulated by multiple pro-inflammatory cytokines including TNF-alpha, interferon-gamma and interleukin-1 alpha. Greater TP expression in the lining of inflamed KOTs may promote conversion of 5-FU to active metabolites, including FdUMP, and thereby enhance destruction of residual KOT inadvertently left behind after enucleation. The low TS and high TP expression in inflamed KOTs suggest that procedures such as prior incisional biopsy, marsupialization, and intraoperative enucleation and curettage, which induce inflammation may increase the efficacy of 5-FU treatment of KOTs. 5-FU itself has been suggested to induce an intense inflammatory reaction when applied topically to skin within the first 24 hours. Previous studies have suggested increased DPD expression may result in decreased efficacy of systemic 5-FU treatment. This is likely related to metabolic inactivation of systemic 5-FU by hepatic DPD and/or tumor DPD. The topical application of 5-FU in our study avoids the problem of inactivation via hepatic DPD metabolism. The effect of increased DPD expression in inflamed KOTs on the efficacy of 5-FU treatment is unclear.
Other than providing a targeted therapy for KOTs, 5-FU may be more ideal than MC due to its ready availability, technical ease, shorter operating time, similar efficacy, and decreased morbidity compared to MC. 5-FU is simply coated onto ¼ inch ribbon gauze and packed into the residual bony cavity in a manner that allows for easy retrieval at 24 hours post-op. In contrast, there is substantially increased operating time when MC is used, due to the need for multiple precautions as described previously. There were no KOT recurrences in the patients treated with 5-FU. Conversely, the 19.0% recurrence rate observed with MC in this study is slightly lower compared to a recent report,¹² which may be explained by the addition of a peripheral ostectomy as a procedural adjunct in our study cohort. Our mean recurrence time of 26.3 months is also in line with prior studies.⁴¹ MC may result in significant local tissue destruction if not carefully handled. When MC is used to cauterize and fix the perilesional cavity, the blood components and bone turn black, which is likely due to protein precipitation and reaction with ferric chloride.⁴² Contact of MC with peripheral nerves causes damage to the perineural tissues when following the 3-minute application protocol defined by Frerich and colleagues.¹⁴ In agreement with prior studies,¹³ and ⁴³ a large majority of the patients with mandibular KOTs treated with MC in our study developed post-operative paresthesia and a substantial number had permanent neurosensory deficits.

This is the first study that demonstrates the efficacy and versatility of topical 5-FU application by packing the surgical site with 5-FU-impregnated ribbon gauze. This technique can be used for hard-to-treat areas of cortical perforation, in contrast to the relative contraindications for MC use in areas of cortical perforation. Similarly, 5-FU may be more amenable than MC for lesions in the posterior maxilla in close proximity to major vessels of the head and neck, orbital contents and the maxillary sinus, where there are concerns of vascular injury, neurovascular injury and sinus necrosis. Peri-orbital connective tissues also seem to be unaffected by twice daily application of topical 5-FU when used to treat ocular surface squamous neoplasia.⁴⁴ No studies to date have shown direct application of topical 5-FU to major blood vessels; however, twice weekly application of topical 5% 5-FU for 4 weeks following medial maxillectomy and sphenoethmoidectomy for ethmoidal adenocarcinoma had no mention of adverse effects on the infraorbital nerve nor the remaining sinus mucosa.⁴⁵ and ⁴⁶
There were no adverse effects from topical application of 5-FU in our study. However, systemic administration of 5-FU may result in adverse responses including: mucositis, granulocytopenia, neuropathy, cardiac toxicities, nausea, vomiting, pallor, hypotension, general malaise and death.\textsuperscript{47} and \textsuperscript{48} Approximately 3-5\% of the population is partially DPD deficient which can cause an intense systemic toxicity when 5-FU is used in any treatment. This is most prevalent in African-American females with up to 12\% of this particular demographic reported to be DPD deficient; therefore caution should be exercised when treating with 5-FU. The benefit of topical application of 5-FU in a controlled fashion as demonstrated in our study is the avoidance of untoward side effects.

3.6 Conclusion

5-Fluorouracil is a novel, effective, targeted treatment for KOTs with lower recurrence rates and less morbidity compared to Modified Carnoy’s solution. Inflamed KOTs may be more likely to respond to 5-FU treatment based on our immunohistochemical findings. The advantages of topical 5-FU include decreased post-operative morbidity, lower risk of re-operation, lower cost, and simple technique. It is also a known, accessible and well-studied drug. Further molecular characterization and prospective clinical trials are suggested for the treatment of KOTs with 5-FU.

3.7 Acknowledgements

We thank Dr. Jing Xu of the Applied Molecular Profiling Laboratory at the Princess Margaret Cancer Centre for her technical assistance. This study was supported by a \textit{Canadian Association of Oral and Maxillofacial Surgeons Research Grant} and the \textit{Bertha Rosenstadt Endowment Fund}. 
3.8 References


Chapter 4

Discussion

We demonstrated for the first time that 5-fluorouracil (5-FU) is an effective, novel treatment for KOTs. Topical application of 5% 5-FU cream (Efudex ®) for 24 hours, following enucleation and peripheral ostectomy, effectively treated KOTs and resulted in normal bony healing with no adverse systemic effects. In addition, 5-FU treatment was technically easier, cost effective; resulted in less morbidity and had lower recurrence rates than conventional treatment with Modified Carnoy’s solution (MC).

4.1 Topical 5-Fluorouracil

5-FU not only targeted the KOT at a molecular level, but by virtue of leaving the impregnated ribbon gauze in situ, it took aim at microscopic tumour remnants that may have been left in the hollowed bony cavity and also targeted those hard to treat areas where cortical perforation arose. Our findings suggested that KOTs with cortical perforations may have been more effectively treated when applying the 5% 5-FU cream compared to MC solution. Using this medication as a treatment option may be an excellent adjunct for lesions where cortical perforation arises because the medication can be placed directly onto these areas to destroy remaining lesional tissues and daughter cells in conjunction with appropriate soft tissue or periosteum excision. Similarly, 5-FU may be more amenable for lesions in the posterior maxilla in close proximity to major vessels of the head and neck, orbital contents and any areas where the maxillary sinus is involved to avoid complications of vascular injury, neural injury and sinus necrosis. Our study had 2 cases of 5-FU-treated KOTs in the maxilla, 1 anterior and 1 posterior. There were no issues with post-operative sinusitis, necrosis nor injury to the infraorbital nerve. Peri-orbital connective tissues were previously demonstrated to be unaffected by twice daily application of topical 5-FU when used to treat ocular surface squamous neoplasia (Parrozzani et al., 2011). No studies to date have shown direct application of topical 5-FU to major blood vessels; however, twice weekly application of topical 5% 5-FU for 4 weeks following maxillectomy and sphenoethmoidectomy for ethmoidal adenocarcinoma had no adverse effects on the infraorbital nerve nor the remaining sinus mucosa (Knegt et al., 2001 and Mackie et al., 2010). Studies suggested that KOTs in the maxilla recur more frequently than KOTs in the mandible due to the
greater degree of marrow spaces and therefore higher degree of difficulty in removing these lesions in their entirety (Stoelinga, 2001). The findings from our study suggested that application of topical 5-FU may have been ideal for these lesional cavities in regions with greater concern for morbidity and recurrences. Its technical ease of use and adaptiveness to these situations should give surgeons a further tool to help eradicate these lesions more effectively.

There were no adverse responses to topical application of 5-FU in our study. Systemic administration of 5-FU may result in adverse responses including: mucositis, granulocytopenia, neuropathy, cardiac toxicities, nausea, vomiting, pallor, hypotension, general malaise and death (Johnson et al, 1999; Papanastasopoulos and Stebbing, 2014). Approximately 3-5% of the population is partially DPD enzyme deficient which can cause an intense systemic toxicity when 5-FU is used in any treatment. This is most prevalent in African-American females with up to 12% of this particular demographic group reported to be DPD deficient, therefore caution should be exercised when treating with 5-FU. Manifestations reported included prolonged pancytopenia with signs and symptoms of sepsis, gastrointestinal toxicities, diarrhea, nausea/vomiting and mucositis (Papanastasopoulos and Stebbing, 2014). The benefit of topical application of 5-FU as demonstrated in our study avoids any possible untoward side effects. However, one case report described severe systemic toxicity with a standard dose of twice daily application of topical application of 5% 5-FU cream in a 76 year-old woman treated for a BCC of the scalp who had evidence of profound DPD deficiency (Johnson et al, 1999). Although it is extremely unlikely for anyone, even DPD deficient individuals, to experience systemic toxicities because of the exceedingly low dose and a one-time application in our study, caution should be exercised with anyone who has experienced severe systemic reactions to chemotherapeutic regimens involving 5-FU.

Older studies in animal models demonstrated safety of 5-FU when applied topically on syrian hamster oral mucosa at a concentration of 0.5 mg/day for 14 days with white blood cell count, red blood cell count, hematocrit, and peripheral blood cell differentials remaining stable (Cherrick and McKelvy, 1974). An ingestion study of topical 5% 5-FU is described in the veterinary literature when accidentally ingested by canines (Dorman et al, 1990). In 12/26 dogs, toxicosis occurred after consuming greater than 20 mg/kg with the dogs developing symptoms of: seizures, vomiting, tremors, diarrhea, ataxia, depression and death. Clinical signs developed within 1 hour of consumption and death occurred at 6-16 hours for all dogs that swallowed
greater than an estimated 43 mg/kg of topical 5-FU (Dorman et al, 1990). Since the 5-FU-coated ribbon gauze was placed into the lesional cavity and closed with our methodology, there was minimal to no risk for ingestion of 5-FU in our study. Accordingly, we did not observe any adverse local or systemic effects.

Other than providing a targeted therapy for KOTs, 5-FU may be more ideal than MC due to its technical ease, shorter operating time, similar efficacy, and decreased morbidity compared to MC. 5-FU was simply coated onto ¼ inch ribbon gauze and packed into the lumen in a manner that allowed for easy retrievability at 24 hours post-op. In contrast, with MC, there was substantially increased operating time due to the necessary precautions taken to prevent exposure to normal tissues as described in our methods.

4.2 KOT Recurrences and Post-Operative Nerve injury

Multiple hypotheses exist on why KOTs recur such as incomplete removal of all tumour lining, growth of daughter cysts after enucleation and alternatively, there may be possible de novo development of a KOT in the adjacent connective tissues (Stoelinga, 2001). There were no KOT recurrences in the patients treated with 5-FU in our study. As such, we accept our first hypothesis that KOTs treated with enucleation, peripheral ostectomy and topical 5% 5-FU had a lower recurrence rate than treatment with conventional enucleation, peripheral ostectomy and application of MC solution. However, there was a 19.0% recurrence rate observed with MC. This recurrence rate with MC in our study is slightly lower compared to a recent report (Dashow et al, 2015), which may be explained by the addition of a peripheral ostectomy as a procedural adjunct in our study cohort.

MC could result in significant local tissue destruction when not carefully handled. When used to cauterize and fix the hollowed perilesional cavity, the blood products and bone turn black as the blood reacts with the solution which was likely due to protein precipitation and reaction with ferric chloride (Saulacic et al, 2009). Contact of MC with peripheral nerves causes damage to the perineural tissues when following the 3-minute application protocol defined by Frerich and colleagues (1994). In agreement with prior studies (Roberio Junior et al, 2012 and Leung et al, 2016), a large majority of the cases treated in the mandible with MC in our study developed post-operative paresthesia and a substantial number had permanent neurosensory deficits. A recent
long-term retrospective study identified post-operative neurosensory deficits in the distribution of the inferior alveolar nerve at 30.1% with 16% of cases resulting in a permanent deficit (Leung et al., 2016). Their protocol used enucleation and application of the original CS. Another study found the paresthesia incidence to be 18.2% following enucleation, peripheral ostectomy and application of the original CS in 22 KOTs, although they did not distinguish between permanent versus temporary paresthesia (Riberio Junior et al., 2012). The authors in this study identified the main reason for post-operative paresthesia as exposure of the nerve.

Other than our study, there have been no studies to date that report the incidence of MC-induced nerve injury nor the effects of topical 5-FU application directly to peripheral sensory nerves. Our results suggested a higher degree of temporary paresthesia with similar numbers of long-term nerve dysfunction with MC, compared to CS. However, more importantly, our findings suggested that MC resulted in significantly higher permanent post-operative IAN damage than 5-FU when treating mandibular KOTs. None of the individuals in the 5-FU study cohort sustained permanent nerve injury. Therefore, we accept our second hypothesis that the rates of inferior alveolar nerve injury were decreased when using 5-FU compared to MC.

4.3 KOT Proliferation

All KOTs in our study demonstrated moderate proliferation through the evaluation of Ki-67 and p53 with IHC, which is in agreement with previous studies (Alur et al., 2014 and Melling et al., 2015). This suggested that KOTs are amenable to treatment by inhibition of DNA synthesis with an antimetabolite agent like 5-FU because these lesions were shown to be proliferating and being able to stop the production of DNA (i.e. with 5-FU) will ultimately destroy these lesions.

4.4 Expression of TS, TP and DPD

The overall low expression of TS in KOTs suggested particular susceptibility to 5-FU. Low expression of TS may have been advantageous when a TS inhibiting substance, such as 5-FU, was applied because less enzyme is available for DNA and RNA repair for the lesion to continue with proliferation. Gene amplification in human colorectal tumors with repeated exposure to 5-FU led to TS gene amplification and an overexpression of the TS protein which caused resistance of the tumour cells to treatment with 5-FU (Copur et al., 1995). However, with a one
time exposure of topical 5-FU to KOTs, it was unlikely for gene amplification to occur in these lesions.

One study found that low colorectal tumour expression of TP correlated with advanced disease (Koumarianou et al., 2014). High TP expression in the tumour lining of inflamed KOTs should have promoted conversion of 5-FU to active metabolites including FdUMP and therefore destroy remaining KOT daughter cells and lining inadvertently left behind after the initial removal.

Previous studies have suggested increased DPD expression may have resulted in decreased efficacy to systemic 5-FU treatment (Li et al., 2013). This was likely related to metabolism of systemic 5-FU by hepatic DPD and/or intratumoral DPD. However, despite the increased expression of intratumoral DPD in KOTs, topical application of 5-FU in our study avoided systemic metabolism of 5-FU via hepatic DPD metabolism, contributing to the effectiveness of 5-FU. As such, we accept our third hypothesis that the therapeutic efficacy of 5-FU is supported by immunohistochemical expression of TS, TP and DPD in KOTs.

4.5 Inflammation in KOTs

Inflamed KOTs showed particular promise when treated with adjunctive 5-FU. Inflammation of the KOT lining was present in the majority of the analyzed cases (10/14) in our study and was likely induced by prior incisional biopsy. There was no change in the low expression of TS in inflamed KOT linings but there was increased expression of both TP and DPD. TP may be up-regulated by multiple pro-inflammatory cytokines including TNF-alpha, interferon-gamma and interleukin-1 alpha (Toyoda et al., 2014 and Eda et al., 1993). Greater TP expression in the epithelial lining of inflamed KOTs may have promoted conversion of 5-FU to active metabolites, including FdUMP, and thereby enhance further destruction of remaining KOT daughter cysts and lining inadvertently left behind after the surgical removal. The low TS and high TP expression in inflamed KOTs suggested that procedures such as prior incisional biopsy, marsupialization, and intraoperative enucleation and curettage, which induce inflammation may increase the efficacy of 5-FU treatment of KOTs. 5-FU itself has been suggested to induce an intense inflammatory reaction when applied topically to skin within the first 24 hours (Costa et al., 2015). We suggested that a pre-operative biopsy +/- a period of marsupialization would likely
cause sufficient inflammation of the KOT for the upregulation of these markers and promote increased efficacy of 5-FU in treating KOTs.

4.6 Study Limitations and Future Studies

KOTs are reasonably rare, which makes having large-scale prospective studies difficult to achieve. As such, our study was limited by the number of cases and follow-up times in both the MC and 5-FU groups. A simple power calculation suggests that 80 cases should be included to better extrapolate the findings to the general population. An ambispective study with a heavy retrospective component had disadvantages including selection bias and information bias. It is often difficult to assess temporal relationships among the study variables. We could not control exposure nor outcome and unfortunately must rely on previous record keeping. Therefore, it can be difficult to make accurate comparisons between the exposed and non-exposed variables. Retrospective studies also require large sample sizes to establish significance to extrapolate to the general population.

There were many unknowns that surrounded the use of both MC solution and 5% 5-FU cream for the treatment of KOTs. Optimal concentrations, application times, safety and further understanding the mechanism of 5-FU interaction with KOTs should be elucidated in order to proceed with other clinical studies. Laboratory studies documenting KOT epithelium turnover time are important so that we can determine proliferation of these lesions. Taking fresh specimens and utilizing cell culture techniques for KOT cell growth and proliferation in vitro may provide further information. This may help to evaluate optimal treatment times and guide surgeons on the optimal time frames in which to apply the 5-FU cream. Furthermore, to understand the safety of 5-FU it would be useful to study the uptake of this medication using Carbon$^{14}$ labelled 5-FU to help quantify the systemic absorption of topically applied 5% 5-FU (Johnson et al, 1999) to bone marrow cavities. To better understand the depth of penetration of topical 5-FU, permeation studies may be undertaken. By labeling KOT preparations with $^3$H-labelled-5-Fluorouracil (Levy et al, 2001), we could effectively measure the flux and KOT lesional penetration of 5-FU. This may also be useful as a guide to measuring penetration of surrounding hard tissues. This information could aid in the selection of the optimal concentration of 5-FU and application times for treatment.
Given that we have shown preliminary evidence that suggested susceptibility of KOTs to treatment with 5-FU on a molecular level, this medication may be able to eradicate the lesion without further surgical techniques. Simply applying topical 5-FU to a biopsied KOT without enucleation may be efficacious to evaluate tumour responsiveness in vivo.

Reports of KOT recurrences have been noted up to 25 years after the initial treatment (Stoelinga, 2001), therefore it is prudent that well designed, long term, prospective, randomized, double-blinded controlled clinic trials are developed to truly analyze these factors and determine optimal sequences for treatment and follow up based on outcomes and recurrence patterns. Future studies comparing the efficacy of 5-FU with new medication classes such as SHH inhibitors may be warranted.
Chapter 5
Conclusions

5-FU is a novel, effective, targeted treatment for KOTs with lower recurrence rates and less morbidity compared to conventional adjunctive treatment with MC. We accept our alternate hypotheses to be true:

1) KOTs treated with enucleation, peripheral ostectomy and 5-FU have a lower recurrence rate than treatment with conventional enucleation, peripheral ostectomy and application of MC solution.

2) Rates of inferior alveolar nerve injury are decreased when using 5-FU compared to MC.

3) The therapeutic efficacy of 5-FU is likely via molecular interactions with TS, TP and DPD in KOTs

In particular, inflamed KOTs may be more likely to respond to 5-FU treatment based on our immunohistochemical findings. The advantages of topical 5-FU include decreased morbidity, lower cost, simple technique; known accessible and well-studied drug; molecularly targeted medication. We present for the first time, successful treatment of KOTs with topical 5% 5-FU cream (Efudex®). Further molecular characterization and prospective, randomized clinical trials with long-term follow up are suggested for the treatment of KOTs with 5-FU.
References


S. Levy, K. Furst, W. Chern. **A comparison of the skin permeation of three topical 0.5% fluorouracil formulations with that of 5% formulation** Clinical Therapeutics, 23 (2001), p. 901-907.


L. Lo Muzio. **Nevoid basal cell carcinoma syndrome (Gorlin syndrome).** Orphanet J Rare Dis, 32 (2008), p. 1-16.


Appendices

Appendix A.

Topical 5-Fluorouracil – A Novel Targeted therapy for the Keratocystic Odontogenic Tumour

Submitted for publication in the Journal of Oral and Maxillofacial Surgery
Abstract: Purpose
The antimetabolite drug, 5-fluorouracil (5-FU), is used in the treatment of various cancers including basal cell carcinomas (BCCs). We hypothesized that Keratocystic Odontogenic Tumors (KOTs) respond to 5-FU treatment due to their similarities in etiopathogenesis to BCCs. We: (1) tested the efficacy of KOT adjunctive treatment with 5-FU compared to conventional treatment with Modified Carnoy's solution (MC), and (2) assessed KOTs for expression of markers that may predict response to 5-FU treatment.

Methods
We conducted an ambispective cohort study of the treatment efficacy of KOTs treated with topical application of either 5% 5-FU or MC following enucleation and peripheral ostectomy, at the University of Toronto from 2006-2014. Outcome variables included KOT recurrence, time to recurrence, and peripheral nerve injury. KOT specimens in these patients were immunostained with p53, KI-67, thymidylate synthetase (TS), thymidylate phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) antibodies. Semi-quantitative staining scores were calculated for all immunohistochemistry sections examined. Descriptive statistics were computed and the p value was set at 0.05.

Results
5-FU was an effective adjunctive treatment for KOTs with no adverse side effects. There were no KOT recurrences in the 5-FU group (n=11); whereas there were 4 recurrences in the MC group (n=21, p=0.190). There was a significantly lower incidence of inferior alveolar nerve paresthesia with 5-FU treatment (p=0.039). Immunohistochemical staining revealed up-regulation of TP (p<0.0001) and DPD (p<0.0001), and no change in TS (p>0.05) in inflamed KOTs.

Conclusions
5-FU effectively treats KOTs with less post-operative morbidity than conventional treatment with MC. The therapeutic efficacy of 5-FU is
likely via molecular interactions with TP, DPD and TS in KOTs. Low TS and
up-regulated TP in inflamed KOTs suggests increased 5-FU efficacy in
inflamed KOTs. Topical 5-FU is a novel therapy for KOTs and provides a
targeted molecular approach to treatment.
Cover Letter

In consideration of the Journal of Oral and Maxillofacial Surgery taking action in reviewing and editing our submission, the authors undersigned hereby transfer, assign, or otherwise convey all copyright ownership to the American Association of Oral and Maxillofacial Surgeons in the event that such work is published in the JOURNAL OF ORAL AND MAXILLOFACIAL SURGERY. The undersigned authors understand that if the manuscript is accepted, the Editors reserve the right to determine whether it will be published in the print edition or solely in the Internet edition of the Journal. Articles accepted for publication are subject to editorial revision.

We have no conflicts of interest and no disclosures.

Nicholas J. Ledderhof

Marco F. Caminiti

Grace Bradley

David K. Lam
Financial Relationships Disclosure Form

For Faculty, Authors, Committee/Board Members, Reviewers and Staff

Organizations accredited by the American Dental Association Continuing Education Recognition Program (ADA CERP) and Accreditation Council for Continuing Medical Education (ACCME) are required to identify and resolve all potential conflicts of interest with any individual in a position to influence and/or control the content of CDE/CME activities. A conflict of interest will be considered to exist if: (1) the individual has a 'relevant financial relationship;' that is, he/she has received financial benefits of any amount, within the past 12 months, from a 'commercial interest' (an entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients), and (2) the individual is in a position to affect the content of CDE/CME regarding the products or services of the commercial interest.

All individuals in a position to influence and/or control the content of AAOMS CDE/CME activities are required to disclose to the AAOMS, and subsequently to learners: (1) any relevant financial relationship(s) they have with a commercial interest, or (2) if they do not have a relevant financial relationship with a commercial interest.

Failure to provide disclosure information in a timely manner prior to the individual's involvement will result in the disqualification of the potential Faculty, Author, Committee/Board Member, or Staff, from participating in the CDE/CME activity.

Type of CME activity: JOJS Manuscript Submission

Title of Submission: Topical 5-Fluorouracil - A Novel Targeted Therapy for the Keratocystic Odontogenic Tumor

Name: David K. Lam

Please check one to indicate your role:

- Faculty  X Author  ___ Committee Member (specify: )  ___ Board of Trustees
- ___ Reviewer  ___ Staff  ___ Other (specify: )

E-mail(required):

DISCLOSURE OF FINANCIAL RELATIONSHIPS WITHIN 12 MONTHS OF DATE OF THIS FORM

X  NO-Neither I, nor any member of my immediate family, has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.

OR

___ YES-I have had an immediate family member has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients. The financial relationships are identified as follows (if needed, attach an additional list):

<table>
<thead>
<tr>
<th>Commercial Interest(s) (any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.)</th>
<th>Research Grant (including funding to an institution for contracted research)</th>
<th>Speakers' Bureau</th>
<th>Stock/Bonds (excluding Mutual Funds)</th>
<th>Consultant</th>
<th>Other (Identify)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I affirm that the foregoing information is complete and truthful, and I agree to notify the AAOMS immediately if there are any changes or additions to my relevant financial relationships. During my participation in this activity, I will wholly support the AAOMS' commitment to conducting CDE activities with the highest integrity, scientific objectivity, and without bias. I agree that I will not accept any honoraria, additional payments or reimbursements beyond what has been agreed upon to be paid directly by the AAOMS in relation to this educational activity.

Electronic Signature*:  

Date: May 3, 2016

Corresponding author

*Electronic signature required from corresponding author only. It is the responsibility of the corresponding author to collect and submit all relevant conflicts of interest (or lack thereof) of all contributing authors at the time of the submission.
**DISCLOSURE OF FINANCIAL RELATIONSHIPS WITHIN 12 MONTHS OF DATE OF THIS FORM**

**1st Co-Author (if applicable)**
Name: Nicholas J. Ledderhof

<table>
<thead>
<tr>
<th>Commercial Interest(s)</th>
<th>Research Grant (including funding to an institution for contracted research)</th>
<th>Speakers' Bureau</th>
<th>Stock/Bonds (excluding Mutual Funds)</th>
<th>Consultant</th>
<th>Other (Identify)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**2nd Co-Author (if applicable)**
Name: Marco F. Caminiti

<table>
<thead>
<tr>
<th>Commercial Interest(s)</th>
<th>Research Grant (including funding to an institution for contracted research)</th>
<th>Speakers' Bureau</th>
<th>Stock/Bonds (excluding Mutual Funds)</th>
<th>Consultant</th>
<th>Other (Identify)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**3rd Co-Author (if applicable)**
Name: Grace Bradley

<table>
<thead>
<tr>
<th>Commercial Interest(s)</th>
<th>Research Grant (including funding to an institution for contracted research)</th>
<th>Speakers' Bureau</th>
<th>Stock/Bonds (excluding Mutual Funds)</th>
<th>Consultant</th>
<th>Other (Identify)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Topical 5-Fluorouracil – A Novel Targeted Therapy for the Keratocystic Odontogenic Tumor

Nicholas J. Ledderhof DDS, a Marco F. Caminiti DDS, MEd, FRCD(C), b Grace Bradley DDS, MSc, FRCD(C), c and David K. Lam MD, DDS, PhD, FRCD(C). d

a Chief Resident, Oral & Maxillofacial Surgery, Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

b Assistant Professor, Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

c Professor and Head, Oral Pathology & Oral Medicine, Faculty of Dentistry, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

d Assistant Professor and Head, Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Toronto, Ontario, Canada M5G 1G6

Corresponding Author: David K. Lam

Oral & Maxillofacial Surgery, University of Toronto, 124 Edward Street, Room 143, Toronto, Ontario, Canada M5G 1G6

Telephone: 416-979-4922 Ext 4329

Facsimile: (416) 979-4936

Email: david.lam@utoronto.ca
Abstract

Purpose

The antimetabolite drug, 5-fluorouracil (5-FU), is used in the treatment of various cancers including basal cell carcinomas (BCCs). We hypothesized that Keratocystic Odontogenic Tumors (KOTs) respond to 5-FU treatment due to their similarities in etiopathogenesis to BCCs. We: (1) tested the efficacy of KOT adjunctive treatment with 5-FU compared to conventional treatment with Modified Carnoy’s solution (MC), and (2) assessed KOTs for expression of markers that may predict response to 5-FU treatment.

Methods

We conducted an ambispective cohort study of the treatment efficacy of KOTs treated with topical application of either 5% 5-FU or MC following enucleation and peripheral ostectomy, at the University of Toronto from 2006-2014. Outcome variables included KOT recurrence, time to recurrence, and peripheral nerve injury. KOT specimens in these patients were immunostained with p53, Ki-67, thymidylate synthetase (TS), thymidylate phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) antibodies. Semi-quantitative staining scores were calculated for all immunohistochemistry sections examined. Descriptive statistics were computed and the p value was set at 0.05.

Results

5-FU was an effective adjunctive treatment for KOTs with no adverse side effects. There were no KOT recurrences in the 5-FU group (n=11); whereas there were 4 recurrences in the MC group (n=21, p=0.190). There was a significantly lower incidence of inferior alveolar nerve paresthesia with 5-FU treatment (p=0.039). Immunohistochemical staining revealed up-regulation of TP (p<0.0001) and DPD (p<0.0001), and no change in TS (p>0.05) in inflamed KOTs.
Conclusions

5-FU effectively treats KOTs with less post-operative morbidity than conventional treatment with MC. The therapeutic efficacy of 5-FU is likely via molecular interactions with TP, DPD and TS in KOTs. Low TS and up-regulated TP in inflamed KOTs suggests increased 5-FU efficacy in inflamed KOTs. Topical 5-FU is a novel therapy for KOTs and provides a targeted molecular approach to treatment.
**Introduction**

Keratocystic odontogenic tumors (KOTs) are benign lesions occurring in the maxilla and/or mandible with potential for significant morbidity. Reports of bone erosion, orbital invasion, skull base extension, and temporal fossa violation demonstrate the aggressive nature of this lesion. Previously known as Odontogenic Keratocysts (OKCs), KOTs were subsequently reclassified as a tumor by the World Health Organization (WHO) to better represent the neoplastic nature of this lesion.

Historically, treatment of the KOT involved simple enucleation; however, this was suboptimal due to a high recurrence rate. Other treatment options include marsupialization, curettage, peripheral ostectomy, adjunctive solution application, removal of overlying mucosa, or resection, either alone or in combination. Enucleation alone resulted in recurrence rates as high as 56%, whereas resection resulted in recurrence rates closer to 0%. Adjunctive application of a chemical fixative called Carnoy’s solution (absolute alcohol, glacial acetic acid, chloroform, ferric chloride) was shown to decrease the rate of recurrence after enucleation. However, chloroform was removed from Carnoy’s solution due to its carcinogenicity, giving rise to Modified Carnoy’s solution (MC). A recent study demonstrated that the use of MC was associated with significantly higher recurrence rates than with the original Carnoy’s solution (CS). Rates of peripheral nerve injury after the application of CS are estimated at 18.2% after direct application of the solution onto the nerve with the 3-minute protocol defined by Frerich and colleagues. Liquid nitrogen following enucleation of the lesion resulted in recurrence rates of 11.5%, which is similar to recurrence rates with CS.

A targeted approach to KOT treatment has been proposed, based on current understanding of the molecular genetics of KOTs. Molecular studies focusing on the PTCH tumor suppressor gene pathway yields a targeted treatment approach for basal cell carcinomas (BCCs). It is known that KOTs develop via PTCH gene mutations, similar to basal cell carcinomas (BCCs). Mutations in PTCH1 causes smoothened (SMO) activation and Sonic Hedgehog (SHH) signaling resulting in neoplastic growth.
More recently Rui and colleagues\textsuperscript{20} demonstrated SMO gene alterations likely play an important role in KOT development. This finding suggests that SHH signaling pathway antagonism may be an efficient way to molecularly target KOTs through SMO inhibition and suppression of SHH transcription factors.\textsuperscript{21} A recent study has shown that orally administered Vismodegib, a SHH inhibitor, may help to decrease the number and morbidity of multiple BCCs and KOTs in patients with Nevoid Basal Cell Carcinoma Syndrome (NBCCS).\textsuperscript{22}

The antimetabolite drug, 5-fluorouracil (5-FU) was shown to induce apoptosis by inhibiting SHH in hepatocellular carcinoma cells.\textsuperscript{23} 5-FU has a variety of applications in treatment of malignant disease including topical application to treat superficial BCCs.\textsuperscript{24} Salonga and colleagues\textsuperscript{25} showed that thymidylate synthetase (TS), dihydropyrimidine dehydrogenase (DPD), and thymidine phosphorylase (TP) are independent predictive measures of tumor responsiveness to 5-FU treatment. Increases in TS mRNA have been shown as a marker of resistance to 5-FU.\textsuperscript{26, 27, 28, 29 and 30} Downregulation of TS results in increased efficacy of 5-FU in colorectal cancer cell lines.\textsuperscript{31} DPD is an enzyme involved in uracil and thymidine catabolism and is responsible for the breakdown of 5-FU into its excretory metabolites.\textsuperscript{32} Low expression of DPD suggests an improved response to 5-FU treatment since DPD is used to break down 5-FU.\textsuperscript{25} Conversely, increased expression of TP suggests improved responsiveness to 5-FU because of increased FdUMP, an active metabolite of 5-FU.\textsuperscript{33}

Due to similarities in the molecular etiopathogenesis of BCCs and KOTs, KOTs may similarly respond favorably to treatment with topical application of 5-FU. We hypothesized that: (1) 5-FU is efficacious in the treatment of KOTs; (2) KOTs treated with 5-FU have a similar or lower recurrence rate than treatment with conventional application of MC; (3) rates of trigeminal nerve injury are decreased when using 5-FU compared to MC; and (4) KOTs express molecular markers that predict responsiveness to 5-FU.
Methods

Study Population and Design

An ambispective study of patients treated with topical application of 5-FU, or MC, following enucleation and peripheral ostectomy of KOT at the University of Toronto and Mount Sinai Hospital (Toronto, Canada) from 2006-2014 was performed. Ethics approval was obtained from the Mount Sinai Hospital (protocol #: 15-0011-E) and the University of Toronto (protocol #: 31638) Research Ethics Boards. Patient records were located via a retrograde search of operating room case lists, and by searching cyst enucleation codes for procedures performed in the clinic as set out by the Ontario Dental Association 2014 fee guide, for all attending Oral and Maxillofacial Surgeons at Mount Sinai Hospital from 2006-2014. Key words to identify and locate charts included: cyst, enucleation, Carnoy’s solution, 5-Fluorouracil, KOT, OKC, Keratocyst, Keratocystic Odontogenic Tumor, and Odontogenic Keratocyst. Operative notes, pathology reports and associated clinical records were reviewed.

Inclusion criteria for patients in the study were (1) biopsy-proven KOT/OKC, (2) complete history and clinical examination prior to definitive surgical intervention, and (3) completed surgical intervention for KOT. Exclusion criteria were (1) having a diagnosed psychiatric condition, (2) multiple KOTs or diagnosed Gorlin-Goltz Syndrome, (3) recurrent KOT, (4) prior trigeminal nerve injury or existing paresthesia, and (5) patients with a diagnosis of orthokeratinizing odontogenic cyst or odontogenic keratocyst – orthokeratinized variant.

Clinical Examination and Oral Biopsy

A comprehensive history and examination was performed on all patients to rule out a history of medical conditions or disorders that may alter their trigeminal sensory perception. Oral biopsy specimens of all patients meeting the inclusion criteria were evaluated by the Mount Sinai Hospital (Toronto, Canada) or the University of Toronto
Oral Pathology Biopsy Service to confirm the diagnosis of KOT. Demographic information was collected for each patient including age, sex, lesion location, radiographic appearance and tumor size.

**Topical application of 5-FU**

Following enucleation and peripheral ostectomy of the KOT lesion, sterile quarter-inch ribbon gauze was coated with 5% 5-FU (Efudex®, Valeant Inc., Laval, Quebec, Canada) and packed into the surgical wound. The wound was then closed per usual manner, leaving a small distal end (approx. 1 cm) of gauze exposed to allow gauze removal at 24 hours post-operatively (Figure 1).

**Topical application of MC**

Following intraoperative enucleation and peripheral ostectomy of the KOT lesion, the surrounding soft tissues were protected with multiple sterile petroleum jelly-coated neuro patties. MC solution-saturated neuro patties were then carefully placed in the surgical wound so that every discernable surface of the lesional cavity was exposed to MC for 3 minutes followed by thorough normal saline irrigation. All instruments exposed to MC were then removed from the operative field, and the surgical team re-gowned and gloved in order to prevent possible injury to healthy tissues by the caustic MC solution during wound closure.

**Immunohistochemistry**

Immunohistochemical staining was performed to evaluate the expression of markers that may predict response to 5-FU. Proliferative activity and DNA damage response were assessed by staining for Ki-67 (mouse monoclonal against MIB-1, M7240, Dako North America Inc., Carpintaria, California, USA) and p53 (DO-1 mouse monoclonal M7001, Dako North American Inc.) respectively. Responsiveness to 5-FU was assessed by staining for TS (mouse monoclonal M3614, Dako North America Inc.), TP (mouse monoclonal ab3151, Abcam, Toronto, Ontario, Canada) and DPD (rabbit monoclonal ab134922, Abcam). In 14 of the 32 study cases, paraffin blocks with sufficient tissue were available for immunohistochemical analyses, using a similar methodology that we
and others\textsuperscript{35} used previously. Four \(\mu\)m thick sections of formalin-fixed, paraffin embedded tissues were placed on charged slides (VWR Superfrost Plus, Cat. No.48311-703), dried at 60\(^\circ\)C for 1 hour, deparaffinized and rehydrated through graded alcohols. Immunohistochemical staining was performed according to the manufacturer’s guidelines using the BenchMark XT automated slide stainer (Ventana Medical System) with standard antigen retrieval (CC1,Tris/Borate/EDTA pH8.0, #950-124). The dilution and incubation time for each primary antibody are shown in Table 1. Positive controls were selected according to information published in the Human Protein Atlas (http://www.proteinatlas.org) and included tonsil (TS, TP, p53), spleen (DPD) and colon (Ki-67/MIB-1). A Ventana Ultraview Universal DAB Detection Kit (#760-500), containing a cocktail of enzyme-labeled secondary antibodies that locate the bound primary antibody, was used. The complex was then visualized with hydrogen peroxide substrate and 3, 3’-diaminobenzidine tetrahydrochloride (DAB) chromogen, which produced a dark brown reaction product. The slides were counterstained with Gill modified hematoxylin, dehydrated in graded alcohol, cleared in xylene, and coverslipped in Permount.

Ki-67, p53, TS, TP and DPD labeling indices (LI) were calculated as percentages of positive cells among at least 500 epithelial cells in 5 randomly selected fields, comparing them to their respective positive controls.\textsuperscript{35} A Leica DM2500 microscope equipped with DFC320 camera and application suite 4.4.0 (build:454) software was used to obtain photomicrographs at 200X. A semi-quantitative scoring system was used initially. 0-10\% staining was considered negative (-), 11-50\% was considered positive (+) and 51-100\% was considered strongly positive (++).\textsuperscript{36} For comparing non-inflamed and inflamed fields of KOTs, a simplified scoring system was used where positive and strongly positive staining were scored as 1 and negative stains were assigned as 0. All LI were analyzed independently by 2 blinded reviewers (NL and DL) and results compared. Any differences were resolved by direct comparison together at the microscope. The percentage of immunoreactive positive cells from LI were summarized as mean percentage.
Data analysis

Data are reported as mean ± SE; Fishers and Chi squared exact tests were used as appropriate (p<0.05 considered to reflect statistical significance) using SPSS version 22.0 software for analysis.

Results

Patient Demographics

A total of 32 KOTs were reviewed, with 41% in women and 59% in men. The mean age at diagnosis was 42 years, 2 months ± 2.9 years. Mandibular lesions accounted for 27/32 KOTs with the remaining 5/32 found in the maxilla. A total of 21 KOTs were treated with enucleation, peripheral ostectomy and topical application of MC, and 11 KOTs were treated by enucleation, peripheral ostectomy and topical application of 5% 5-FU cream (Table 2). There were no significant differences in patient demographics between the two treatment groups (p>0.05).

KOT Recurrences

KOTs measured a mean of 34.8 x 44.5 ± (3.9 x 4.8) mm in the MC group and 28.4 x 30.1 ± (4.1 x 6.1) mm in the 5-FU group (p>0.05). In the MC group (n=21), there were 4 (19.0%) recurrences with a mean recurrence time of 26.3 ± 1.8 months and a mean follow up time of 41.3 ± 3.8 months. In contrast, there were no recurrences in the 5-FU group (n=11) with a mean follow up time of 35.0 ± 8.5 months (p=0.19, Table 3). All 5-FU-treated cases demonstrated normal bony healing (Figure 3).

Patient Morbidity

There were no adverse local or systemic events in response to 5-FU or MC application. In 14/18 mandibular cases (77.8%) treated with MC, post-operative inferior alveolar nerve paresthesia was noted with a mean recovery time of 29.0±10.6 weeks. Four of these cases
(22.2%) resulted in permanent paresthesia. In contrast, only 3 cases (33.3%) of 5-FU-treated patients had transient paresthesia that resolved in a mean time of 42.0±10.0 weeks ($p=0.039$, Table 4).

**Immunohistochemistry**

Ki-67 and p53 immunopositivity was demonstrated in the basal and suprabasal nuclei of KOT epithelium with LI of 18.64% and 16.56%, respectively (Figure 2).

TS and DPD staining was observed in the cytoplasm; whereas TP demonstrated both nuclear and cytoplasmic staining (Figure 2). Sum scores calculated for TS, TP and DPD demonstrated mainly negative TS immunostaining, while positive TP and DPD staining was seen in 8 of 14 cases (Table 5).

Of the 14 cases evaluated, 10/14 (71.4%) showed inflammation in the cyst lining in at least 1 area of all sections examined. Inflamed areas of KOT showed significantly higher expression of TP ($p<0.0001$) and DPD ($p<0.0001$) in the epithelial lining compared with non-inflamed areas (Figure 2). TS expression was low in both non-inflamed and inflamed areas (Figure 2). There were no differences in the LI for Ki-67 or p53 between non-inflamed and inflamed fields ($p>0.05$).

All inflamed TP (27/27) and DPD (24/24) stained fields were positive; whereas TS (16/26) stained minimally in the inflamed fields. This is in contrast to the non-inflamed fields where minimal positive staining was observed for all 3 markers [TS (18/44), TP (7/43) and DPD (6/41)] (Table 6, Figure 2).
Discussion

We demonstrate for the first time that 5-FU is an effective and novel, targeted treatment for KOTs. Topical application of 5-FU, following enucleation and peripheral ostectomy, effectively treats KOTs resulting in normal bony healing with no adverse local or systemic effects. In addition, 5-FU is more readily available, technically easier, and results in less morbidity than conventional treatment with MC.

We examined 14 representative study cases by immunohistochemical staining for markers that may predict responsiveness to 5-FU treatment. All KOTs in our study demonstrated a moderate proliferation index, which is in agreement with previous studies and suggests that KOTs are amenable to treatment by inhibition of DNA synthesis with an antimetabolite agent such as 5-FU. The overall low expression of TS in KOTs is suggestive of susceptibility to 5-FU treatment. Studies in cancer cell lines have shown an inverse relationship between TS expression and efficacy of 5-FU treatment.

The expression of TP and DPD was markedly altered by the presence of inflammation. Areas of inflammation in the cyst wall were seen in the majority of the cases (10/14) in our study and was likely induced by prior incisional biopsy. There was no change in the low expression of TS in inflamed KOT linings but there was increased expression of both TP and DPD. TP may be up-regulated by multiple pro-inflammatory cytokines including TNF-alpha, interferon-gamma and interleukin-1 alpha. Greater TP expression in the lining of inflamed KOTs may promote conversion of 5-FU to active metabolites, including FdUMP, and thereby enhance destruction of residual KOT inadvertently left behind after enucleation. The low TS and high TP expression in inflamed KOTs suggest that procedures such as prior incisional biopsy, marsupialization, and intraoperative enucleation and curettage, which induce inflammation may increase the efficacy of 5-FU treatment of KOTs. 5-FU itself has been suggested to induce an intense inflammatory reaction when applied topically to skin within the first 24 hours. Previous studies have suggested increased DPD expression may result in decreased efficacy of systemic 5-FU treatment. This is likely related to metabolic inactivation of systemic 5-FU by hepatic DPD and/or tumor DPD. The topical application of 5-FU in our study avoids the problem
of inactivation via hepatic DPD metabolism. The effect of increased DPD expression in inflamed KOTs on the efficacy of 5-FU treatment is unclear.

Other than providing a targeted therapy for KOTs, 5-FU may be more ideal than MC due to its ready availability, technical ease, shorter operating time, similar efficacy, and decreased morbidity compared to MC. 5-FU is simply coated onto ¼ inch ribbon gauze and packed into the residual bony cavity in a manner that allows for easy retrieval at 24 hours post-op. In contrast, there is substantially increased operating time when MC is used, due to the need for multiple precautions as described previously. There were no KOT recurrences in the patients treated with 5-FU. Conversely, the 19.0% recurrence rate observed with MC in this study is slightly lower compared to a recent report,\textsuperscript{12} which may be explained by the addition of a peripheral ostectomy as a procedural adjunct in our study cohort. Our mean recurrence time of 26.3 months is also in line with prior studies.\textsuperscript{41} MC may result in significant local tissue destruction if not carefully handled. When MC is used to cauterize and fix the perilesional cavity, the blood components and bone turn black, which is likely due to protein precipitation and reaction with ferric chloride.\textsuperscript{42} Contact of MC with peripheral nerves causes damage to the perineural tissues when following the 3-minute application protocol defined by Frerich and colleagues.\textsuperscript{14} In agreement with prior studies,\textsuperscript{13,43} a large majority of the patients with mandibular KOTs treated with MC in our study developed post-operative paresthesia and a substantial number had permanent neurosensory deficits.

This is the first study that demonstrates the efficacy and versatility of topical 5-FU application by packing the surgical site with 5-FU-impregnated ribbon gauze. This technique can be used for hard-to-treat areas of cortical perforation, in contrast to the relative contraindications for MC use in areas of cortical perforation. Similarly, 5-FU may be more amenable than MC for lesions in the posterior maxilla in close proximity to major vessels of the head and neck, orbital contents and the maxillary sinus, where there are concerns of vascular injury, neurovascular injury and sinus necrosis. Peri-orbital connective tissues also seem to be unaffected by twice daily application of topical 5-FU when used to treat ocular surface squamous neoplasia.\textsuperscript{44} No studies to date have shown direct application of topical 5-FU to major blood vessels; however, twice weekly
application of topical 5% 5-FU for 4 weeks following medial maxillectomy and sphenoethmoidectomy for ethmoidal adenocarcinoma had no mention of adverse effects on the infraorbital nerve nor the remaining sinus mucosa.\textsuperscript{45} and \textsuperscript{46}

There were no adverse effects from topical application of 5-FU in our study. However, systemic administration of 5-FU may result in adverse responses including: mucositis, granulocytopenia, neuropathy, cardiac toxicities, nausea, vomiting, pallor, hypotension, general malaise and death.\textsuperscript{47} and \textsuperscript{48} Approximately 3-5\% of the population is partially DPD deficient which can cause an intense systemic toxicity when 5-FU is used in any treatment. This is most prevalent in African-American females with up to 12\% of this particular demographic reported to be DPD deficient; therefore caution should be exercised when treating with 5-FU. The benefit of topical application of 5-FU in a controlled fashion as demonstrated in our study is the avoidance of untoward side effects.

\textbf{Conclusion}

5-Fluorouracil is a novel, effective, targeted treatment for KOTs with lower recurrence rates and less morbidity compared to Modified Carnoy’s solution. Inflamed KOTs may be more likely to respond to 5-FU treatment based on our immunohistochemical findings. The advantages of topical 5-FU include decreased post-operative morbidity, lower risk of re-operation, lower cost, and simple technique. It is also a known, accessible and well-studied drug. Further molecular characterization and prospective clinical trials are suggested for the treatment of KOTs with 5-FU.

\textbf{Acknowledgements}

We thank Dr. Jing Xu of the Applied Molecular Profiling Laboratory at the Princess Margaret Cancer Centre for her technical assistance. This study was supported by a \textit{Canadian Association of Oral and Maxillofacial Surgeons Research Grant} and the \textit{Bertha Rosenstadt Endowment Fund}. 
References

1. T. Emerson, R. Whitlock, J. Jones

Involvement of soft tissues by odontogenic keratocyst (primordial cyst)

2. R. Chuong, R. Donoff, W. Guralnick

The odontogenic keratocyst

3. I. Jackson, Z. Potparic, M. Fasching

Penetration of the skull base by dissecting keratocyst


Unusual extraosseous extension of jaw lesion into the temporal fossa

5. L. Barnes, J. Eveson, P. Reichart, D. Sidransky, editors.

Pathology and genetics of head and neck tumours.

6. R. Bell, E. Dierks

Treatment options for the recurrent odontogenic keratocyst
7. G. Ghali, M. Connor

**Surgical management of the odontogenic keratocyst**


**Systematic review of the treatment and prognosis of the odontogenic keratocyst**


**Keratocystic Odontogenic Tumor (KCOT/OKC) – Clinical Guidelines for Resection**


**Two modifications in the treatment of keratocystic odontogenic tumors (KCOT) and the use of Carnoy's solution (CS)—a retrospective study lasting between 2 and 10 years**


**Management of odontogenic keratoctys of the jaws: a ten-year experience with 120 consecutive lesions**


Significantly Decreased Recurrence Rates in Keratocystic Odontogenic Tumor with Simple Enucleation and Currettage Using Carnoy’s Versus Modified Carnoy’s Solution


Keratocystic odontogenic tumors and Carnoy’s solution: results and complications assessment


14. B. Frerich, C. Cornelius, H. Weitholter

Critical Time of Exposure of the Rabbit Inferior Alveolar Nerve to Carnoy’s Solution


15. B. Schmidt, M. Pogrel

The use of enucleation and liquid nitrogen cryotherapy in the management of odontogenic keratocysts


16. D. Beach, R. Somer

Novel approach to Gorlin syndrome: a patient treated with oral capecitabine


17. C. Ren, H. Amm, P. DeVilliers, et al.
Targeting the Sonic Hedgehog Pathway in Keratocystic Odontogenic Tumor


Underestimated PTCH1 mutation rate in sporadic keratocystic odontogenic tumors

Oral Oncology, 51 (2015), p. 40–45

19. R. Toftgard

Hedgehog signaling in cancer


20. Z. Rui, P. Li-Ying, Q. Jia-Fei, et al.

Smoothened gene alterations in keratocystic odontogenic tumors

Head & Face Medicine, 10 (2014), p. 1-7


Inhibition of SHH signaling pathway: molecular treatment strategy of odontogenic keratocyst


22. P. Booms, M. Harth, R. Sader et al.

Vismodegib hedgehog-signaling inhibition and treatment of basal cell carcinomas as well as keratocystic odontogenic tumors in Gorlin syndrome

23. Q. Wang, S. Huang, L. Yang, et al.

*Down-regulation of Sonic hedgehog signaling pathway activity is involved in 5-fluorouracil-induced apoptosis and motility inhibition in Hep3B cells.*


24. K. Gross, L. Kircik and G. Kricorian

5% 5-Fluorouracil Cream for the Treatment of Small Superficial Basal Cell Carcinoma: Efficacy, Tolerability, Cosmetic Outcome, and Patient Satisfaction


*Colorectal tumors responding to 5-fluorouracil have low gene expression levels of dihydropyrimidine dehydrogenase, thymidylate synthase, and thymidine phosphorylase*


*Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines*


*Thymidylate synthase gene amplification in human colon cancer cell lines resistant to 5-fluorouracil*

28. S. Popat, A. Matakidou, R. Houlston

*Thymidylate synthase expression and prognosis in colorectal cancer: a systematic review and meta-analysis*


*TYMS serves as a prognostic indicator to predict the lymph node metastasis in Chinese patients with colorectal cancer*


*Cell-cycle Distribution and Thymidilate Synthatase (TS) Expression Correlate With 5-FU Resistance in Head and Neck Carcinoma Cells*


*Gossypol sensitizes the antitumor activity of 5-FU through down-regulation of thymidylate synthase in human colon carcinoma cells*


*Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile*


**Increased cytotoxicity and bystander effect of 5-fluorouracil and 5-deoxy-5-fluorouridine in human colorectal cancer cells transfected with thymidine phosphorylase**

Br J Cancer, 80 (1999), p. 1726–1733

34. M. Hardt, D. Lam, B. Schmidt

**Surveying proteolytic processes in human cancer microenvironments by microdialysis and activity-based mass spectrometry**


**Thymidylate synthase and dihydropyrimidine dehydrogenase expression in oral squamous cell carcinoma: An immunohistochemical and clinicopathologic study**


**Expression of Thymidylate Synthase and Dihydropyrimidine Dehydrogenase in Primary Oral Squamous Cell Carcinoma and Corresponding Metastases in Cervical Lymph Nodes: Association with the Metastasis Suppressor CD82**

Anticancer Research, 31 (2011), p. 3521-3526

Ki-67 and p53 expression in solitary sporadic, syndrome associated and recurrent keratocystic odontogenic tumor


Thymidine Phosphorylase Regulates the Expression of CXCL10 in Rheumatoid Arthritis Fibroblast-like Synoviocytes


How to treat actinic keratosis? An update

40. L. Li, H. Dong, F. Zhao, et al.

The upregulation of dihydropyrimidine dehydrogenase in liver is involved in acquired resistance to 5-fluorouracil

41. S. Apajalahti, J. Hagstrom, C. Lindqvist, et al.

Computerized tomography findings and recurrence of keratocystic odontogenic tumor of the mandible and maxillofacial region in a series of 46 patients

**Effects of Carnoy’s solution on blood vessels of the axillary fossa of rats**


43. Y. Leung, S. Lau, K. Tsoi, et al.

**Results of the treatment of keratocystic odontogenic tumours using enucleation and treatment of the residual bony defect with Carnoy’s solution**


**Topical 1% 5-fluorouracil in ocular surface squamous neoplasia: a long-term safety study**


**Adenocarcinoma of the Ethmoidal Sinus Complex Surgical Debulking and Topical Fluorouracil May Be the Optimal Treatment**


46. S. Mackie, T. Malik, and H. Khalil

**Endoscopic Resection and Topical 5-Fluorouracil as an Alternative Treatment to Craniofacial Resection for the Management of Primary Intestinal-Type Sinonasal Adenocarcinoma.**


Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil


48. P. Papanastasopoulos, J. Stebbing

Molecular Basis of 5-Fluorouracil-related Toxicity: Lessons from Clinical Practice

Figure Legend

Figure 1. 5-Fluorouracil application technique for the Keratocystic Odontogenic Tumor (KOT). (A) Panorex and computed tomographic (CT) imaging are used as necessary in the pre-operative work up and evaluation which includes incisional biopsy. The patient with a biopsy-confirmed KOT is brought to OR and undergoes enucleation and peripheral ostectomy in the standard fashion. B) Topical 5% 5-Fluorouracil cream (Efudex®) is applied generously to a ¼ inch ribbon gauze, and (C) packed into the entire wound covering all surfaces. The wound is closed in a standard fashion leaving approximately 1 cm of ribbon gauze out of the wound. The entire ribbon gauze is removed 24 hours post-operatively. No further lavage or rinsing of the surgical site is given in the post-operative phase.

Figure 2. Representative immunohistochemical staining of non-inflamed and inflamed areas of Keratocystic Odontogenic Tumors (KOTs) for p53, Ki-67, Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP), and Dihydropyrimidine Dehydrogenase (DPD) at 200x magnification. p53 and Ki-67 staining was observed in the nuclei of basal and suprabasal epithelial cells. Minimal to no staining for TS was seen in either non-inflamed or inflamed areas, TP showed positive nuclear and cytoplasmic staining in the epithelial lining of inflamed areas and DPD showed positive cytoplasmic staining in the epithelial lining of inflamed areas.

Figure 3. Representative example of Keratocystic Odontogenic Tumor (KOT) treated with 5-fluorouracil. (A) Pre-operative panorex with a biopsy-confirmed KOT involving the right mandibular body, ramus and coronoid process. (B) 2-year post-operative panorex demonstrating well-healed, tumor-free right mandible treated with enucleation, peripheral ostectomy and topical application of 5% 5-fluorouracil cream (Efudex®).
Table Legend

Table 1. Dilution and incubation times for primary antibodies.

Table 2. Demographics of Keratocystic Odontogenic Tumor cases treated using Modified Carnoy’s (MC) or 5-Fluouracil (5-FU).

Table 3. Recurrences in Keratocystic Odontogenic Tumor (KOT) treated with Modified Carnoy’s (MC) or 5-Fluouracil (5-FU).

Table 4. Inferior alveolar nerve injury data for mandibular cases treated with Modified Carnoy’s (MC) or 5-Fluouracil (5-FU) (* = p < 0.05).

Table 5. Immunohistochemical staining scores for predictors of 5-Fluouracil response in Keratocystic Odontogenic Tumors (KOTs). Sum scores calculated by counting total positive cells among 500 cells in 5 randomly selected fields (200x magnification) for Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP), and Dihydropyrimidine Dehydrogenase (DPD): negative (-) = 0-10%, positive (+) = 11-50%, and highly positive (++) = 51-100%.

Table 6. Immunohistochemical staining scores for predictors of 5-Fluouracil response according to the presence of inflammation in Keratocystic Odontogenic Tumors (KOTs). Thymidylate Synthetase (TS), Thymidine Phosphorylase (TP) and Dihydropyrimidine Dehydrogenase (DPD) staining was assessed in a total of 5 fields (200x magnification) for each case, counting 100 cells/field. Non-inflamed and inflamed fields were separated for each case. Positive and highly positive staining were both scored as 1 and negative staining was scored as 0. The proportion of positively stained fields was indicated for non-inflamed and inflamed fields (*** = p < 0.001).
Figure 1A
Click here to download high resolution image
Figure 1C
Click here to download high resolution image
Figure 2

<table>
<thead>
<tr>
<th></th>
<th>Non-inflamed</th>
<th>Inflamed</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Host</th>
<th>Product #</th>
<th>Dilution</th>
<th>Incubation time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>Dako</td>
<td>mouse</td>
<td>M7240</td>
<td>1:100</td>
<td>60</td>
</tr>
<tr>
<td>p53</td>
<td>Dako</td>
<td>mouse</td>
<td>M7001</td>
<td>1:250</td>
<td>32</td>
</tr>
<tr>
<td>Thymidylate Synthetase</td>
<td>Dako</td>
<td>mouse</td>
<td>M3614</td>
<td>1:50</td>
<td>60</td>
</tr>
<tr>
<td>Thymidine Phosphorylase</td>
<td>Abcam</td>
<td>mouse</td>
<td>ab3151</td>
<td>1:1000</td>
<td>60</td>
</tr>
<tr>
<td>DPD</td>
<td>Abcam</td>
<td>rabbit</td>
<td>ab134922</td>
<td>1:2000</td>
<td>60</td>
</tr>
</tbody>
</table>
### Table 2

<table>
<thead>
<tr>
<th></th>
<th>MC</th>
<th>5-FU</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Cases</strong></td>
<td>21</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td><strong>Age (+/- SE years)</strong></td>
<td>42y 3m (3.7)</td>
<td>42y 1m (4.8)</td>
<td>42y 2m (2.9)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandibular body</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>mandibular ramus</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>mandibular condyle</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anterior mandible + body</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mandibular body + ramus</td>
<td>12</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Mandibular body + ramus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ coronoid process</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Anterior Maxilla</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Maxillary premolar + molar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior maxilla + maxillary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>premolar + molar</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Radiographic Appearance</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilocular</td>
<td>12</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Multilocular</td>
<td>9</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td><strong>Mean lesion size</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(width x height mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+/- SE width x height)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC</td>
<td>34.8 x 44.5 (3.9 x 4.8)</td>
<td>28.4 x 30.1 (4.1 x 6.1)</td>
<td>31.6 x 37.3 (2.9 x 3.9)</td>
</tr>
</tbody>
</table>
Table 3.

<table>
<thead>
<tr>
<th></th>
<th>MC (n=21)</th>
<th>5-FU (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recurrences</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Mean time to recurrence in months (±SE)</td>
<td>26.25 (1.8)</td>
<td>N/A</td>
</tr>
<tr>
<td>Follow up time in months (±SE)</td>
<td>41.3 (3.8)</td>
<td>35.0 (8.5)</td>
</tr>
</tbody>
</table>
Table 4.

<table>
<thead>
<tr>
<th></th>
<th>MC</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cases</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Post-operative nerve injury cases</td>
<td>14</td>
<td>3*</td>
</tr>
<tr>
<td>Average neurosensory recovery time in weeks (±SE)</td>
<td>29.0 (10.6)</td>
<td>42.0 (10.0)</td>
</tr>
<tr>
<td>Permanent nerve injury cases</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5.

<table>
<thead>
<tr>
<th>Case</th>
<th>TS</th>
<th>TP</th>
<th>DPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 6.

<table>
<thead>
<tr>
<th>Case</th>
<th>TS</th>
<th></th>
<th>TP</th>
<th></th>
<th>DPD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non Inflamed</td>
<td>Inflamed</td>
<td>Non Inflamed</td>
<td>Inflamed</td>
<td>Non Inflamed</td>
<td>Inflamed</td>
</tr>
<tr>
<td>1</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>2</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>3</td>
<td>1/5</td>
<td>-</td>
<td>1/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2/3</td>
<td>2/2</td>
<td>0/2</td>
<td>3/3</td>
<td>1/2</td>
<td>3/3</td>
</tr>
<tr>
<td>5</td>
<td>4/5</td>
<td>-</td>
<td>2/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0/3</td>
<td>0/2</td>
<td>0/3</td>
<td>2/2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>7</td>
<td>0/5</td>
<td>-</td>
<td>1/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>2/2</td>
<td>1/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>9</td>
<td>1/2</td>
<td>2/3</td>
<td>1/2</td>
<td>3/3</td>
<td>3/3</td>
<td>2/2</td>
</tr>
<tr>
<td>10</td>
<td>3/4</td>
<td>1/1</td>
<td>1/4</td>
<td>1/1</td>
<td>0/4</td>
<td>1/1</td>
</tr>
<tr>
<td>11</td>
<td>1/2</td>
<td>2/3</td>
<td>1/2</td>
<td>3/3</td>
<td>1/2</td>
<td>3/3</td>
</tr>
<tr>
<td>12</td>
<td>0/2</td>
<td>2/3</td>
<td>0/2</td>
<td>3/3</td>
<td>0/2</td>
<td>3/3</td>
</tr>
<tr>
<td>13</td>
<td>1/2</td>
<td>0/3</td>
<td>0/2</td>
<td>3/3</td>
<td>1/2</td>
<td>3/3</td>
</tr>
<tr>
<td>14</td>
<td>3/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
</tbody>
</table>

Total | 18/44 | 16/26 | 7/43 | 27/27*** | 6/41 | 24/24***
**Cover Letter**

In consideration of the Journal of Oral and Maxillofacial Surgery taking action in reviewing and editing our submission, the authors undersigned hereby transfer, assign, or otherwise convey all copyright ownership to the American Association of Oral and Maxillofacial Surgeons in the event that such work is published in the JOURNAL OF ORAL AND MAXILLOFACIAL SURGERY. The undersigned authors understand that if the manuscript is accepted, the Editors reserve the right to determine whether it will be published in the print edition or solely in the Internet edition of the Journal. Articles accepted for publication are subject to editorial revision.

We have no conflicts of interest and no disclosures.

Nicholas J. Ledderhof

Marco F. Caminiti

Grace Bradley

David K. Lam
Financial Relationships Disclosure Form

For Faculty, Authors, Committee/Board Members, Reviewers and Staff

Organizations accredited by the American Dental Association Continuing Education Recognition Program (ADA CERP) and Accreditation Council for Continuing Medical Education (ACCME) are required to identify and resolve all potential conflicts of interest with any individual in a position to influence and/or control the content of CDE/CME activities. A conflict of interest will be considered to exist if: (1) the individual has a 'relevant financial relationship'; that is, he/she has received financial benefits of any amount, within the past 12 months, from a 'commercial interest' (an entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients), and (2) the individual is in a position to affect the content of CDE/CME regarding the products or services of the commercial interest.

All individuals in a position to influence and/or control the content of AAOMS CDE/CME activities are required to disclose to the AAOMS, and subsequently to learners: (1) any relevant financial relationship(s) they have with a commercial interest, or (2) if they do not have a relevant financial relationship with a commercial interest.

Failure to provide disclosure information in a timely manner prior to the individual's involvement will result in the disqualification of the potential Faculty, Author, Committee/Board Member, or Staff, from participating in the CDE/CME activity.

Type of CME activity: JOMS Manuscript Submission

Title of Submission: Topical 5-Fluorouracil - A Novel Targeted Therapy for the Keratoctytic Odontogenic Tumor

Name: David K. Lam

Please check one to indicate your role:

Faculty
Author
Committee Member (specify: )
Board of Trustees
Reviewer
Staff
Other (specify: )

E-mail (required):

DISCLOSURE OF FINANCIAL RELATIONSHIPS WITHIN 12 MONTHS OF DATE OF THIS FORM

☐ NO—Neither I, nor any member of my immediate family, has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.

OR

☐ YES—I have or an immediate family member has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients. The financial relationships are identified as follows (if needed, attach an additional list):

<table>
<thead>
<tr>
<th>Commercial Interest(s) (any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.)</th>
<th>Research Grant (including funding to an institution for contracted research)</th>
<th>Speakers' Bureau</th>
<th>Stock/Bonds (excluding Mutual Funds)</th>
<th>Consultant</th>
<th>Other (Identify)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I affirm that the foregoing information is complete and truthful, and I agree to notify the AAOMS immediately if there are any changes or additions to my relevant financial relationships. During my participation in this activity, I will wholly support the AAOMS' commitment to conducting CDE activities with the highest integrity, scientific objectivity, and without bias. I agree that I will not accept any honoraria, additional payments or reimbursements beyond what has been agreed upon to be paid directly by the AAOMS in relation to this educational activity.

Electronic Signature: __________________________ Date: May 3, 2016

Corresponding author

*Electronic signature required from corresponding author only. It is the responsibility of the corresponding author to collect and submit all relevant conflicts of interest (or lack thereof) of all contributing authors at the time of the submission.
1st Co-Author (if applicable)
Name: Nicholas J. Ledderhof

DISCLOSURE OF FINANCIAL RELATIONSHIPS WITHIN 12 MONTHS OF DATE OF THIS FORM

X NO—Neither I, nor any member of my immediate family, has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.

OR

YES—I have or an immediate family member has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients. The financial relationships are identified as follows (if needed, attach an additional list):

<table>
<thead>
<tr>
<th>Commercial Interest(s) (any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.)</th>
<th>Relevant Financial Relationship(s) Related to Your Content (Check all that apply)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Research Grant (including funding to an institution for contracted research)</td>
</tr>
<tr>
<td></td>
<td>Speakers’ Bureau</td>
</tr>
<tr>
<td></td>
<td>Stock/Bonds (excluding Mutual Funds)</td>
</tr>
<tr>
<td></td>
<td>Consultant</td>
</tr>
<tr>
<td></td>
<td>Other (Identify)</td>
</tr>
</tbody>
</table>

2nd Co-Author (if applicable)
Name: Marco F. Caminiti

DISCLOSURE OF FINANCIAL RELATIONSHIPS WITHIN 12 MONTHS OF DATE OF THIS FORM

X NO—Neither I, nor any member of my immediate family, has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.

OR

YES—I have or an immediate family member has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients. The financial relationships are identified as follows (if needed, attach an additional list):

<table>
<thead>
<tr>
<th>Commercial Interest(s) (any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.)</th>
<th>Relevant Financial Relationship(s) Related to Your Content (Check all that apply)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Research Grant (including funding to an institution for contracted research)</td>
</tr>
<tr>
<td></td>
<td>Speakers’ Bureau</td>
</tr>
<tr>
<td></td>
<td>Stock/Bonds (excluding Mutual Funds)</td>
</tr>
<tr>
<td></td>
<td>Consultant</td>
</tr>
<tr>
<td></td>
<td>Other (Identify)</td>
</tr>
</tbody>
</table>

3rd Co-Author (if applicable)
Name: Grace Bradley

DISCLOSURE OF FINANCIAL RELATIONSHIPS WITHIN 12 MONTHS OF DATE OF THIS FORM

X NO—Neither I, nor any member of my immediate family, has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.

OR

YES—I have or an immediate family member has a financial relationship or interest (currently or within the past 12 months) with any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients. The financial relationships are identified as follows (if needed, attach an additional list):

<table>
<thead>
<tr>
<th>Commercial Interest(s) (any entity producing, marketing, re-selling, or distributing health care goods or services consumed by, or used on, patients.)</th>
<th>Relevant Financial Relationship(s) Related to Your Content (Check all that apply)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Research Grant (including funding to an institution for contracted research)</td>
</tr>
<tr>
<td></td>
<td>Speakers’ Bureau</td>
</tr>
<tr>
<td></td>
<td>Stock/Bonds (excluding Mutual Funds)</td>
</tr>
<tr>
<td></td>
<td>Consultant</td>
</tr>
<tr>
<td></td>
<td>Other (Identify)</td>
</tr>
</tbody>
</table>