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An immunohistochemical study on BCL-6 expression in odontogenic cysts

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An immunohistochemical study on BCL-6 expression in odontogenic cysts

Nadeem Karachi

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Dentistry

in the branch of Maxillofacial and Oral Surgery

Johannesburg, 2013

DECLARATION

I, Nadeem Karachi, declare that this research report is my own work. It is being submitted for the degree of Master of Dentistry in the branch of Maxillofacial and Oral Surgery to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

Nadeem Karachi

_30th___ day of April 2013

DEDICATION

I dedicate this work to my heroes, my parents, Ally and Ayesha Karachi, who have given me everything…

ABSTRACT

In 2005 the World Health Organisation classified the odontogenic keratocyst (OKC) as a benign cystic neoplasm, using the term keratocystic odontogenic tumour. Significantly higher expression of proto-oncogenes and loss of expression of tumour suppressor genes have been demonstrated in the OKC when compared to more indolent jaw counterparts. This together with the higher mitotic activity in the epithelial lining of OKC could explain the aggressive behaviour of the OKC consistent with that of benign neoplasms. The BCL-6 proto-oncogene was identified in 1993 as a transcription factor whose deregulated expression is associated with B-cell non-Hodgkin lymphomas. In epithelial neoplasms, the BCL-6 transcription factor is associated with continuous growth and its absence triggers apoptosis. It has been suggested that BCL-6 may also participate prominently in the process of differentiation of epithelial cells. The aim of the study was to evaluate BCL-6 protein expression in the dentigerous cyst (n=10), radicular cyst (n=10) and OKC (n=20) by immunohistochemistry. Expression of BCL-6 was significantly higher in the OKC than in the dentigerous cyst and radicular cyst. In the OKC BCL-6 further showed a distinct predilection for the suprabasal compartment while in the dentigerous and radicular cysts the staining tended to be uniform throughout all the cell layers of the cyst lining. The study findings suggest that BCL-6 may play a role in the regulation of the suprabasal proliferative compartment of the OKC and in the keratinising epithelial cell differentiation of the OKC.

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PRESENTATIONS ARISING FROM THIS STUDY

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CHAPTER 1

1.0 INTRODUCTION

Since the time of its first description, the odontogenic keratocyst (OKC) has been a subject of active research particularly amongst oral pathologists and maxillo-facial and oral surgeons. The OKC presents with an aggressive clinical course and high recurrence rates compared to most other odontogenic cysts. The aggressive clinical course of the OKC, high recurrence rate and association with the nevoid basal cell carcinoma syndrome prompted researchers to try to find possible differences in the biological mechanisms of growth between the OKC and other odontogenic cysts.[1-3] More than thirty years of study, investigating the pathogenesis, histology, high recurrence rate and intrinsic higher growth potential compared to other odontogenic cysts has led to the reclassification of the OKC as a benign cystic neoplasm, designated as keratocystic odontogenic tumour (KCOT).[4] Subsequently, most studies on the OKC are focused on the underlying molecular mechanisms of growth and differentiation that distinguish the OKC from other jaw cysts that show no potential for recurrence following conservative enucleation of the lesion.[5]

Researchers studying the molecular pathogenesis of OKC have demonstrated significantly higher expression of proto-oncogenes and loss of expression of tumour suppressor genes in the OKC when compared to its more indolent jaw counterparts.[6] The complex process of neoplasia is known to involve the interplay between several classes of genes, many of which remain unidentified in the OKC. The BCL-6 proto-

oncogene was identified in 1993 as a transcription factor whose deregulated expression is associated with a subset of diffuse large B-cell non-Hodgkin lymphomas.[7] Recent studies have, however, shown that expression of the transcription factor initially identified as B-Cell Lymphoma 6 protein (BCL-6) is not restricted to lymphoma cells and may play a role in the histogenesis of neoplasms derived from various epithelial tissues.[8] This study therefore aimed to investigate whether the newly discovered BCL-6 protein is expressed by the epithelial lining constituting the OKC and if so whether or not there exists significant differences in BCL-6 expression between the OKC and clinically more indolent odontogenic cysts.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1. Historical background

The OKC was first described by Phillipsen in 1956,[1,2,6,9] and accounts for 8 to 11 percent of all jaw cysts.[1,9] It may occur in any age group, with a peak incidence in the second and third decades of life.[1,9] OKCs are usually asymptomatic and are chance findings during routine dental radiographic examination.[1,2,9] When OKCs are symptomatic, the most common signs are swelling and intra-oral drainage. Maxillary lesions are more prone to infection, even when small, hence presenting with symptoms usually at an earlier stage than mandibular lesions.[2] OKCs are more common in the mandible than in the maxilla. The posterior regions of the mandible are more frequently involved, especially the ramus, angle, and body region.[1,2,9] Extra-osseous lesions are exceedingly rare.[2,9] OKCs display intramedullary growth antero-posteriorly. It is possibly for this reason, that unlike ameloblastomas, OKCs infrequently cause cortical expansion.[2] Long standing, symptomatic lesions may cause cortical expansion.

Radiographically the OKC appears as a unilocular or multilocular radiolucency with distinct margins.[1,2,9] The margins are usually not sclerotic. Scalloping of the margins may, or may not be present. Scalloped margins are indicative of an OKC, but may be seen in other odontogenic lesions.[1] In 25 to 40 percent of cases an unerupted tooth may be involved.[2]

Histologically the OKC consists of an epithelial lining and a thin fibrous capsule.[1,2,6,10] The epithelial lining is 4 to 8 cell layers thick and consists of a parakeratinised stratified squamous epithelium, with corrugation of the parakeratin surface and palisaded nuclei of the basal cell layer.[1,2,6] The fibrous capsule is thin and often discontinuous in histological sections. There may be daughter cysts present in the fibrous capsule.[1,2,6] Daughter cysts are believed to arise due to budding off of cells from the basal layer and become incorporated into the fibrous capsule forming microcysts.[1,2] Sometimes odontogenic rests are seen in the fibrous capsule as well.

The first two World Health Organisation (WHO) classifications of odontogenic lesions in 1971 and 1992 put the OKC into the category of developmental odontogenic cysts.[1] Due to the accumulated evidence regarding the biological behaviour, clinico-pathological features, proliferative behaviour, and genetic and epigenetic alterations; the OKC was reclassified as a benign cystic neoplasm. According to the 2005 WHO classification the OKC is now categorised as a benign cystic odontogenic tumour, and it is called KCOT.[2,6,9,10] This contention is based on research that has been conducted on the molecular profile of the OKC. Different types of odontogenic tumours and odontogenic cysts arise from different epithelial remnants of the embryological dental lamina. The potential for further proliferation and differentiation of these epithelial remnants is different for the various odontogenic tumours and odontogenic cysts. These differences are speculated to be the underlying mechanism of the variations in biological behaviour and molecular expression in the different types of odontogenic cysts and tumours.[2,6]

The aggressive biological behaviour and the tendency of the OKC to recur after treatment may be attributed to cell kinetics in the lining epithelium.[2] Numerous research reports have shown a greater proliferative potential of the lining of the OKC, as well as a greater mitotic index, as compared to other types of odontogenic cysts.[2] Numerous growth factors influence cell kinetics and proliferative behaviour. The consistent finding of a higher proliferative and mitotic index in the lining cells of the OKC support the idea that the OKC may indeed be a neoplasm.[1,5]

Immunohistochemical studies by different groups of researchers have been done on the expression of proliferating cell nuclear antigen (PCNA), p53 and Ki-67 in odontogenic cysts. These immunocytochemical markers all have in common expression in actively dividing cells. A definite trend has emerged among the different research results. This trend is that PCNA, p53 and Ki-67 are expressed more frequently as well as more intensely in the OKC than in other types of odontogenic cysts. The positivity is also located in the suprabasal compartment in OKC, whilst in other odontogenic cysts positivity is located mainly in the basal cell layer.[2,11] Keratinocyte growth factor, which is responsible for epithelial proliferation and differentiation has also been shown to be expressed more in the OKC than in other types of odontogenic cysts.[10] Other studies have shown positive expression of epidermal growth factor receptor (EGFR), carcinoembryonic antigen (CEA), and cyclin D1.[5] The results suggest that there are differences in cell cycle kinetics between the OKC and other types of odontogenic cysts, particularly dentigerous and radicular cysts, as these were the cyst types that had been more commonly compared in previous studies.[5]

2.2. Molecular studies on the OKC

2.2.1. Expression of proto-oncogenes in the OKC

Any mutation or abnormal activation of genes that control the cell cycle and mitosis results in abnormal cell proliferation. These genes are called proto-oncogenes and their abnormal counterparts, oncogenes. Certain checkpoints of the cell cycle are regulated by cyclin-dependant kinases and their obligate activating partners, the cyclins. The CCND1 gene encodes for the cyclin D1, also known as BCL1. Cyclin D1 is located on chromosome 11q13. It controls the transition of the cell cycle from the G1 phase to the S phase.[12] Overexpression of cyclin D1 is implicated to play an important role in tumourigenesis. Cyclin D1 is expressed and easily detected in various types of malignancies, whereas in benign lesions or normal tissue, staining is generally weak.[5,12] Researchers have shown expression of cyclin D1 to be significantly higher in OKC than in other types of odontogenic cysts, particularly dentigerous cysts and radicular cysts.[5] Research has also shown that the expression of cyclin D1 is significantly higher in OKC than in ameloblastomas.[5] The expression of cyclin D1 is found mainly in the suprabasal compartment in OKC, whilst expression in other odontogenic cysts and in ameloblastomas it exhibits a more diffuse pattern.[5,12] This pattern of cyclin D1 expression in the OKC is thought to effect cellular proliferative activity specifically occurring in the suprabasal cell layers of the OKC.

In a study by Li *et al.*[13] in 1994 which compared PCNA in OKC, dentigerous cysts, radicular cysts and proliferating epithelial rests of Malassez in periapical inflammatory lesions, the expression of PCNA was significantly higher in OKC. The PCNA positive cells were found mainly in the suprabasal compartment; whereas in the other lesions

with significantly lower PCNA positivity, positive expression was detected mainly in the basal layer of the epithelium.[13] The findings suggest a unique proliferative and differentiation process that occurs within the epithelial lining of the OKC as compared to other odontogenic lesions. The suprabasal location of PCNA positivity in OKC could represent an intermediate stage between normality and dysplasia.[13]

In an immunohistochemical comparative study of OKCs and other odontogenic lesions by Di-Vicente *et al.*[5] in 2010, it was shown that Ki-67 positivity was significantly greater in OKC when compared to dentigerous cysts and radicular cysts, and comparable to the positivity seen in ameloblastomas. These results indicate a higher mitotic index for OKC and are suggestive of an intrinsic growth potential and its unique biological behaviour.[5]

The p63 gene may be regarded as a proto-oncogene and a gene that is responsible for the regulation of apoptosis. The reason for this is that the p63 gene exists in more than six isoforms. The TAp63 isoform is involved in apoptosis, whilst the ΔNp63 isoform participates in cellular proliferation and can be regarded as a proto-oncogene.[14] The ΔNp63 isoform has been shown to inhibit wild type p53 and therefore plays a role in blocking apoptosis.[14] These actions may facilitate the proliferative potential of epithelial tissue, play a role in the differentiation of epithelial lining cells of the OKC and favour tumourigenesis. Researchers have shown positive expression of p63 in the OKC and in up to 50% of cases positive expression has been demonstrated in a suprabasal pattern.[14]

Parathyroid hormone related protein has parathyroid hormone-like bioactivity and is a factor which regulates cell growth and differentiation locally.[15] In 1997 Li *et al.*[15] studied the expression of parathyroid hormone related protein in odontogenic cysts. The results showed significantly higher levels of parathyroid hormone related protein in OKC when compared to dentigerous cysts and radicular cysts. These findings suggest that parathyroid hormone related protein may modulate growth and bone resorption in odontogenic cysts.[15]

In 2011 Gadbail *et al.*[16] conducted a study to assess and compare angiogenesis with proliferative activity in OKC and dentigerous cysts. The study revealed significantly higher levels of Ki-67 in OKC as compared to dentigerous cysts and normal oral mucosa.[16] Gadbail *et al.*[16] further showed a significant correlation between Ki-67 and mean vascular density, total vascular area and mean vascular area. The findings of their study support the hypothesis that the stroma of the OKC should not only be regarded as a form of structural support of the OKC, but as playing a part in the neoplastic behaviour of the OKC.[16]

2.2.2. Expression of tumour suppressor genes in the OKC

Clonal allelic losses of tumour suppressor genes are seen in many benign and malignant neoplasms, and they have been found to accumulate in a temporal fashion. Knudson's hypothesis of tumour suppressor genes states that measurable DNA loss type mutations are accompanied by an immeasurable mutation in the copy of the gene that remains.[17] Methylation is an example of an immeasurable mutation. Methylation of p16, p21 and p27 has been shown in the OKC.[18] Methylation of p16 was, however, also found in

dental follicles and normal oral mucosa in the study by Moreira *et al*.[18] while in the same study 30% of OKCs showed methylation in the p21 gene. The expression of p21 is normally induced by wild type p53 leading to apoptosis or cell cycle arrest at the G1 checkpoint of the cell cycle. Loss of p21 protein could therefore lead to defective cell cycle regulation and also facilitate the actions of other oncogenic pathways. Similar to the methylation status of p16 in the group of samples examined by Moreira *et al.*,[18] methylation of p27 gene was also found in dental follicles and normal oral mucosa samples. The authors could not fully explain the functional relevance of these unexpected findings but explained that their investigation was a pilot study and suggested that some of the positive results could be due to partial methylation, monoallelic methylation or due to contamination of stromal cells.[18]

Among tumour suppressor genes the p53 protein is a common target for mutations and is seen as the common step in the pathogenesis of many human tumours.[19] Mutation of the p53 gene often results in an increase in stability of the p53 protein.[19] Two types of p53 protein exist, a wild type and a mutated type. The wild type acts as a tumour suppressor gene, whilst the mutated type is involved in tumourigenic activity. Expression of p53 is usually associated with neoplastic disease.[20]

In a study by Ogden *et al*.[19] in 1992, p53 protein expression was assessed in dentigerous cysts, radicular cysts and OKC. Ogden *et al*.[19] found that p53 expression was detected in almost half of the OKC samples and none of the other cyst type samples. It is believed that this was the first report to identify p53 expression in benign cystic epithelium.[19] Numerous studies have been done by different research groups over the

years on p53 expression, amongst other immunohistochemical markers, in odontogenic lesions.[14,20-22] The results have shown that the OKC exhibits a significantly higher expression of p53 when compared to dentigerous cysts and radicular cysts.[14,20-22]

A study by Slootweg *et al.*[22] compared p53 expression in the OKC, ameloblastoma, odontogenic carcinomas, radicular cysts and dentigerous cysts. The study findings showed similar expression of p53 in the OKC, ameloblastoma and odontogenic carcinoma.[22] The results of the study by Slootweg *et al.*[22] also showed a significantly higher expression of p53 in the OKC, ameloblastomas and odontogenic carcinomas, as compared to radicular and dentigerous cysts.[22] The biological mechanism associated with the expression of the p53 protein in the lining of the OKC has not been defined.[14] There still exists a lack of clarity regarding whether or not the accumulation of p53 in the OKC is due to mutated p53 or an accumulation of normal p53 protein.[14,20,22]

Another tumour suppressor gene of particular interest in the development of the OKC is the Patched (PTCH) gene.[23] It is involved in the sonic hedgehog signalling pathway and serves a role as a potent activator of cellular proliferation.[23] The sonic hedgehog signalling pathway has been implicated in embryogenesis as well as tumourigenesis. Binding of sonic hedgehog (Hh) to its receptor Patched can occur. In the absence of Hh, PTCH prevents the overexpression of a seven membrane spanning receptor, called smoothened (SMO), thereby preventing the activation of hedgehog signalling which culminates in cellular proliferation.[9,24] Genetic linkage studies have shown that the PTCH gene has been mapped to the same locus as the nevoid basal cell carcinoma syndrome gene and it has also been identified as the gene responsible for the nevoid

basal cell carcinoma syndrome.[25,26] Earlier research demonstrated the presence of mutations in the PTCH gene in OKCs in the nevoid basal cell carcinoma syndrome.[25,26] Some reports showed an incidence of over 75% of nevoid basal cell carcinoma syndrome associated OKCs harbouring PTCH gene mutations.[26] Later, research demonstrated PTCH mutations in non-syndromic sporadic OKCs.[24,26,27] Diniz *et al*.[28] stated in a report that PTCH has different isoforms. These different isoforms code for different functions, expression profiles, and transcriptional regulation.

Vered *et al*.[27] in a study in 2009 showed that solid ameloblastomas, unicystic ameloblastomas, primary OKCs, recurrent OKCs, as well as nevoid basal cell carcinoma associated OKCs expressed sonic hedgehog pathway related proteins which includes PTCH, in a similar pattern. The same study also showed that sonic hedgehog pathway proteins were also expressed in a similar pattern in orthokeratinised jaw cysts, dentigerous cysts and radicular cysts.[27] Based on these results the current evidence suggests that PTCH/Hh signalling alterations are not specifically associated with OKC development but may also drive the formation of other odontogenic cysts and tumours.[28]

2.2.3. Expression of genes that regulate apoptosis in the OKC

Apoptosis is described as a process of programmed cell death where there is a proteolytic cascade, condensation and shrinkage of the cell, and an alteration of the cell surface which allows it to be phagocytosed.[29] Apoptosis regulates cellular turnover and proliferation in normal and neoplastic tissues.[30] It is modulated by many proteins such as p53, BCL-2, Fas antigen, Fas ligand and caspases.[12]

The BCL-2 gene functions as a proto-oncogene by supressing apoptosis.[6] The BCL-2 protein is normally expressed in the basal layers of the skin and most other epithelia.[6] Expression of BCL-2 has also been reported in some low grade tumours.[11] The ability of BCL-2 to inhibit apoptosis has been regarded as one of the most common pathways in tumourigenesis.[11] When comparing BCL-2 expression between OKCs, dentigerous cysts and radicular cysts research has shown BCL-2 positivity in OKCs, whereas radicular cysts and dentigerous cysts showed almost complete negativity for BCL-2 expression.[3,30]

Studies have demonstrated that TdT-mediated nick–positive apoptotic cells are found in the superficial cells of the lining epithelium of the OKC.[31] The TUNEL method has been reported as an *in situ* method for detecting apoptotic cells.[31] In a study by Kichi *et al*.[31] in 2005 comparing cell proliferation, apoptosis and apoptosis related factors in OKCs and in dentigerous cysts, TUNEL-positive cells were observed in the lining epithelium of both OKCs as well as dentigerous cysts, however, the number of TUNEL positive cells was significantly greater in OKCs than in dentigerous cysts.[31]

A rationale was drawn as to why the OKC presents as cystic lesion rather than a tumour mass in spite of its biological potential to have a high proliferative index. The reasons cited were that the OKC has prominent proliferative activity in the intermediate layers of its lining epithelium while apoptosis is inhibited in the basal layers of the epithelium by BCL-2. Apoptosis occurs primarily in the superficial layer of the epithelium thereby regulating the thickness of the epithelial layer. p53 may contribute as an apoptosis-

related protein, while cyst cavity expansion may be facilitated by the abundance of keratin production and cellular debris arising from apoptosis.[31]

The biological profile of the OKC is progressively better understood by studies aimed at unravelling the molecular cascade of events that underlie the development of the OKC. These studies will hopefully pave the way to make up the strategy of molecular therapy for the OKC.

2.3. BCL-6

2.3.1. Molecular biological aspects of BCL-6

The BCL-6 protein is a zinc-finger type transcription factor. Zinc finger transcription factors are composed of zinc finger-binding domains. The zinc finger is a protein structural motif whose 3-dimentional architecture is co-ordinated and stabilised by one or more zinc ions. Zinc finger proteins typically function as interaction molecules that bind DNA, RNA or proteins. The BCL-6 protein is structurally made up of 2 important domains; one being the BTB/POZ domain in the amino-terminal end of the protein and the other important domain is that of six Kruppel-type zinc finger motifs in the carboxy-terminal end of the protein.[32,33] The BTB/POZ domain is important for protein-protein interactions. Similar to several other zinc finger proteins, BTB/POZ domains from BCL-6 have been shown to mediate transcriptional repression and to interact with components of histone deacetylase co-repressor complexes.[34] The zinc finger motifs of BCL-6 bind specific DNA sequences of target genes *in vitro*.[33]

Many common biochemical features have been noted for this class of proteins, in particular their ability to self-aggregate, to heteromerise with related but distinct zinc finger proteins and to interact with multi-protein complexes involved in transcriptional regulation. This ability allows BCL-6 to act as a transcriptional regulator.[33] BCL-6 harbours two separable trans-repressing regions. The first one is the N-terminal which is a self-interacting domain, termed BTB/POZ, which mentioned before is important in protein-protein interactions; the other trans-repressing region is one that encompasses a large central part of the protein and contains a short sequence shared by the BCL-6 close relative BAZF.[34]

Many transcription factors can act as both activators and repressors, depending on the promoter context. In a large scale analysis of BCL-6 target genes, BCL-6 appeared to activate the transcription of a subpopulation of target genes, however, the same target genes were repressed under different conditions or in other cell types.[35] The mechanism by which BCL-6 acts as a transcriptional activator under certain conditions is not known, although it is thought that BCL-6 most likely activates transcription by an indirect mechanism.[36] One theory is that BCL-6 may bind to repressor factors and remove them from the transcription machinery, thereby acting as an activator of transcription. BCL-6 may also fundamentally alter the growth cycle of the cell thereby changing the overall balance of multiple transcription factors, resulting in an apparent transcriptional activation of some regulatory elements. Another possibility is that the transcriptional activity of BCL-6 varies with the cell type or even the proliferative state of the cell, such that in some cells BCL-6 is a transcriptional repressor, whereas in other cells BCL-6 is an activator of transcription.[36]

BCL-6 has been shown to be part of the inherent machinery and a regulator of the cell cycle and is therefore being recognised to be intimately involved in the regulation of proliferation and differentiation.[37] Whether BCL-6 induces or inhibits apoptosis is still unclear. The finding that the human programmed cell death-2 (PDCD2) gene is a target for BCL-6 repression provides support for the concept that it is anti-apoptotic.[38] To the contrary Tang *et al*.[39] in 2002 showed that BCL-6 is a pro-apoptotic gene.[39] An examination of potential targets for BCL-6 repression revealed that the gene encoding the anti-apoptotic protein, BCL-XL, contains BCL-6 binding sites.[39] Similarly expression of BCL-6 in certain transformed or cancer cell lines such as HeLa, CV1 and U20S were found to cause cell cycle block in S phase and apoptosis,[32] while an anti-apoptotic role was described for BCL-6 in differentiating mouse myocytes.[40] Hence whether BCL-6 induces or inhibits apoptosis may depend on the levels of BCL-6 and the specific cell type or cellular environment.

The structural features and expression patterns of the BCL-6 protein suggest that it may function as a transcription factor involved in the control of B-cell development.[41] BCL-6 protein expression in B-cells is regulated following antigen encounter. Pre-germinal centre B cells up regulate BCL-6 protein, migrate to the follicular area and initiate germinal centre formation.[35] The BCL-6 protein is found at the highest levels in B-cells and T-cells of the germinal centre immune reaction. The germinal centre is a transient cellular compartment that proliferates after antigenic challenge and comprises rapidly proliferating B-cells. The fact that BCL-6 is expressed at high levels in rapidly proliferating germinal centre centroblast B-cells might suggest that BCL-6 is involved

in and may be partly responsible for B-cell proliferation. However, BCL-6 expression does not always correlate with cell proliferation, since high levels of BCL-6 protein have also been found in quiescent cells such as germinal centre centrocyte B-cells.[36]

Although BCL-6 is involved in the development and function of cells within lymphoid organs, it also has important functions in several other non-lymphoid cells. In non-lymphoid cell types it may function in the control of apoptosis and differentiation of these cells.[34] BCL-6 protein expression has been found in various sites outside the lymphoid system. It is expressed in various epithelial sites in the mouse embryo and in the olfactory bulb.[42] It is also found in the keratinocytes of mature animals where BCL-6 expression is associated with terminal differentiation of this cell type.[43] Furthermore, BCL-6 is expressed in various skin tumours.[44]

2.3.2. BCL-6 expression in epithelial tissues

Haematological malignancies are often characterised by non-random chromosomal translocations and it is from the 3q27 translocation breakpoint of diffuse large B-cell lymphoma that the BCL-6 gene was isolated as described earlier.[32,33] Subsequent to this discovery around the mid-nineties BCL-6 expression was increasingly being recognised in developing and adult non-lymphoid tissue. Yoshida *et al*.[43] analysed BCL-6 expression in keratinocytes by *in situ* hybridisation in epidermis from adult mice. Keratinocytes, which are synonymous with squamous epithelial cells, are known to differentiate from basal cells. It was found that BCL-6 mRNA expression was strong in the spinous and granular cell layer but was undetectable in the basal layer indicating that BCL-6 is up-regulated in keratinocytes after commitment of differentiation.[43]

Using immunohistochemistry, Kanazawa *et al*.[44] later sought to determine how the BCL-6 protein is expressed in human epidermal cells and how this expression mode compares with benign and malignant skin tumours. Of the 2 rabbit polyclonal anti-BCL-6 antibodies used in their study, the anti-3FR-1 reacts with formalin-fixed paraffin embedded tissue specimens.[44] On normal skin tissue cells, the basal layer consisting of basal cells and melanocytes, were unstained or stained very weakly. A large proportion of suprabasal cells showed strong nuclear staining. The granular cells stained weakly. Nuclei in the stratum corneum, when present, were either weakly stained or unstained. In the benign lesions examined, which included squamous papillomas and keratoacanthomas, a large proportion of basal cells were poorly stained while keratinocytes located near the basal layer were much more heavily stained. Keratinocytes in the upper epidermal layers were weakly stained and keratinising cells in the surface layer remained unstained.[44] Although the squamous cell carcinomas showed considerable variation in BCL-6 staining, in some cases the staining pattern was similar to that of the non-neoplastic epidermis and showed that the basally located cells and those around the keratinised foci were less intensely stained.[44]

In a study on BCL-2 and BCL-6 protein expression in normal and malignant transitional epithelium, Lin *et al*.[45] showed that BCL-2 was expressed only on basal layer cells. By contrast, BCL-6 was negative in most normal transitional epithelium with a positive rate of only 10%. When positive, BCL-6 was mainly distributed in the suprabasal layers of transitional epithelium. In transitional cell carcinomas, the expression of BCL-2 was significantly decreased while BCL-6 expression was significantly increased. The

positive rate of BCL-6 expression was 61.7% in transitional cell carcinoma, which was significantly higher than that in normal transitional epithelium (10%).

Yoshida *et al*.[43] and Kanazawa *et al*.[44] reported that BCL-6 protein was expressed in normal squamous cells of the skin and their neoplastic counterparts, and suggested that BCL-6 protein might play a prominent role in the differentiation of epithelial cells.[43,44] Lin *et al*.[45] also suggested that BCL-6 may play a role in the differentiation of urothelial tissue.

In contrast to those studies that demonstrate a positive correlation between BCL-6 expression and differentiation in keratinocytes,[43,44] transitional epithelium,[45] spermatids and skeletal muscle,[43] a study on BCL-6 expression in mammary epithelium suggests a paradoxical role for BCL-6 in this epithelium.[46] In *in vitro* mammary cell cultures, BCL-6 prevented the formation of ducto-alveolar structures that occurs on differentiation of the mammary cell line examined in the study by Logarajah *et al.*[46] From their results the authors put forward some hypotheses of the *in vivo* effects of BCL-6 expression in mammary epithelium suggesting that BCL-6 prevents terminal differentiation, maintains replicative potential and prevents apoptosis of mammary epithelium during the development and maturation of the mammary epithelium.[46]

From the above literature survey it is apparent that BCL-6 expression is progressively being recognised in a variety of non-lymphoid tissues. A physiological function of this protein is still, however, not known and from the studies conducted on BCL-6 thus far it would appear that its role may vary depending on the nature of the tissue wherein it is

expressed. In the field of maxillofacial and oral surgery odontogenic cysts of the jaw are encountered on a regular basis. Further, given the fact that the biological profile of the OKC is progressively better understood by studies aimed at unravelling the molecular cascade of events that underlie the development of the OKC, it seems timely to study BCL-6 expression in a group of odontogenic cysts of varying clinical behaviours and varying types of cyst epithelial linings. One of the characteristic histomorphological features of the OKC is its unique pattern of parakeratinisation. Based on previous studies that emphasise the role of BCL-6 in the differentiation of keratinising epithelium it is hypothesised that this marker would show preferential expression in the OKC as opposed to non-keratinising odontogenic cysts. This study therefore aims to test this hypothesis by analysing BCL-6 protein expression in the OKC, dentigerous cyst and radicular cyst.

CHAPTER 3

3.0 AIM AND OBJECTIVES

3.1. Aim

The overall aim of this study is to investigate BCL-6 protein expression by immunohistochemistry in the OKC, dentigerous cyst and radicular cyst.

3.2. Objectives

3.2.1. To determine whether the OKC lining shows BCL-6 expression

3.2.2. To determine the distribution of the BCL-6 positively stained cells in the OKC

3.2.3. To compare the staining characteristics of BCL-6 in the OKC with dentigerous cysts and radicular cysts

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1. Sample size

The haematoxylin and eosin (H&E) stained histological tissue sections of 40 OKCs, 20 dentigerous cysts (DC) and 20 radicular cysts (RC) were retrieved from the Oral Pathology archives at the University of the Witwatersrand. The H&E sections were examined histologically to confirm the diagnosis in each case and to select representative sections for subsequent immunohistochemical staining for the BCL-6 protein.

4.2. Immunohistochemistry

Sections from the 40 selected formalin-fixed paraffin-embedded tissue blocks were cut at 3 μm and immunostained with a monoclonal antibody that recognises BCL-6 (monoclonal mouse, Clone 4A4; DAKO, Carpinteria, CA, 1:50). Heat-induced epitope retrieval (HIER) was achieved in 10 μL EDTA using steam (pH 8.0). Before staining, all sections were blocked with 3% hydrogen peroxide for 5-10 minutes.

The BCL-6 immunostaining was performed using a DakoCytomation Autostainer (DAKO). Briefly, sections were treated with 2% normal goat serum (Vector Labs, Burlingame, CA) at room temperature for 30 minutes before labelling with primary antibody. After rinsing, sections were then incubated in anti-BCL-6 for 30 minutes at room temperature. Signal amplification for BCL-6 was performed with a horseradish

peroxidase-dextran-goat-antimouse antibody conjugate system at room temperature for 30 minutes (DakoCytomation EnVision+HRP Mouse detection system).

Immunoreactive cells were visualised with the brown colour resulting from incubation with diaminobenzidine (DAB) chromogenic substrate at room temperature for 5-20 minutes. Sections were counterstained blue with haematoxylin for 15 seconds and lithium carbonate for 5 seconds to provide morphologic detail. Brown nuclear staining of cells was recorded as positive. Tonsil was used as positive control tissue while the primary antibody was omitted in the immunohistochemical reactions on the 40 negative case control sections.

4.3. Evaluation of staining

Two observers utilised a double-headed microscope to evaluate BCL-6 expression in the lining of the cyst at a 40X magnification in each case. The number of BCL-6 positive cells (brown staining of the cell nucleus) present on the section of cyst lining represented on the slide was scored with regard to the approximate percentage of positive cells and relative immunostaining intensity. The cyst lining was evaluated for BCL-6 staining in the basal cell layer, suprabasal cell layers and the surface epithelial layer. Each of these 3 compartments of the cyst lining was scored independently, for quantity and intensity, and the data was recorded separately for each case.

The number (quantity) of BCL-6 positive cells was scored semi-quantitatively as follows: 0 = none, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100%; while staining intensity was rated as the percentage of BCL-6 stained cells at the following

levels of intensity: 0 = no staining; 1 = weak; 2 = moderate; and 3 = intense. For example, if the suprabasal cellular compartment of the cyst examined contained 50% of the epithelial cells with moderate intensity (2 x 2 = 4), 25% of epithelial cells with intense immunostaining (1 x 3 = 3), and 25% of cells with weak intensity (1 x 1 = 1), the intensity score would be 4 + 3 + 1 = 8. The maximum possible score was 12.

Inter-examiner reliability of the data was established with the aid of a double-headed microscope. For intra-observer reliability, every second case was re-assessed on a second occasion. The re-counted data was statistically compared using the Sign test and probability levels of < 0.05% were regarded as being significant. After establishing the reliability of the data we compared the results of the BCL-6 staining between the 3 cyst types using statistical tests for ordinal, categorical data.

4.4. Collection of data and analysis of results

The raw data was entered onto an Excel spread sheet and statistically analysed using Stata software, version 11.

4.5. Ethics

Ethics approval was granted to the Division of Oral Pathology by the Committee for Research on Human Subjects of the University of the Witwatersrand (Clearance Certificate number: M080850) for the use of stored paraffin wax embedded blocks in histopathology studies (Appendix 1).

CHAPTER 5

5.0 RESULTS

5.1. Analysis of count–recount data to determine intra-observer reliability

Since the data are ordinal the paired sample t-test could not be applied. The Sign test, which makes very few assumptions about the nature of the distributions of the data, was therefore used to test the hypothesis that there is no difference in the count-recount paired sample data for both quantity and intensity of BCL-6 staining. For the count-recount data on quantification of BCL-6 staining the test yielded *p*-values of 1.0, 0.08 and 0.72 for the basal, suprabasal and surface layer respectively. For the count-recount data on intensity of BCL-6 staining the test yielded *p*-values of 0.34, 0.61 and 0.42 for the basal, suprabasal and surface layer respectively indicating no significant differences between the count-recount data.

5.2. Comparison of BCL-6 staining within the cyst linings of the OKC, DC and RC

5.2.1. To determine whether there is a significant quantitative difference in BCL-6 expression in the basal cell layer, suprabasal cell layers and surface epithelial layer between the 3 cyst types (Figures 3, 5, 7, 9, 11), statistical tests for ordinal data were applied. The chi-square test was discarded as > 33% of the cells had counts of < 5, which invalidates the chi square test percentage. An example of one of these frequency tables is shown in Table 1.

Table 1. Frequency table depicting semi-quantitative data for BCL-6 expression in the suprabasal layers of the 3 cyst types

Summary Frequency Table (Stats1) Marked cells have counts > 10 (Marginal summaries are not marked)				
Suprabasal Q	Group 1	Group 2	Group 3	Row Totals
1	0	8	1	9
2	1	1	6	8
3	14	1	3	18
4	5	0	0	5
All Grps	20	10	10	40

0 = none, 1 = 1-25%, 2 = 26-50%, 3 = 51-75% and 4 = 76-100%

Cyst type comprised 3 groups:

Group 1 = odontogenic keratocyst, Group 2 = dentigerous cyst, Group 3 = radicular cyst

A 3x2 Fisher's exact test was then done for each of the layers of the epithelial lining of the 3 cysts (Tables 2 - 4). The number (quantity) of BCL-6 positive cells was scored semi-quantitatively as follows: BCL-6 positivity in ≤50% of cells and BCL-6 positivity in > 50% of cells. There was no significant difference in BCL-6 expression of the basal cells between the 3 cyst types (Figures 4, 6, 8, 10, 12).

Table 2. Fisher's exact table for semi-quantitative analysis of BCL-6 expression in the basal layer of the cysts

Score	**OKC**	**Dentigerous cyst**	**Radicular cyst**
≤ 50% BCL-6+ cells	17	10	8
> 50% BCL-6+ cells	3	0	2

The statistical test yielded a *P* value of 0.57.

Table 3. Fisher's exact table for semi-quantitative analysis of BCL-6 expression in the suprabasal layer of the cysts

Score	OKC	Dentigerous cyst	Radicular cyst
≤ 50% BCL-6+ cells	1	9	7
> 50% BCL-6+ cells	19	1	3

The statistical test yielded a *P* value of < 0.0001.

The OKC showed significantly greater expression of BCL-6 in the suprabasal cells compared to the dentigerous and radicular cyst (Figures 1, 4, 6, 8, 10, and 12).

Table 4. Fisher's exact table for semi-quantitative analysis of BCL-6 expression in the surface layer of the cysts

Score	OKC	Dentigerous cyst	Radicular cyst
≤ 50% BCL-6+ cells	20	9	7
> 50% BCL-6+ cells	0	1	3

The statistical test yielded a *P* value of 0.03.

The OKC showed significantly less BCL-6 expression in the surface layer of cells compared to the dentigerous cyst and radicular cyst (Figures 1, 4, 6, 8, 10, and 12).

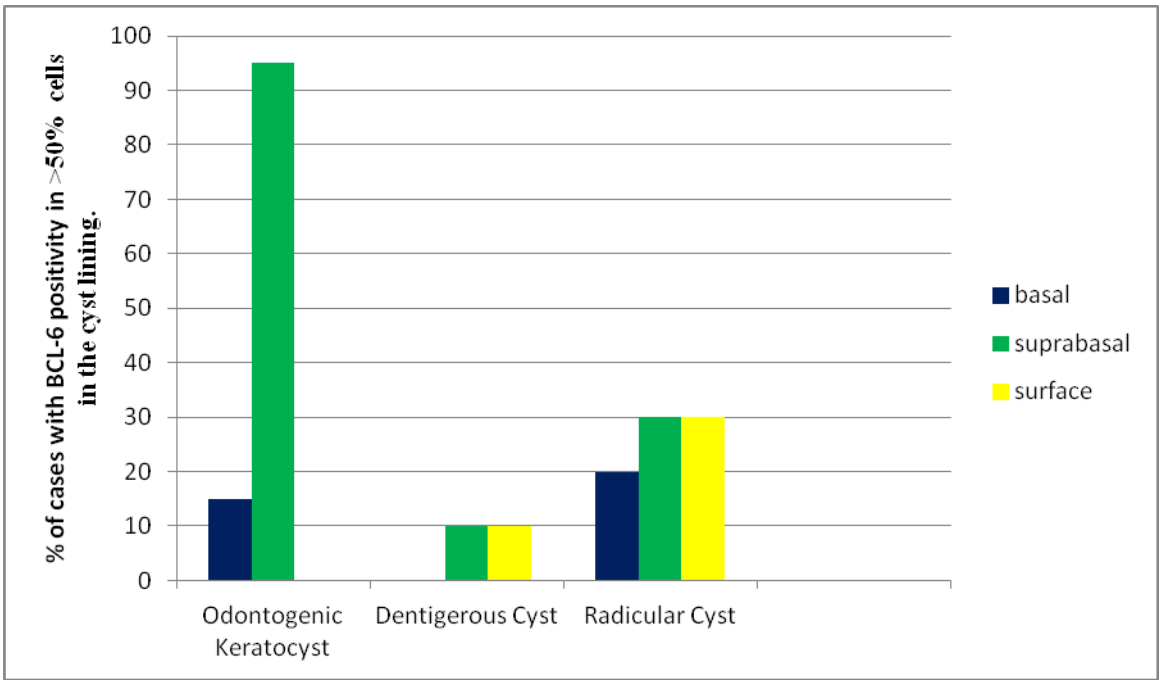


Figure 1. Histogram illustrating semi-quantitative comparisons of BCL-6 expression in the basal, suprabasal, and surface epithelial layers of the odontogenic keratocyst, dentigerous cyst and radicular cyst

5.2.2. In order to determine whether there is a significant difference in the intensity of BCL-6 expression in the basal cell layer, suprabasal cell layers and surface epithelial layer between the 3 cyst types, statistical tests for ordinal data were applied. For intensity, the scores varied from 0 to a maximum score of 12. An example of one of these frequency tables is shown in Table 5.

Table 5. Frequency table depicting intensity of BCL-6 expression in the basal cell layer of the 3 cyst types

Summary Frequency Table (Stats1) Marked cells have counts > 10 (Marginal summaries are not marked)				
Basal I	Group 1	Group 2	Group 3	Row Totals
0	2	0	0	2
4	7	6	2	15
5	3	3	2	8
6	1	0	3	4
7	2	0	1	3
8	3	0	0	3
9	1	0	1	2
10	1	0	0	1
11	0	1	0	1
12	0	0	1	1
All Grps	20	10	10	40

Cyst type comprised 3 groups:

Group 1 = odontogenic keratocyst, Group 2 = dentigerous cyst, Group 3 = radicular cyst

Due to the wide range of scores obtained over relatively small sample sizes in each cyst category, the intensity of staining was divided into scores of < 6 and scores of ≥ 6.

3x2 Fisher's exact tests were then applied (Tables 6 - 8).

Table 6. Fisher's exact table for intensity of BCL-6 expression in the basal layer of the cysts

Intensity	OKC	Dentigerous cyst	Radicular cyst
< 6	12	9	4
≥ 6	8	1	6

The statistical test yielded a *P* value of 0.08.

Table 7. Fisher's exact table for intensity of BCL-6 expression in the suprabasal layer of the cysts

Intensity	OKC	Dentigerous cyst	Radicular cyst
< 6	0	9	7
≥ 6	20	1	3

The statistical test yielded a *P* value of < 0.0001.

The intensity of BCL-6 staining was significantly greater in the OKC compared to the dentigerous and radicular cyst (Figures 2, 4, 6, 8, 10, and 12).

Table 8. Fisher's exact table for intensity of BCL-6 expression in the surface layer of the cysts

Intensity	OKC	Dentigerous cyst	Radicular cyst
< 6	11	9	7
≥ 6	9	1	3

The statistical test yielded a *P* value of 0.02.

Although the surface parakeratinocytes of the OKC showed significant fewer BCL-6 positive cells (Figure 1), when BCL-6 staining was present in the surface layer of the OKC, the intensity of staining was significantly greater than in the surface cells of the dentigerous and radicular cysts (Figure 2).

All raw data are shown in Appendix B-E.

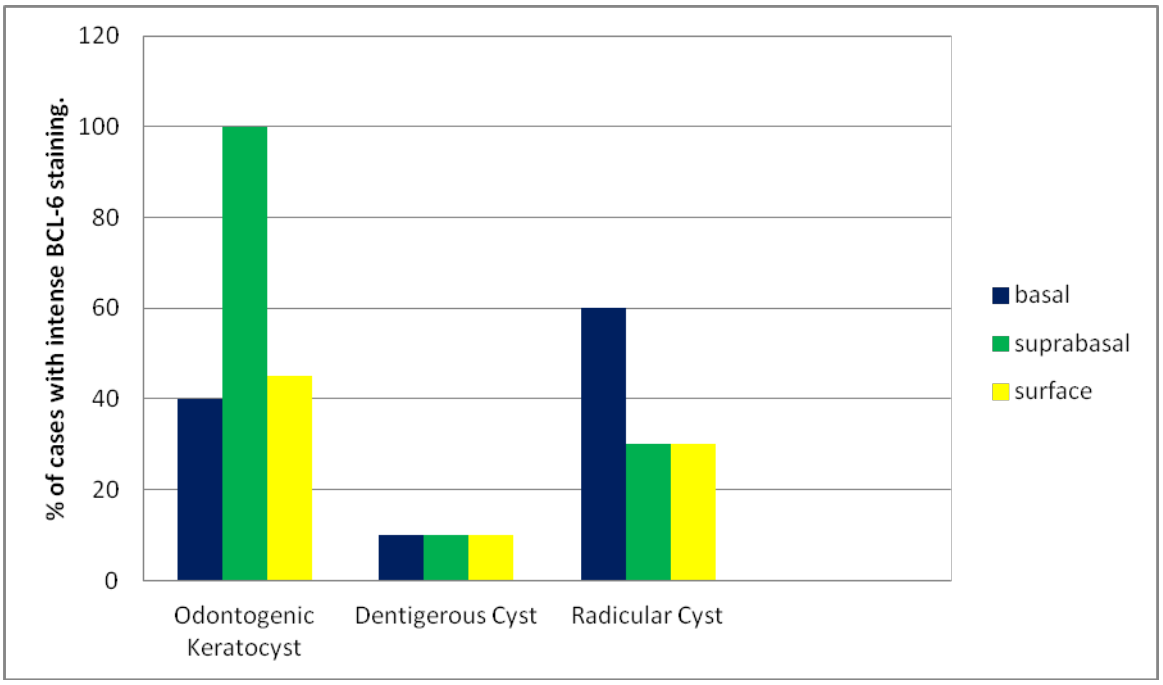


Figure 2. Histogram illustrating BCL-6 intensity in the basal, suprabasal, and surface epithelial layers of the odontogenic keratocyst, dentigerous cyst and radicular cyst

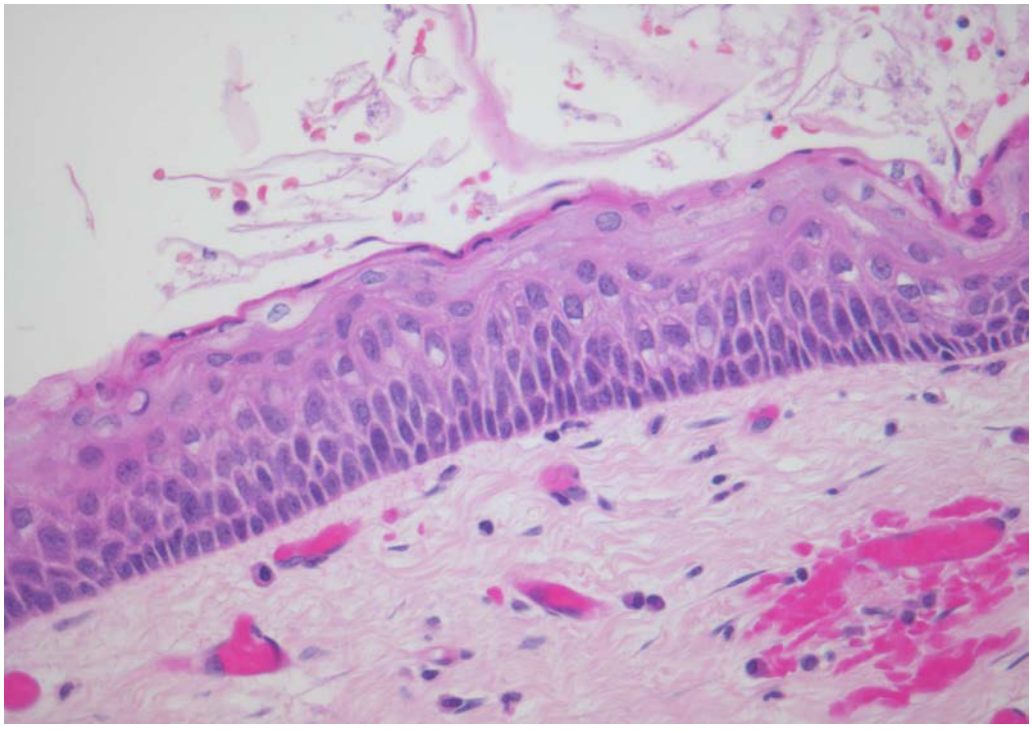


Figure 3. Photomicrograph of odontogenic keratocyst depicting the corrugated surface layer of parakeratinocytes (H&E; 400X)

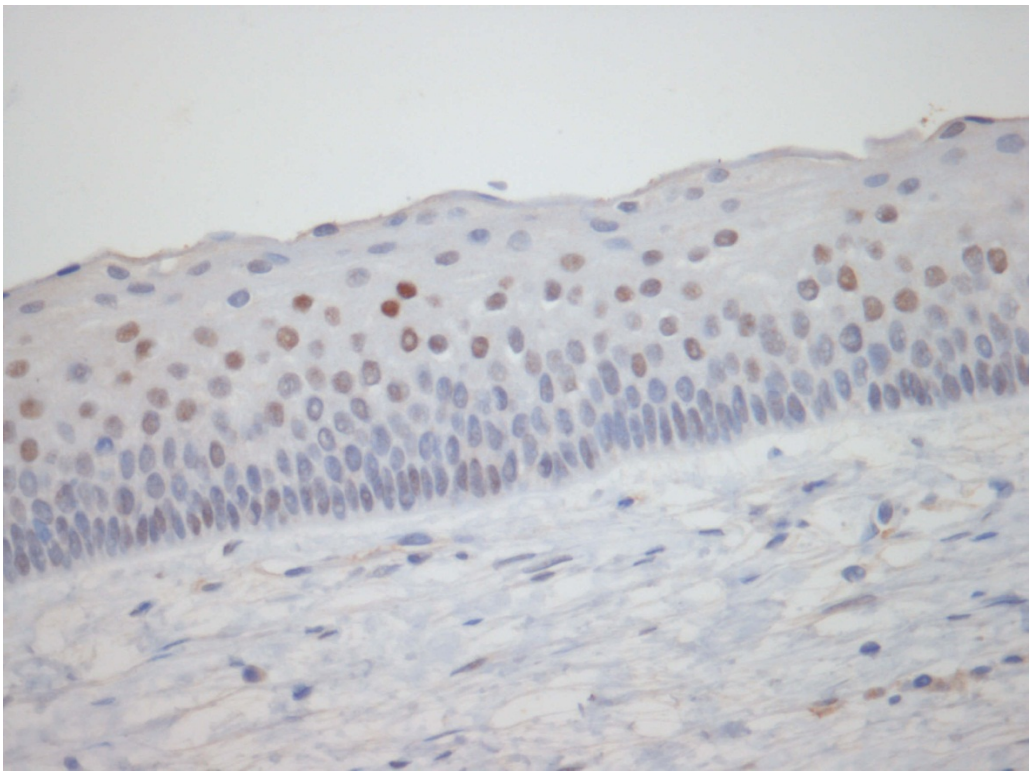


Figure 4. Photomicrograph of odontogenic keratocyst showing nuclear BCL-6 expression localised predominantly in the suprabasal cell layer of the cyst lining (BCL-6; 400X)

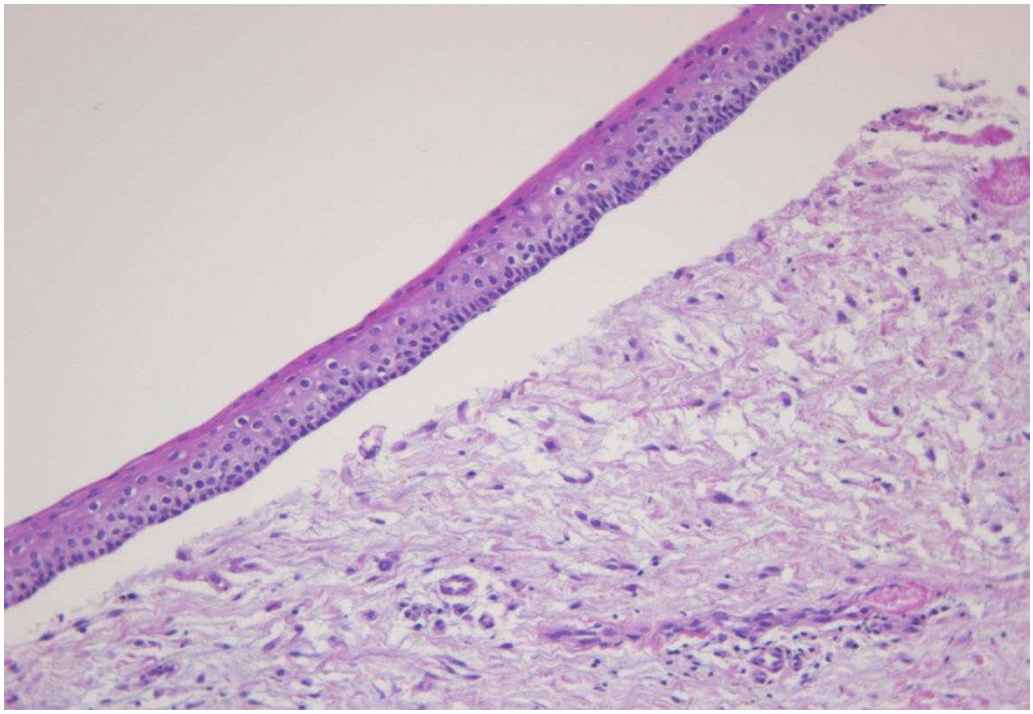


Figure 5. Photomicrograph of odontogenic keratocyst. Note separation of the epithelial lining from the underlying connective tissue cyst wall (H&E; 200X)

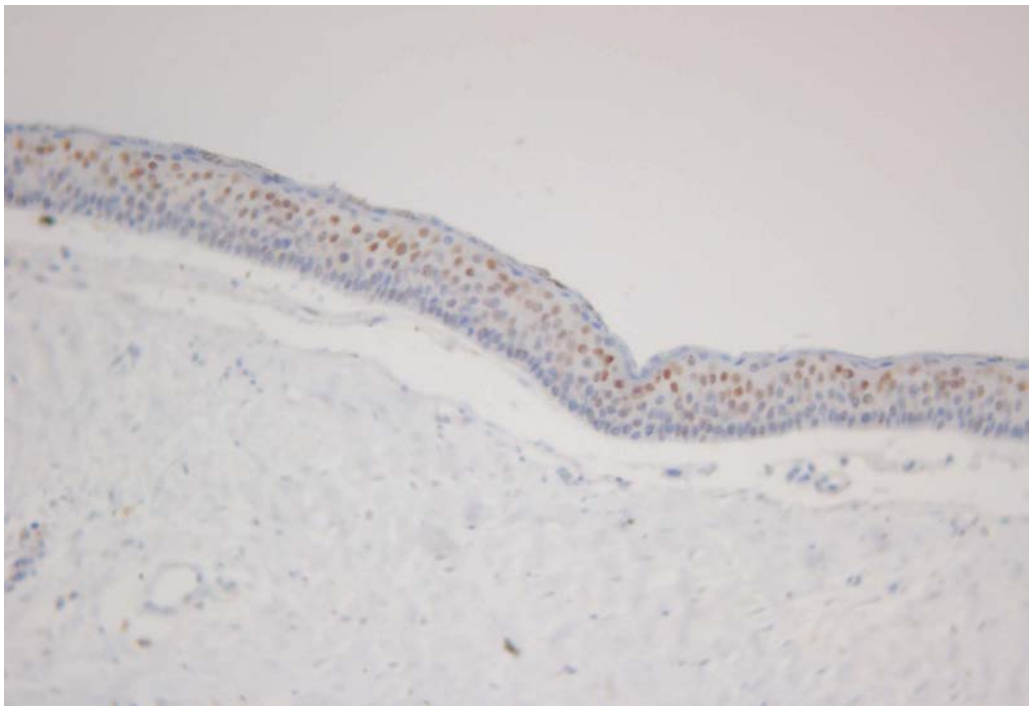


Figure 6. Photomicrograph of odontogenic keratocyst showing intense BCL-6 staining of the suprabasal cells, while the basal cells and cells at the luminal surface show comparatively less BCL-6 expression (BCL-6; 200X)

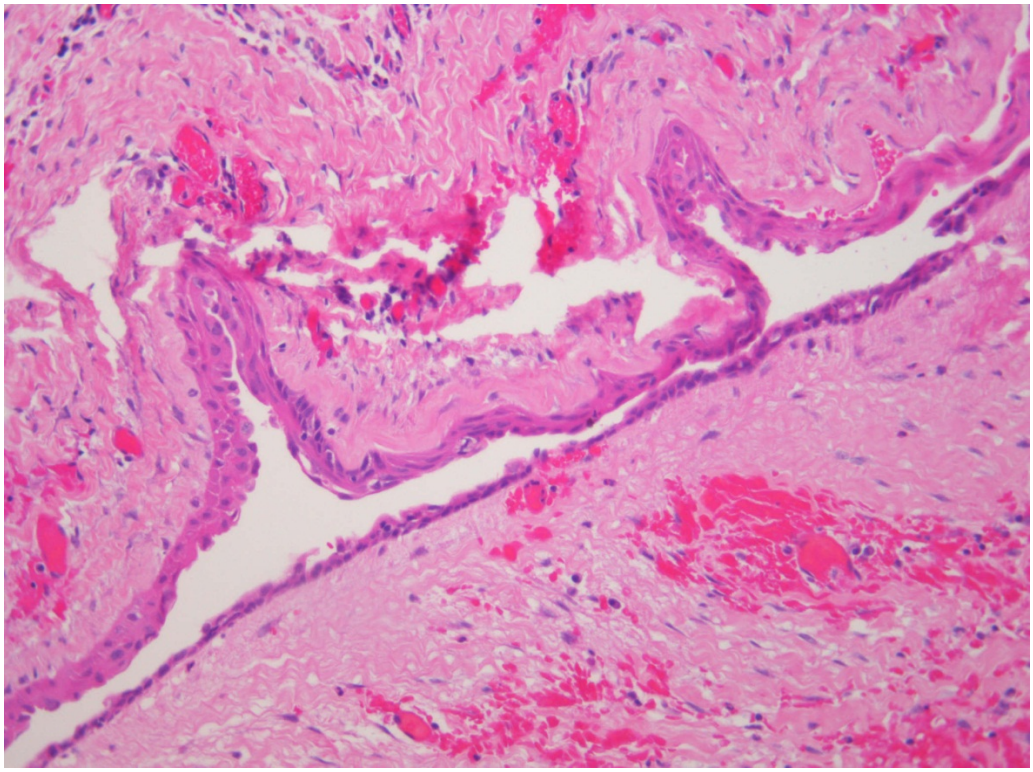


Figure 7. Photomicrograph of dentigerous cyst with non-keratinised cyst lining that resembles the reduced enamel epithelium (H&E; 400X)

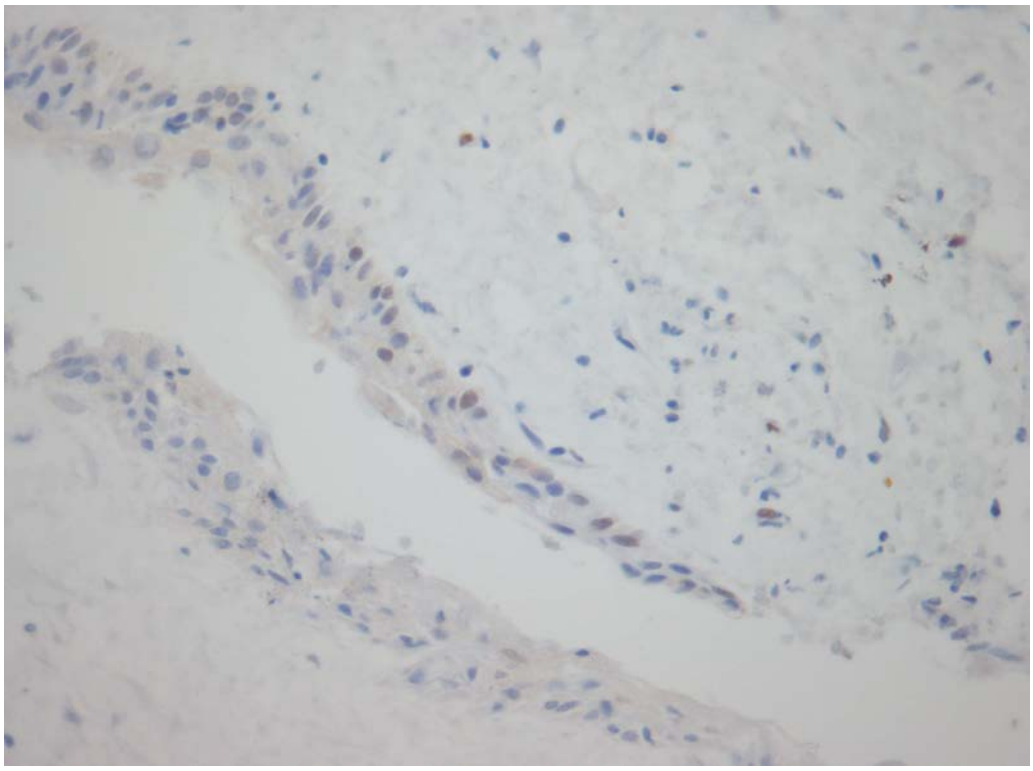


Figure 8. Photomicrograph of dentigerous cyst showing only scattered cells in the cyst lining showing faint BCL-6 expression (BCL-6; 400X)

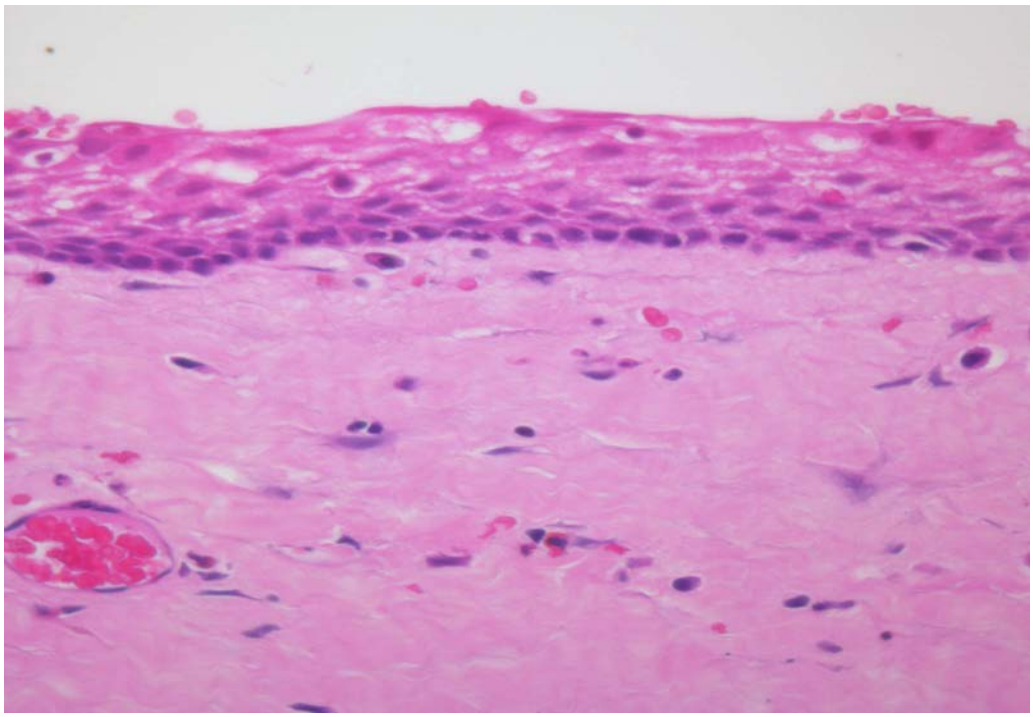


Figure 9. Photomicrograph of dentigerous cyst showing squamous metaplasia of the cyst lining (H&E; 400X)

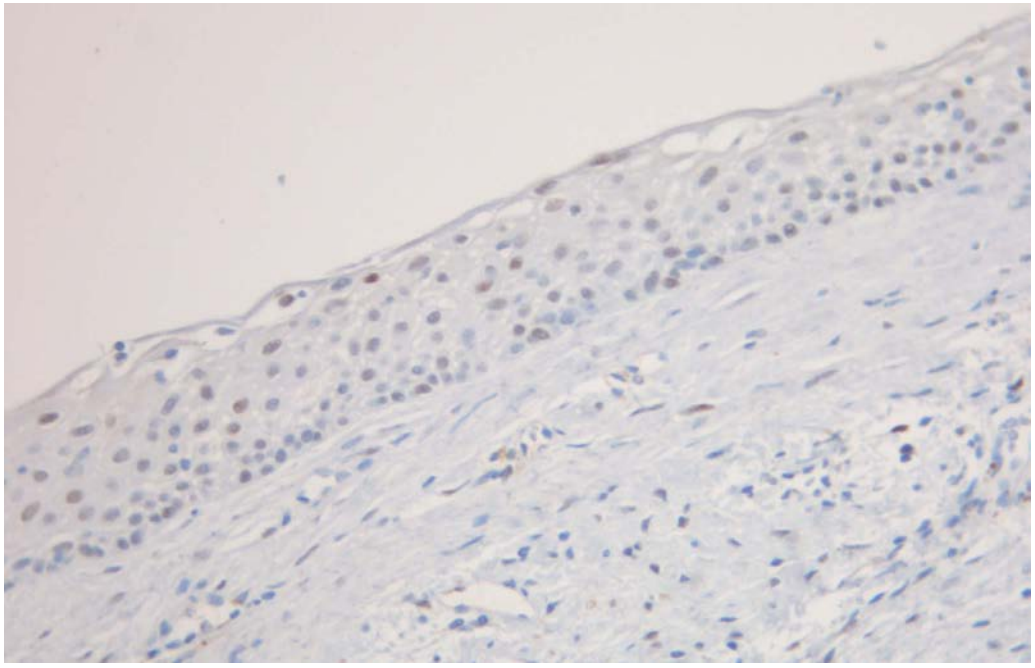


Figure 10. Photomicrograph of dentigerous cyst showing BCL-6 staining in occasional cells in the basal, suprabasal and surface epithelial cells (BCL-6; 200X)

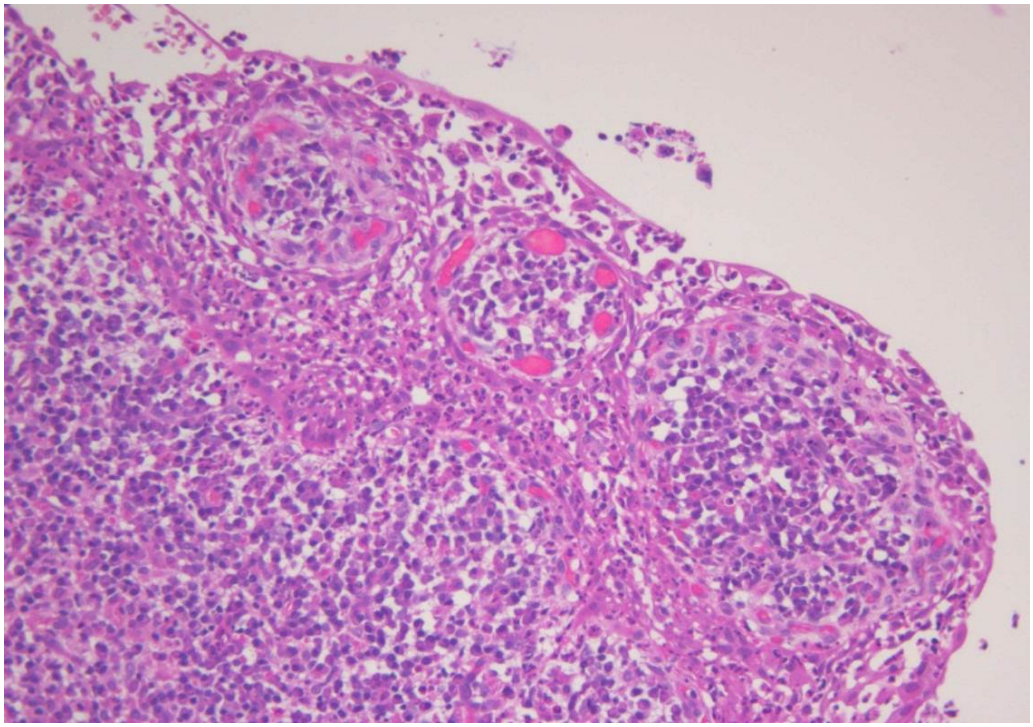


Figure 11. Photomicrograph of radicular cyst showing arcading of the cyst lining surrounding cores of inflamed granulation tissues in the cyst wall (H&E; 200X)

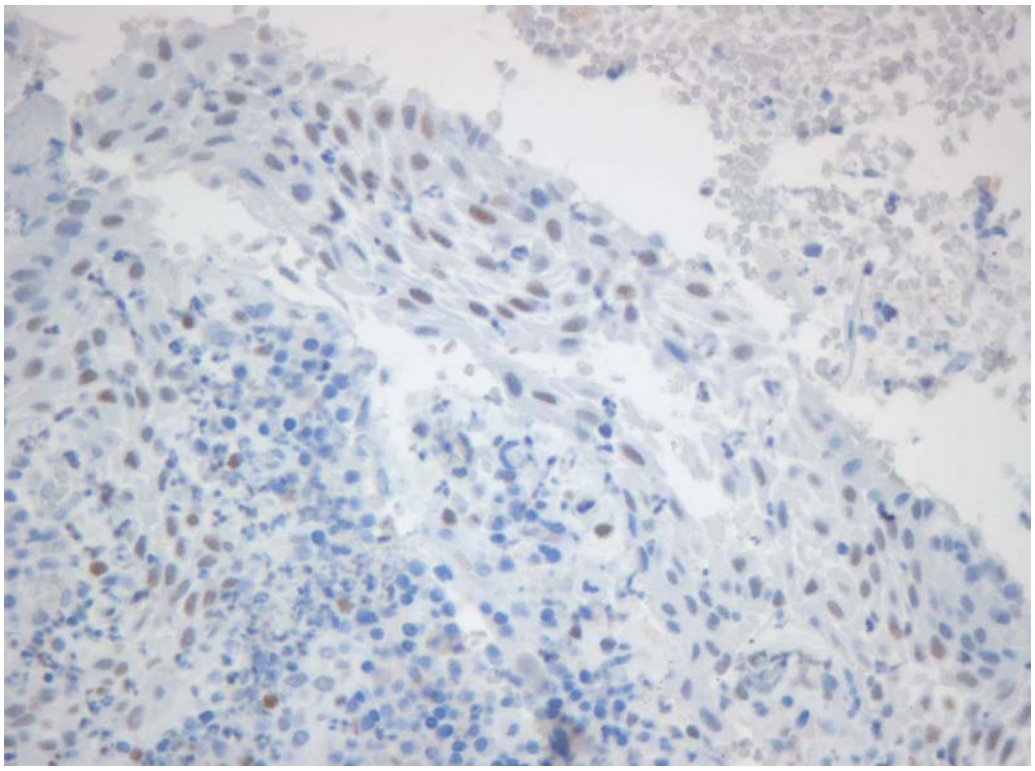


Figure 12. Photomicrograph of radicular cyst showing BCL-6 expression in the cyst lining epithelium (BCL-6; 400X)

CHAPTER 6

6.0 DISCUSSION

The first two classifications of odontogenic lesions of the World Health Organisation in 1971 and 1992 put the OKC into the category of developmental odontogenic cysts.[1] According to the 2005 World Health Organisation classification, the OKC is now categorised as a benign cystic neoplasm – also referred to as Keratocystic Odontogenic Tumour (KCOT).[2,6,9,10] This contention is based on research that has been conducted on the molecular profile of the OKC, indicating that the proliferative potential and genetic alterations seen in the OKC are more akin to neoplastic lesions than developmental odontogenic cysts.

After reviewing the English literature, this appears to be the first study of BCL-6 expression in odontogenic cysts. This study identifies the presence of BCL-6 protein in odontogenic epithelium with significant differences found between the OKC and other clinically indolent odontogenic cysts. The BCL-6 protein is a zinc finger-type transcription factor.[32] It is prominently expressed in B-cells within the germinal centre and is required for the formation of germinal centres in lymphoid tissue.[41] BCL-6 expression is low in immature B-cells, it increases as these cells differentiate through the germinal centre and is turned off when B-cells terminally differentiate into plasma cells.[35] Centroblasts in the germinal centre have the unique ability, among normal non-neoplastic cells, to survive the genomic instability that occurs when the B-cell gene is rearranged as B-cells mature in the germinal centre.[35] This is due in part to BCL-6

mediated repression of the ATR and p53 transcription that otherwise would block B-cell proliferation and induce apoptosis of the cell.[41] BCL-6 can support neoplastic transformation by maintaining the proliferative potential of cells with DNA damage. In fact the BCL-6 gene was first identified from chromosomal breakpoints involving 3q27 (the location of the BCL-6 gene in the human genome) in some non-Hodgkin lymphomas, particularly diffuse large B-cell non-Hodgkin lymphoma of germinal centre phenotype.[32,33]

Although BCL-6 is primarily associated with normal and neoplastic lymphoid development, its presence outside the lymphoid system in various epithelial tissues has been described. Yoshida *et al*.[43] reported that BCL-6 protein is expressed in normal squamous epithelium of the skin and in its neoplastic counterparts, while Lin *et al*.[45] and Huang *et al*.[47] studied BCL-6 expression in the normal transitional epithelium of the bladder and in urothelial carcinoma. BCL-6 protein was expressed intensely in the nuclei of a large proportion of prickle cells in the stratum spinosum while nuclei in the stratum corneum and the basal cells were usually not stained.[43] In the normal transitional epithelium, BCL-6 was also expressed intensely in the nuclei of the suprabasal cell layers, but not in the basal cell layer.[45,47] Mirroring to these findings, all 3 odontogenic cysts examined in the present study showed negative or weak BCL-6 staining of the basal cells. It has been suggested that this distribution pattern of BCL-6 staining in epithelia may be indicative that the BCL-6 protein may play a role in the differentiation of these epithelial tissues.[43] Of note, the OKC lining undergoes a characteristic pattern of keratinisation as opposed to the other 2 cysts, which are lined by a non-keratinised epithelium that does not undergo any form of distinctive

differentiation. Further molecular studies are needed to elucidate which gene/s in the suprabasal cells of the OKC could be the target of BCL-6 mediated transcription.

The preferential expression of BCL-6 in the suprabasal compartment of the OKC is an intriguing finding and may suggest a role for BCL-6 in the pathogenesis and behaviour of the OKC. The OKC develops from remnants of the dental lamina in the jaw and differs behaviourally from the dentigerous and radicular cyst in its tendency to recur if inadequately excised.[2,6,9,10] An important reason for this difference in behaviour is the intrinsic growth potential of the OKC epithelium, which has not been demonstrated in other jaw cysts.[2,6] The high proliferative activity of the OKC is regarded as its main mechanism of growth and molecular markers of cell proliferation such as Ki-67 and cyclin D1 have been studied in the OKC.[5,11,12] These studies have shown that Ki-67 and cyclin D1 expression is significantly higher in the OKC compared to dentigerous and radicular cysts.[5,11,12] Previous studies have demonstrated that the proliferative centre of the OKC lining epithelium lies in the suprabasal layer thereby suggesting that this epithelial lining represents a unique pattern of cellular proliferation and differentiation not shared by any other odontogenic cyst.[5,12] Kimi *et al.*[12] showed that cyclin D1 expression is most frequently located in the suprabasal cell layers of the OKC lining. The molecular mechanism of cyclin D1 upregulation in OKC is not yet explained. From the findings in this study it is tempting to speculate that BCL-6 may upregulate/ mediate transcription of cyclin D1 in a similar fashion as BCL-6 has been shown to activate cyclin D1 expression in other cell types. The function of BCL-6 in mammary epithelium was previously addressed by Logarajah *et al.*[46] and Bos *et al.*[48] These

studies showed that BCL-6 and cyclin D1 overexpression are associated in breast cancer and that this may be the result of BCL-6 mediated transcription of cyclin D1.[46,48]

The third observation that was made in this study was the significantly reduced BCL-6 expression in most of the terminally differentiated parakeratinocytes of the OKC. Many studies have shown that the OKC has the molecular characteristics of a neoplasm but maintains a cystic configuration and is therefore categorised as a cystic neoplasm.[31] A higher rate of apoptosis in the surface layer of the OKC is logically expected as this would allow it to remain cystic instead of becoming a solid tumour. Previous studies confirming apoptotic reactions by TUNEL assay suggest that the OKC epithelium is characterised by an increase in both cell proliferation and apoptosis in comparison with the dentigerous cyst.[31] The luminal cells of the OKC would appear to require down-regulation of the BCL-6 protein for apoptosis to occur, with the OKC further appearing to show greater dependency on BCL-6 mediated apoptosis than dentigerous and radicular cysts.

Published work on the role of BCL-6 in proliferation and apoptosis is very controversial. In *in vitro,* studies overexpression of BCL-6 was found to cause cell cycle block and apotosis,[39] while an anti-apoptotic role was described for BCL-6 in differentiating mouse myocytes,[40] and germinal centre B-cells.[35] From the distinctive pattern of BCL-6 expression demonstrated in the OKC in this study, BCL-6 would appear to yield opposing effects in the different cellular compartments of the OKC where it may play a role in the maintenance of the suprabasal compartment of the OKC while the abrupt loss of BCL-6 staining noted in the surface parakeratinised cells, which

is similar to that observed in the stratum corneum of skin, may suggest that its loss of expression in surface keratinised cells mediates apoptosis with consequent sloughing off of these cells at the surface of the epithelium. Further research is necessary to investigate the hypothesised role of bcl-6 in the kinetics of the OKC lining epithelium and the potential therapeutic implications of these new findings.

CHAPTER 7

7.0 CONCLUSIONS

7.1 Odontogenic keratocysts show positive expression for BCL-6. BCL-6 is mainly distributed in the suprabasal layer of the OKC.

7.2 There are significant differences in the staining characteristics of the epithelial lining of the OKC when compared to the epithelial linings of dentigerous and radicular cysts. The suprabasal layer of the OKC has statistically significantly higher values of BCL-6 staining, as compared to the dentigerous and radicular cysts.

7.3 BCL-6 may effect differentiation of the epithelial lining of OKC particularly in the suprabasal layer and may influence cellular proliferative activity. BCL-6 may therefore be a factor in the aggressive nature and unique biological behaviour of the odontogenic keratocyst.

CHAPTER 8

8.0 APPENDICES

8.1 Appendix A: Ethics certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Altini

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080850**
PROJECT	Approval for Research Conducted on Archived Paraffin Wax Embedded Tissue Blocks (Original Class Approval M00/08/29)
INVESTIGATORS	Prof M Altini
DEPARTMENT	Dept of Oral Pathology
DATE CONSIDERED	08.09.10
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 23.09.08 **CHAIRPERSON** .....
pp (Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Altini

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

8.2 Appendix B: Raw data - BCL-6 expression in OKC

Cyst No	Basal Q	Suprabasal Q	Surface Q	Basal I	Suprabasal I	Surface I
11017/10	1	3	1	3X1+1X2	4 X 3	2X1+2X2
33704/10	1	3	0	4X1	2X3+1X2+1X1	0
6575/11	1	3	0	4X1	1X3+3X2	0
38123/11	0	3	1	0	2X3+2X2	4X1
LP4235/12	0	2	0	0	2X3+2X2	0
LP84075/11	1	3	1	4X1	2X3+1X2+1X1	2X3+2X2
LP18732/12	1	4	0	4X1	3X3+1X2	0
LP12923/12	1	3	1	3X1+1X2	2X2+1X1+1X3	4X3
LP19760/12	1	3	1	4X1	2X2+1X1+1X3	4X2
LP33155/12	2	4	1	3X1+1X2	2X3+1X2+1X1	3X1+1X2
LP39093/12	1	3	1	4X1	3X3+1X2	4X1
LP39426/12	2	3	1	3X2+1X1	2X3+2X2	4X1
LP42765/12	2	3	0	2X2+2X3	3X3+1X2	0
LP41390/12	2	3	1	2X2+1X1+1X3	2X3+2X2	4X3
LP44283/12	3	4	1	3X2+1X3	3X2+1X2	4X3
LP44579/12	3	3	1	2X2+2X3	2X3+1X2+1X1	4X2
44390/12	1	3	0	2X2+1X3+1X1	2X3+1X2+1X1	0
46108/12	2	4	1	2X2+2X1	3X3+1X2	4X2
48226/12	3	3	1	3X2+1X1	2X3+2X2	4x3
49482/12	2	4	1	4X1	2X3+1X2+1X1	4X2

8.3 Appendix C: Raw data - BCL-6 expression in DC

Cyst No	BasalQ	SuprabasalQ	SurfaceQ	Basal Int	Suprabasal Int	Surface Int
LP27193/12	2	1	0	3X3+1X2	1X3+1X2+2X1	0
LP25934/12	1	2	0	4X1	2X2+2X1	0
LP25918/12	1	1	0	4X1	1X2+3X1	0
LP27026/12	1	1	0	4X1	4X1	0
LP29132/12	2	3	3	3X1+1X2	2X2+2X1	3X3+1X2
LP29673/12	1	1	0	4X1	4X1	0
LP31692/12	1	1	0	3X1+1X2	3X1+1X2	0
LP27742/12	1	1	0	4X1	4X1	0
LP31685/12	1	1	0	3X1+1X2	4X1	0
LP31013/12	1	1	0	4X1	4X1	0

8.4 Appendix D: Raw data - BCL-6 expression in RC

Cyst No	Basal Q	Suprabasal Q	Surface Q	Basal Int	Suprabasal Int	Surface Int
LP36241/12	2	2	2	3X2+1X1	3X2+1X1	3X2+1X1
LP39476/12	3	3	3	3X3+1X3	3X3+1X3	3X3+1X3
LP34817/12	2	2	2	4X1	4X1	4X1
LP33424/12	1	1	1	4X1	4X1	4X1
LP42675/12	2	2	2	1X3+3X2	1X3+3X2	1X3+3X2
46653A/12	2	3	3	2X2+2X1	2X3+2X2	2X2+2X1
46653B/12	3	3	3	2X2+2X1	2X2+2X1	2X2+2X1
47411/12	2	2	2	3X1+1X2	3X1+1X2	3X1+1X2
48392/12	2	2	2	3X1+1X3	3X1+1X3	3X1+1X3
48336/12	2	2	2	3X1+1X2	3X1+1X2	3X1+1X2

8.5 Appendix E: Raw data - BCL-6 recount data

Cyst type	Basal Q	Suprabasal Q	Surface Q	Basal Int	Suprabasal Int	Surface Int
OKC						
11017/10	1	3	1	4X1	2X3+2X2	4X2
33704/10	1	2	1	4X1	2X2+1X3+1X1	2X2+2X1
6575/11	1	2	1	4X1	2X2+1X3+1X1	0
LP4235/12	1	2	1	4X1	2X3+2X2	4X2
LP84075/11	1	3	1	4X1	2X2+1X3+1X1	4X2
LP18732/12	2	4	0	4X1	3X3+1X2	0
LP12923/12	1	2	1	4X1	2X2+1X3+1X1	4X2
LP19760/12	1	2	0	4X1	3X3+1X1	0
LP39093/12	1	3	0	4X1	3X3+1X2	0
38123/11	1	3	1	4X1	2X2+1X3+1X1	4X2
DC						
LP25918/12	1	1	2	4X1	4X1	2X2+2X1
LP27193/12	3	1	0	4X2	4X1	0
LP27026/12	1	1	0	4X1	4X1	0
LP29132/12	1	2	3	4X1	2X1+1X2+1X3	3X3+1X2
LP29673/12	1	1	0	4X1	4X1	0
RC						
LP36241/12	2	2	2	2X2+2X1	2X2+2X1	2X2+2X1
LP39476/12	2	2	1	3X1+1X2	3X1+1X2	3X1+1X2
LP34817/12	1	1	1	4X1	4X1	3X1+1X2
LP33424/12	1	1	1	4X1	4X1	4X1
LP46653B/12	3	3	3	1X3+2X2+1X1	1X3+2X2+1X1	1X3+2X2+1X1

CHAPTER 9

9.0 REFERENCES

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