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Expression of proliferation markers Ki-67 and cyclin D1 in the odontogenic keratocyst and orthokeratinising jaw cyst

Johannesburg 2013

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9/17/2013

A research report submitted to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg, in partial fulfillment of the degree of Master of Science in the branch of Dentistry.

Declaration

I, Fatima Gani, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Dentistry in the University of Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination at this or any other University. The study was conducted in the Department of Oral Pathology, School of Oral Health Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg.

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F. Gani

September 2013

Dedication

To all my friends and family that supported me and spurred me on to complete this project.

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Presentations and publications arising from this study

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Abstract

Orthokeratinised jaw cyst (OJC) is an entity distinct from odontogenic keratocyst (OKC) but that has not been fully characterised at the molecular level. The aim of this study was to compare the proliferative activity between the epithelial linings of OKC and OJC by immunohistochemical staining for Ki-67 and cyclin D1. The total numbers of Ki-67 and cyclin D1 positively (+) stained cells/10 consecutive lengths of a light microscope calibration ruler were counted for each case (OKC, n=15; OJC, n=15) and statistically compared in the basal compartment, suprabasal compartment and full thickness of the cyst lining between the 2 cyst types. OJC showed significantly fewer Ki-67+ cells and cyclin D1+ cells than OKC, consistent with the clinically more indolent behaviour of the OJC. Ki-67 expression was mainly detected in the suprabasal compartment in OKC. Expression of Ki-67 was more uniform in OJC and notably without a significant predilection for the suprabasal compartment. The accumulation of Ki-67 positive cells suprabasally in OKC raises the possibility that a process of asymmetrical cell division may be operational in OKC. Expression between Ki-67 and cyclin D1 differed significantly both quantitatively and by distribution pattern in OKC and OJC, which suggests that the presence of the cyclin D1 protein may not necessarily reflect production of this molecule by cycling cells in OKC and OJC.

TABLE OF CONTENTS

Declaration ii
Dedication iii
Acknowledgements iii
Presentations and publications arising from this study v
ABSTRACT vi
TABLE OF CONTENTS vii
List of Figures ix
List of Tables x
1.0. Introduction 1
2.0. Literature review 3
2.1. Background information on the OKC and OJC 3
2.1.1. Frequency 3
2.1.2. Clinico-pathological features 3
2. 2. Review of molecular studies on the OKC and OJC with emphasis on Ki-67 and cyclin D1 6
2.2.1. Ki-67 6
2.2.1.1. Structure and function 6
2.2.1.2. Ki-67 immunohistochemistry 8
2.2.1.3. Ki-67 expression in the OKC 9
2.2.1.4. Ki-67 expression in OJC 15
2.3. Cyclin D1 in the normal cell cycle and neoplasia 19
2.3.1. Cyclin D1 expression in odontogenic cysts and tumours 23
2.4. Summary of literature review 26
3.0. AIM AND OBJECTIVES 28
4.0. MATERIALS AND METHODS 30
4.1. Tissue samples 30

4.2. Inclusion criteria 30
4.3. Exclusion criteria 32
4.4. Immunohistochemistry 33
4.5. Evaluation of staining 34
4.6. Statistical analysis 35
5.0. RESULTS 36
6.0. DISCUSSION 43
7.0. CONCLUSIONS 50
APPENDIX A: Ethics certificate 51
APPENDIX B 52
APPENDIX B1: Raw data on Ki-67 expression in OKC lining epithelium 52
APPENDIX B2: Raw data on Ki-67 expression in OJC lining epithelium 55
APPENDIX B3: Raw data on cyclin D1 expression in OKC lining epithelium 60
APPENDIX B4: Raw data on cyclin D1 expression in OJC lining epithelium 64
8.0 REFERENCES 67

List of Figures

Figure 1. Role of cyclins and cyclin dependent kinases (CDKs) in the regulation of the cell cycle…….....21

Figure 2. Histomorphological appearance of the OKC…….**.....**31

Figure 3. Histomorphological appearance of the OJC……32

Figure 4. Gaussian distribution of data for Ki-67+ cell count in the OKC and OJC …….37

Figure 5. Immunohistochemical expression of Ki-67 in the OKC **...**…….**....**40

Figure 6. Immunohistochemical expression of Ki-67 in the OJC……..40

Figure 7. Immunohistochemical expression of cyclin D1 in the OKC ……..41

Figure 8. Immunohistochemical expression of cyclin D1 in the OJC……42

List of Tables

Table 1. Ki-67 expression in odontogenic keratocysts……...10

Table 2. Molecular marker expression in orthokeratinised jaw cysts and other odontogenic lesions…….....16

Table 3. Cyclin D1 expression in odontogenic cysts and tumours……23

Table 4. Ki-67 and cyclin D1 expression in OKC and OJC lining epithelium.....38

CHAPTER 1

1.0. Introduction

Traditionally all developmental jaw cysts which showed either parakeratin, orthokeratin or mixed patterns of keratinisation were classified together as odontogenic keratocysts (OKC). Mounting evidence has shown distinct differences in biological behaviour between those cysts lined by parakeratin and those lined by orthokeratin. The former are referred to as OKC while the latter are called orthokeratinising odontogenic cysts or orthokeratinised jaw cysts (OJC).[1] The biological behaviour of cysts lined by both ortho- and parakeratinised epithelium is as yet not fully known.[1] It has been suggested that if part of the lining is parakeratinised epithelium the cyst should be regarded as an OKC, however, other authors have placed emphasis on the basal cell features rather than the pattern of keratinisation in the OKC.[2-5]

OKC is a distinctive odontogenic cyst that exhibits specific histopathological features and aggressive clinical behaviour. It is thought to arise from cell rests of the dental lamina.[1] Several studies suggest that it should be regarded as a benign cystic neoplasm rather than a developmental cyst.[2,5,6] Multiple OKCs may be associated with nevoid basal cell carcinoma syndrome (NBCCS), also known as Gorlin syndrome.[6]

OJC is regarded as a separate clinical entity, with a less aggressive behaviour and representing 5,2-16,8% of all keratinising jaw cysts.[3] The clinical behaviour of this cyst is similar to the dentigerous cyst and radicular cyst, showing little tendency for recurrence following surgical enucleation. Aggressive features such as multiplicity, satellite cysts,

proliferating epithelial islands and association with the NBCCS have not been reported in OJC.[7]

The OKC has been extensively analysed using immunohistochemistry. These studies have been reviewed by Shear[8,9] and Kolar *et al*.[10] Among the antigenic markers, cytokeratins, apoptosis-related proteins and connective tissue components have been analysed.[10] Of interest in these studies are the investigations dealing with expression of cell proliferation markers and cell cycle-related proteins such as Ki-67,[5,10-25] proliferating cell nuclear antigen (PCNA),[8, 9, 26] IPO-38,[27] p53[21] and cyclin D1.[22,28,29]

Unlike OKC, OJC has been less rigorously analysed. Differences between the OKC and OJC in expression of PCNA and of Ki-67 have nevertheless been reported, with both PCNA and Ki-67 positive cells being found more frequently in OKC.[30] Cyclin D1 has not been fully researched in this group of lesions. Only 3 studies have evaluated cyclin D1 expression in the OKC,[22,28,29] while no study has thus far evaluated its expression in the OJC. This study therefore aimed to compare the epithelial linings of the OKC and OJC with respect to Ki-67 and cyclin D1 expression. It was hypothesised that the biologically more aggressive group, that is the OKCs, would show a greater expression of both these proliferation markers.

CHAPTER 2

2.0. Literature review

2.1. Background information on the OKC and OJC

2.1.1. Frequency

A retrospective analysis of the frequency of OKCs registered in the archives of the Department of Oral Pathology at the University of the Witwatersrand revealed a 10,2% prevalence among a total of 3498 cysts of the jaws.[1] This rates the OKC as the third most common cyst of the jaw at the aforementioned institution. By contrast, the OJC is very rare lesion accounting for <1% of all jaw cysts.[31,32]

2.1.2. Clinico-pathological features

Clinico-pathological studies have found no significant differences between the OKC and OJC when age, race, sex, presenting symptoms and the clinical appearances are considered.[2] Radiologically, the OJC has been described as a solitary unilocular lesion that is usually non-expanding and often occurs in the posterior mandible.[3,33] Several authors have commented on the frequent association of OJC with the crowns of unerupted teeth and consequently suggested that some OJCs may represent dentigerous cysts with orthokeratosis.[3,34 35] The favoured location of the OKC is the angle of the mandible, ascending posteriorly into the ramus and anteriorly into the posterior body. The OKC frequently shows scalloped outlines, it seldom causes cortical expansion and approximately 40% present radiographically as radiolucent lesions around the crowns of impacted lower third molars.[1,7]

- **Histology**

The histopathological features of OKCs are characterised by a uniform thickness of stratified squamous epithelium, usually ranging from 6-8 cells thick with a prominent polarised basal layer of cells often described as having a "picket fence" appearance. The epithelium and connective tissue interface is usually flat, and rete ridge formation is inconspicuous. The luminal surface shows flattened parakeratotic epithelial cells which exhibits a corrugated appearance. Satellite cysts or odontogenic epithelial islands may be found within the cyst fibrous wall, however, when there is inflammation present within the wall the cystic lining changes from the classical pattern described above to a non-keratinised stratified squamous lining characteristic of other inflammatory jaw cysts.[13]

The OJC does not demonstrate the same microscopic features as the OKC. The OJC has a uniform epithelial lining with an average thickness of 4-8 cells and a well-developed granular layer. The spinous layer is made up of polyhedral to flattened cells with eosinophilic cytoplasm and prominent intercellular bridges. The luminal surface lacks a corrugated appearance and demonstrates thick onion skin-like orthokeratin. Moreover, unlike the OKC, the OJC lacks accentuated basal cells, which in the OJC show little tendency to palisade or polarise.[3,34]

- **Biological behaviour**

Recurrence rates of the OKC vary between 25,0%- 62,5%.[37,38] Time ranges for recurrences may vary from 1 to 23 years but most reports found recurrences within 5 to 7 years after enucleation.[37,38] The recurrence rate for OJC is in the order of 2,0%.[3] Several factors have been cited as possible reasons for the relatively higher rate of local recurrence for the OKC as opposed to most other odontogenic cysts. These include the morphological configuration of the OKC which hinders complete removal following simple enucleation, the OKCs tendency for multiplicity and association with the NBCCS. Recurrence rates may also be dependent on the type of surgical therapy employed and it was found that the combination of surgical enucleation and cryotherapy provided improved outcomes for patients.[20] Furthermore, it has been suggested that the epithelial lining of the OKC demonstrates molecular characteristics that provide some basis for regarding the OKC as a benign cystic neoplasm.[8,9] The term keratocystic odontogenic tumour instead of OKC has been suggested.[38] There is, however, thus far no international consensus on renaming this lesion. Further as the term OKC is so widely used by clinicians and pathologists, it is felt that a good case can be made for retaining this term even if it is agreed that the cyst is indeed neoplastic.[7,38]

The histomorphological features of the lining epithelia of the OKC and OJC suggest that characteristics of the cyst linings probably play a dominant role in determining their biological behaviour. Consequently, studies examining the proliferative capacity of these lesions are aimed at providing a molecular scientific basis for the difference in clinical behaviour between these two entities.

2. 2. Review of molecular studies on the OKC and OJC with emphasis on Ki-67 and cyclin D1

2.2.1. Ki-67

2.2.1.1. Structure and function

Ki-67 was originally described by Gerdes *et al*.[39] in 1983 in the city of Kiel in Germany. It was identified in the 96th well of the original culture plate.[39] Initially Ki-67 was not identified as a protein, due to the absence of obvious homology with other proteins and was referred to as Ki-67 antigen.[40] It was first identified as a non-histone protein by the screening of a c-DNA expression library in 1991.[41] The Ki-67 protein is encoded in humans by the MK167 gene.[42] MIB1 is the commonly used monoclonal antibody that detects the Ki-67 antigen.[41]

The Ki-67 protein is a marker of cell proliferation.[40] Ki-67 is expressed in all proliferating cells at G1, S, G2 and M stages (active stages) of the cell cycle, and is not detected in resting cells in the G0 stage.[43] The expression of Ki-67 increases with cell cycle progression, rising during the latter half of S phase and reaching its peak in the G2 and M phase.[43] It is reportedly an excellent operational marker to determine the growth fraction (the number of cells in the cell cycle) of a given cell population.[44] The results of expression studies of Ki-67 in the G1 phase are contradictory. Lopez *et al.*[45] found that cells in the initial G1 phase were negative for Ki-67, whereas Gerdes *et al*.[40] reported that Ki-67 was detectable in the late G_1 phase. Some authors similarly report an increase of Ki-67 staining starting in the late G1 phase,[46,47] and yet others found a decrease until the onset

of DNA synthesis.[45,48] Van Dierendonck *et al.*[49] found that Ki-67 expression was undetectable in cells entering S phase, which has been disputed by many authors and is not generally accepted. [50,51] Du Manoir *et al.*[50] attempted to explain these inconsistencies in 1991 describing three different pathways during G1 phase. The Ki-67 decrease pathway is characterised by a decline in Ki-67 staining thereby leading to the exit of cells from the active cell cycle and entry into G0.[50] If these cells on this pathway are then stimulated by growth factors, they can then enter the Ki-67 increase pathway that pushes the cells back into G1 phase. The Ki-67 stable pathway, occurs in optimal local growth conditions and shows a constant intensity of Ki-67 staining during G1.[44]

Ki-67 protein has a unique structure dissimilar to other proteins.[52] It is a nuclear non-histone protein with two differentially spliced mRNA isoforms that were initially described by Schluter *et al*.[53] with sizes of 8688 and 9768 base pairs (based on the presence or absence of the region encoded by exon 7) and molecular weights of 320 kD and 359 kD respectively. Each contains a large central region with 16 concatenated direct repetitive elements (Ki-67 repeats) encoded by a single exon 13 of 6845 base pairs.[41,44,53] The two cDNA isoforms represent different mRNAs formed from the same gene by alternative mRNA splicing.[53] The entire gene locus of the Ki-67 protein has been sequenced and comprises of almost 30,000 base pairs in the human genome.[54] This protein undergoes phosphorylation and dephosphorylation during mitosis and is susceptible to proteolytic pathways, so that the amount of Ki-67 protein that is present at any time during the cell cycle is highly regulated with an estimated half-life of 60 to 90 minutes.[55] Although its exact function has not yet been fully identified, it is strictly associated with cell

proliferation.[52] Hofmann and Bucher[56] in 1995 located a forkhead-associated domain in the terminal amino region of the Ki-67 protein; which is a common feature for a number of proteins known to be involved in the regulation of the cell cycle.[44,52,56] Recently researchers have speculated that it is associated with ribosomal RNA transcription and is an essential factor in the synthesis of ribosomes within the nucleolus during cell division. This premise is supported by the observation that Ki-67 protein especially correlates with the rate of protein synthesis which is a function of ribosomes.[57]

2.2.1.2. Ki-67 immunohistochemistry

One of the major disadvantages of the original Ki-67 antibody was that it could be used only on fresh or frozen tissue.[44] This was overcome by the use of a Ki-67 equivalent monoclonal antibody MIB-1 (Molecular Immunology Borstel), which can be used on paraffin sections after antigen retrieval by microwave-processing. This therefore also allows for retrospective studies to be undertaken using archived histology material.[58]

The distribution of Ki-67 in normal tissues reflects their known cell kinetics. For example, it stains the germinal centre cells in tonsils and the basal cells of epithelium.[58] Furthermore, unlike utilising a count of mitotic figures to determine the proliferation index of a particular tissue, the Ki-67 labelling index identifies cells in all active phases of the cell cycle while mitotic counts only reflect one part (the mitotic or M phase) of the cell cycle.[44] It is nevertheless important to realise that proliferation rate comprises, the growth

fraction (assessable by Ki-67) and the time taken for the cell to complete the cell cycle (not assessable by Ki-67).[43]

2.2.1.3. Ki-67 expression in the OKC

The findings of previous studies on Ki-67 expression in the OKC are summarised in Table 1.

Table 1. Ki-67 expression in odontogenic keratocysts

Author	Study findings
Mateus *et al.*[5]	• The proliferative and apoptotic indices: OKC > dentigerous cysts ($p<0.05$). • No differences in cell turnover between sporadic and NBCCS OKCs. • Proliferative activity is > in the basal layer of DC ($p<0.05$) and in the suprabasal layer of OKC ($p<0.05$).
Kolar *et al.*[10]	• Sporadic OKC demonstrated significantly > Ki-67+ basal cells than NBCCS OKC ($p = 0.011$). • OKC has a ↑ proliferative potential than DC and ↑ apoptotic rate.
Slootweg[11]	• ↓ Ki-67+ cells in the DC (dentigerous cyst) and RC (radicular cyst) compared to OKCs. • Ki-67+ cells found mainly suprabasally in OKC and basally in DC and RC. • Positive correlation between Ki-67 and p53 irrespective of cyst type.
Li *et al.*[12]	• In the OKC, normal oral mucosa, DC and RC >90%, 65%, 21% and 19% of the Ki-67+ cells are suprabasal respectively. • No significant difference in Ki-67+ cell count between simple and recurrent OKC. • Significantly ↑ Ki-67+ cell count in NBCCS-related OKC.
De Paula *et al.*[13]	• Total number of Ki-67+ cells ↑ in the presence of inflammation in OKC linings. • Inflamed and non-inflamed OKCs had ↑ numbers of Ki-67+ cells in the suprabasal layer. • Inflamed OKCs had ↑ numbers of basal Ki-67+ cells when compared to non-inflamed OKCs.
Kim *et al.*[14]	• Numbers of Ki-67+ cells were ↑ in OKC > DC. • Ki-67+ cells distributed suprabasally in OKC and basally in DC. • No significant difference in number of Ki-67+ cells in follicular versus extrafollicular OKC and in unilocular versus multilocular OKC.
Kaplan and Hirshberg[15]	• Localised ↑ in Ki-67 expression of OKC in areas with moderate to severe inflammation ($p = 0.036$). • Overall proliferative activity of OKC lining epithelium is unaffected by inflammation.
Saracoglu *et al.*[16]	• The mean numbers of Ki-67 positive cells in normal oral mucosa, RC and DC were similar but the expression of Ki-67 in OKCs was much higher.
Kichi *et al.*[17]	• Ki-67+ cells were highest in the intermediate layer of the OKC ($p<0.001$) compared to DC, although the basal cell layers were similar in OKC and DC.
Gonzalez-Moles *et al.*[18]	• ↑ number of Ki-67+ cells were found suprabasally compared to the basal layer ($p<0.001$). • Ki-67 expression unrelated to the type of OKC i.e. sporadic, NBCCS (nevoid basal cell carcinoma syndrome) associated or recurrent.
Gurgel *et al.*[19]	• Ki-67 expression detected in 97.3% of cases in the suprabasal layers of OKC. • No significant differences in Ki-67, p53 and p63 expression were noted between syndromic versus sporadic or primary versus recurrent lesions. • Ki-67 and p53+ cells show a similar distribution, while p63 was found throughout the cyst lining.
Kuroyanagi *et al.*[20]	• Recurrent OKC had ↑ numbers of Ki-67+ cells in the basal cell layer ($p=0.025$). • Ki-67 immunopositivity can be used as a prognostic marker if >10% labelling of basal cells in terms of recurrent potential. • Daughter cysts and epithelial budding were not associated with higher numbers of Ki-67+ cells.
Gadbail *et al.*[21]	• Growth fraction and growth rate of OKC > normal oral mucosa, RC and DC in suprabasal layers, basal layer and complete epithelium. • In DC, RC and normal oral mucosa Ki-67 labeling index was greater in basal cell than suprabasal cell layers.
De Vicente *et al.*[22]	• Mean % of Ki-67+ cells in OKC, DC, RC and AB are 40%, 17%, 15.5% and 7.8% respectively.
Ayoub *et al.*[23]	• Ki-67 expression significantly ↑ in OKC than RC. • Ki-67+ cells located in basal and suprabasal cells in OKC while staining was mainly in basal cells in RC.
Nadalin *et al.*[24]	• Suprabasal expression of Ki-67 significantly higher in OKC compared to RC and DC.
Gadbail *et al.*[25]	• Ki-67 expression mainly in the suprabasal layer of the epithelial lining of OKC.

OKC=odontogenic keratocyst; NBCCS=nevoid basal cell carcinoma syndrome; AB=ameloblastoma; DC=dentigerous cyst; RC=radicular cyst

From their study sample some authors excluded jaw cysts showing orthokeratinisation,[14,15] NBCCS associated OKCs,[13-15,22,24] recurrent OKCs [13,15] as well as OKCs enucleated following decompression treatment.[15] Other studies also excluded cases where inflammation was present in the OKC lining.[19, 24] Baghaei, *et al.*[30] and Li *et al.*[12] excluded any tissue sample that did not have a basement membrane length greater than 8mm while de Vicente *et al.*[22] stated that sufficient tissue had to be present but did not ascribe a value to it. Cases that did not have a minimum of 1000 cells were not included in the study conducted by Gurgel *et al.*[19] The exclusion criteria were not specified in other studies.[16,21,23,25] The most frequently employed Ki-67 labelling technique was to manually count a minimum of 1000 cells in 10 fields at 400X magnification using a light microscope.[14,15,22,24] Three studies made use of an eyepiece grid,[5,15,30] and in 2 other studies, a calibration micro-ruler inserted into the eyepiece of a light microscope was used to prevent overlap of counting fields.[12,13] Additionally, some studies utilised digital measures to ensure the accuracy of the counting technique such as the use of a digital camera,[19] the use of computer image analysis and histomorphometry.[10,12,13, 17,20, 23, 59] Only 4 studies used semi-quantitative analyses to collect data,[11,18,19,24] while the remaining studies utilised a quantitative method with various formulas to either calculate the proliferation index or the number of Ki-67 positive cells per unit length of basement membrane.[12-14,22,30]

Eight studies independently assessed Ki-67 expression in the basal and suprabasal layers of the epithelium of the OKC.[13,14, 18-21,24, 30] The OKC demonstrated a greater frequency of suprabasal Ki-67 positive cells.[11,12, 17-19, 21, 25] When inflammation was present in the OKC,

de Paula *et al.*[13] showed that the basal cell layer would have higher counts of Ki-67 positive cells compared to a non-inflamed OKC. Kaplan *et al*.[15] however, found that there was no overall difference in Ki-67 expression (proliferative index) between inflamed and non-inflamed OKCs.

The Ki-67 labeling index of the OKC was most commonly compared to the dentigerous cyst (DC), which is a clinically less aggressive lesion than the OKC.[5,11,12, 14, 21- 25] It has been found that the OKC has a proliferation index and apoptotic index that is greater than the dentigerous cyst.[5] The basal cell layer of dentigerous cysts, however, showed greater numbers of Ki-67 positive cells when compared to the basal cell layer of the OKC but the overall number of Ki-67 positive cells was still greater in the OKC.[5,17] By contrast the study by Gadbail *et al.*[21] suggests that the basal cell layer of OKCs had significantly higher expression of Ki-67 than dentigerous and radicular cysts.

In comparative studies between the radicular cyst and the OKC, fewer Ki-67 positive cells were found in the radicular cyst epithelium.[11,12,16,21-24] The distribution of Ki-67 positive cells was also irregular in comparison to the OKC, ranging from areas with a few positive cells and alternating with large numbers of Ki-67 positive cells.[11] A similar number of Ki-67 positive cells were seen in the epithelial linings of normal oral mucosa and the OKC.[16] However, a dramatic difference was observed in the basal cell layer of the OKC, with the normal oral epithelium surpassing the number of basal Ki-67 positive cells compared to the OKC.[16] It has been suggested that the OKC epithelium appears to undergo a maturation

process in which the suprabasal cells increase their proliferative capacity.[18,21] The increased proliferative potential in the suprabasal compartment of the epithelium may further suggest an abnormal control of the cell cycle, which in turn could play a role in the locally aggressive behaviour of the OKC.[21]

Daughter cysts and epithelial budding were found not to be associated with increased Ki-67 expression in the OKC lining.[20] On the basis of this finding it is speculated that satellite cysts in the OKC are a result of cystic breakdown of epithelial islands rather than basal budding.[12] With regard to the follicular variant of OKC, Kim *et al.*[14] described that irrespective of whether the OKC was associated with an impacted tooth or not, this did not make any difference on the level of Ki-67 expression.[14]

As far as primary versus recurrent OKCs are concerned, Gurgel *et al.*[19] and Li *et al.*[12] found no differences in Ki-67 immunostaining between primary and recurrent lesions indicating that recurrence is unlikely to be associated with a subgroup of OKC lesions showing increased proliferation. On the contrary, Kuroyanagi *et al.*[20] found higher numbers of Ki-67 positive cells in the basal cell layer of the epithelium of 4 primary OKCs which recurred. These authors suggested that if the Ki-67 expression in the basal layer of an OKC was greater than 10% then the lesion was more likely to recur.[20]

In NBCCS associated OKCs, Li *et al.*[12] found that syndromic lesions had greater numbers of Ki-67 positive cells compared to sporadic OKCs. The distribution of Ki-67 expression in syndromic OKCs was also found to be more uniformly distributed throughout all the cell layers than that of sporadic lesions,[19] which contrasts the findings of Kolar *et al.*[10] who found that the basal cell layer of syndromic lesions contained significantly fewer positive cells than sporadic lesions. This discrepancy may be related to differences in methodology. Ki-67 expression was assessed semi-quantitatively by Gurgel *et al.*[19] while Kolar *et al.*[10] determined the percentage of Ki-67 positive cells from a minimum of 1000 cells followed by validation using computer image analysis.

Several molecular markers have been examined in association with Ki-67 in the OKC. These include p53,[19,25,30] syndecan 1,[24] p63,[19] laminin-1,[23] bcl-2,[28] TdT-mediated dUTP-biotin nick end labeling (TUNEL),[5,17] and cyclin D1.[22,28,29] The p53 protein is a product of the p53 tumour suppressor gene. p53 protein functions in G1 arrest to allow the repair of DNA damage and to prevent the cell from entering the S phase of the cell cycle or alternatively to guide the damaged cells to apoptosis.[25] p53 immunostaining in OKC was found to be dense and scattered throughout the basal and suprabasal layers coinciding with the distribution of Ki-67 positive cells.[11] Slootweg *et al.*[11] suggested that the overexpression of p53 protein in OKC may not necessarily be the result of p53 gene mutation but may instead be due to stabilisation of wild-type p53 protein. The positive correlation between p53 detection by immunohistochemistry and proliferative activity in the epithelium of OKC would consequently further suggest that the negative growth

regulation of normal p53 protein is suppressed to some extent and so leading to increased proliferation.[11,21]

The apoptotic index of the OKC was evaluated using TUNEL and it was found that the apoptotic index was greatest in the superficial layers of the OKC and dentigerous cysts.[5,17] Although apoptotic cells were found in all layers of the epithelium of OKCs, this finding paired with the high proliferative index of these lesions indicates a high turnover of cells.[5] Comparatively the OKC has a greater apoptotic index when compared to the dentigerous cyst.[17] Bcl-2 is an apoptosis inhibiting protein and in OKCs its expression is particularly noticeable in the basal cell layer.[17]

Among the various treatment modalities that have been advocated for the treatment of OKCs is marsupialisation i.e. enucleation following decompression. The Ki-67 proliferation index before and after decompression of OKCs were compared by Clark *et al*.[60] These authors found no significant difference in the Ki-67 values before and after decompression thereby suggesting that the pronounced clinical shrinkage which occurs in OKC after decompression are not accompanied by changes in proliferative activity.[60]

2.2.1.4. Ki-67 expression in OJC

The OJC was first categorised in 1981 by Wright[34] as a distinct clinical entity. As it is a less aggressive lesion than the OKC it has attracted less attention. Only 3 articles deal with the OJC and the cellular proliferation protein Ki-67.[3,30,59] However, various other markers for cytokeratins, extracellular matrix proteins, genes that regulate apoptosis and tumour

suppressor genes have also been examined and compared between the OJC and OKC.[3,27,30,59,61-63] The findings of these molecular studies are summarised in Table 2.

Table 2. Molecular marker expression in orthokeratinised jaw cysts and other odontogenic lesions

Reference	Molecular marker	Study findings
Li *et al.*[3]	Ki-67	• Ki-67expression restricted to basal layer in OJC, suprabasal Ki-67 expression in OKC. • Ki-67 significantly > in OKC than OJC.
Thosaporn *et al.*[27]	IPO-38	• IPO-38 labeling index was ↑ in decreasing order in ameloblastoma >OKC>OJC>DC. • In OKC and OJC, IPO-38+ cells were located suprabasally, basally in dentigerous cyst and in peripheral columnar cells in ameloblastoma.
Baghaei *et al*. [30]	Ki-67, p53	• ↑ total number of Ki-67+ cells found in the OKC (62.3+ cells/mm basement membrane) versus OJC (29.9+ cells/mm basement membrane) ($p<0.05$). • ↑ number of the Ki-67+ cells found suprabasally than basally in OKC and OJC ($p<0.007$).
Dong *et al.*[59]	Ki-67, p63	• Ki-67 mostly confined to basal layer in OJC, ↑ suprabasally in OKC, significantly higher positive count in OKC. • p63 + in basal and part of suprabasal layer in OJC, in OKC p63+ in all cell layers except parakeratinised layer.
Lo Muzio *et al.*[61]	p63	• Basal cell layer p63+ in 28/28 OJCs and 25/25 OKCs, intermediate cell layers + in 25/25 OKCs and 16/28 OJCs, superficial layers + in 15/25 OKCs and 0/28 OJCs.
Rangiani *et al.*[62]	bcl-2, bax	• Bcl-2 + in basal cells in OKC, completely absent in OJC. • Bax showed no significant difference between OKC and OJC, bax+ in all cell layers of OKC and OJC.
Diniz *et al.*[63]	PTCH gene	• Loss of heterozygosity in Patched (PTCH) gene region found in 5/7 OKC and 4/7 OJC where 75% of OJC were of follicular type.

OKC=odontogenic keratocyst; NBCCS=nevoid basal cell carcinoma syndrome; AB=ameloblastoma; DC=dentigerous cyst; RC=radicular cyst

Overall, the study findings suggest that the OJC has a different molecular profile that is in keeping with its considerably less aggressive clinical behaviour. Of the 3 studies that examined Ki-67 expression in the OJC and OKC,[3,30,59] 2 studies found most of the Ki-67 positive cells located in the basal layer in the OJC [3,59] in contrast to the predominantly suprabasal localisation of Ki-67 positive cells in the OKC. Despite the different methodologies that were used in these 3 studies for quantification of Ki-67 in the cyst linings, all 3 studies reported a significantly greater total number of Ki-67 positive cells in the OKC.[3,30,59]

In addition to Ki-67, the cell proliferation marker IPO-38, has also been examined in a variety of odontogenic cysts and tumours that included the OKC and OJC.[27] IPO-38, otherwise known as non-lineage 116 antigen (N-L116), is an antigen whose expression is noted in the nuclei of proliferating cells.[27] Its expression is constant through most stages of the cell cycle except during mitosis where a 400-fold increase in concentration has been observed.[27] In the study by Thosaporn *et al.*[27] significant differences in the expression of IPO-38 was observed in OKC, OJC, dentigerous cyst and ameloblastoma. IPO-38 labeling index was highest in descending order in ameloblastoma, OKC, OJC and dentigerous cyst. In OKC and OJC, IPO-38 positive cells were located suprabasally, basally in DC and in the peripheral columnar cells in ameloblastoma.[27]

Besides examining markers that promote cell proliferation in the OKC and OJC, molecules that protect cells from apoptosis have also been studied in both these cysts. The bcl-2 family includes cell death suppressors and promoters, however, the bcl-2 protein acts as a

cell death suppressor (anti-apoptotic) whereas bax is pro-apoptotic.[64] The overexpression of bcl-2 inhibits apoptosis.[64] Rangiani *et al.*[62] found bax expression was similar in the 2 cysts, however, the bcl-2 expression was much higher in the OKCs. Immunopositive cells for bcl-2 were detected in the basal layer of all OKCs studied while no cases of OJC demonstrated any staining.[62] The ratio of bcl-2 to bax is the correlating factor in apoptosis and the higher ratio of bcl-2 in the basal layer of OKC would result in a higher cell survival rate and may partly explain its more aggressive behaviour and tendency for recurrence if incompletely removed.[62]

Among the tumour suppressor genes, p53 and Patched (PTCH) protein were examined in the OJC and compared to the OKC.[63] In both cysts, p53 positive cells were mainly detected in the parabasal layer and there was no significant difference in the average number of p53 positive cells between the OKC and OJC.[30] Expression of p53 protein in the OKC and OJC may, however, not indicate p53 gene mutation but may instead be due to stabilisation of wild-type p53 protein. Baghaei *et al.*[30] emphasised that correlation with the methylation status of the p53 gene is required before definitive conclusions can be made on the significance of their findings on immunohistochemistry.

Alteration in the sonic hedgehog (SHH) pathway have been described in OKC due to loss of heterozygosity (LOH) of the Patched (PTCH) gene found at chromosome 9.[65] LOH was found by Diniz *et al.*[63] in 5/7 OKCs and 4/7 OJCs. LOH for PTCH gene has also been described in dentigerous cysts, radicular cysts, ameloblastomas and calcifying epithelial odontogenic tumours; hence it is not a unique feature of the OKC.[63,65,66] It is interesting to

note that 3/4 OJCs with LOH at the PTCH gene region were associated with an impacted tooth in the study by Diniz *et al*.[63] Similar genetic alterations were reported in dentigerous cysts in 2 earlier studies.[65,66] On this premise it is speculated that OJCs associated with impacted teeth may represent dentigerous cysts with metaplastic transformation of the cyst lining to orthokeratinised epithelium.[66]

CK14 and p63, which are both markers of basal cells, have shown differential staining patterns in the OJC and OKC.[33,59,61] Both cysts showed continuous and constant expression for CK14 in the basal layer, while expression in the suprabasal epithelial layers was not uniform in intensity or distribution.[33] By contrast, p63 was mainly confined to the basal layer in OJC while in OKC all cell layers were p63 positive, except the surface parakeratinised layer.[61] p63 is a member of the p53 tumour suppressor gene family that plays an important role in the maintenance of epithelial stem cells, which in stratified squamous epithelium is represented by the basal cells. The findings of studies on p63 in odontogenic cysts may therefore indicate that similar to basal cell carcinoma of the skin the OKC epithelium appear to be composed predominantly of basal cells.[59,61]

2.3. Cyclin D1 in the normal cell cycle and neoplasia

The ultimate goal of all growth-promoting stimuli is the entry of quiescent cells (G0 cells) into the cell cycle.[64] Under physiological conditions this involves the binding of a growth factor to its specific receptor on the cell surface followed by a transient activation of the growth factor receptor which in turn activates signalling molecules in the signal transduction pathway that convey the message from the receptor to the nucleus where

transcription factors bind to DNA and activate the transcription of proteins that cause the cell to enter the cell cycle.[64] The cell cycle is divided into two main phases: interphase and mitosis. During interphase the cell replicates its chromosomes. Interphase is subdivided into three phases; namely gap phase 1 (G1), synthesis of DNA (S) and gap phase 2 (G2). Interphase is followed by nuclear division (mitosis) and cell division (cytokinesis).[67]

Following initiation, usually by a growth factor, the cell progresses through the cell cycle which is regulated by a number of proteins called cyclins, cyclin dependent kinases (CDKs) and their inhibitors known as cyclin dependent kinase inhibitors (CDKI).[68-72] Nine CDKs have thus far been identified, of which at least 5 are active during the cell cycle.[67] The CDKs are enzymes that are present in the cell at all times, however, they require a specific cyclin to bind to them before they become active. When they are activated by a cyclin, the cyclin-CDK complex phosphorylates essential proteins that are required for the cell to enter the next phase of the cell cycle. The cyclin levels usually rise and fall throughout the cell cycle. The cyclins are specific to different cell cycle checkpoints and are present at high concentrations only when the cell is ready to advance to the next phase of the cell cycle.[68,70-72] CDK activity is regulated by two families of CDK inhibitors; those that inhibit the CDKs broadly (p21, p27 and p57) and those that have selective effects on cyclin D complexes (p15, p16, p18 and p19); the so-called INK4 inhibitors.[67,72]

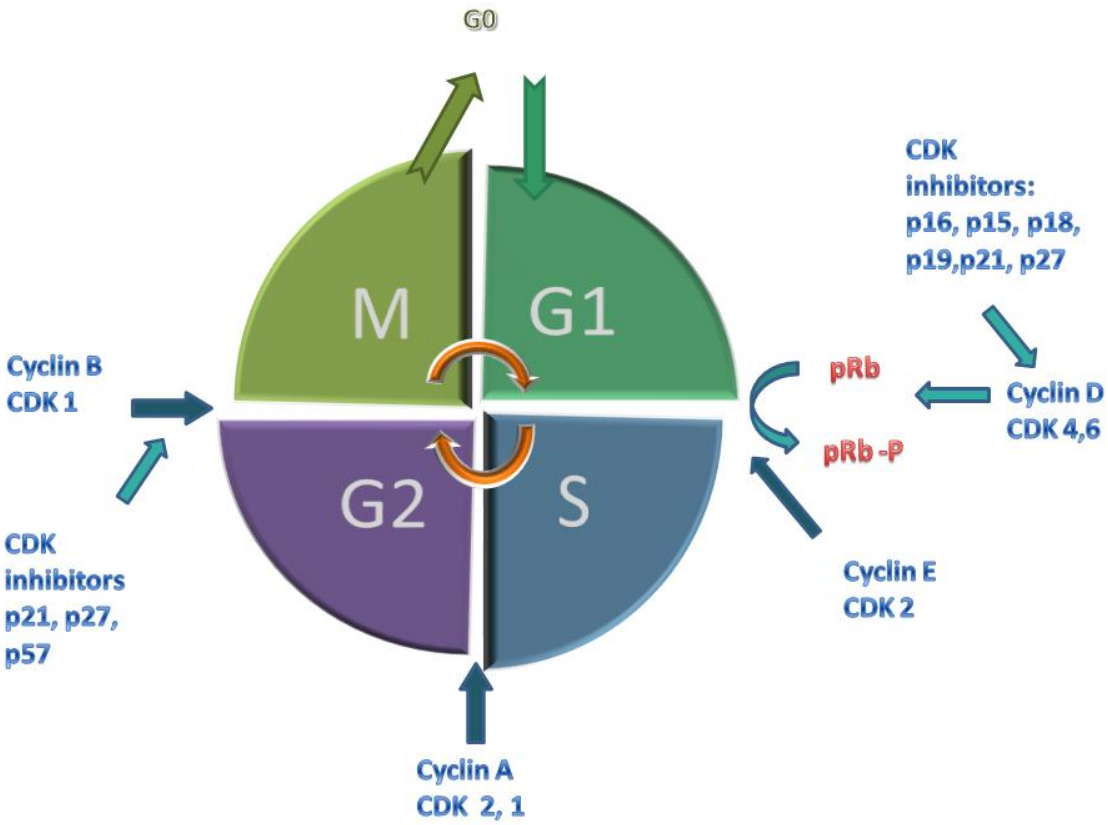


Figure 1. Illustration of the role of cyclins and cyclin dependent kinases (CDKs) in the regulation of the cell cycle (Adapted from Kumar *et al.*[64]).

Cyclins of the D-class are the first cyclins to be induced as quiescent cells in G0 receive growth-promoting signals to enter the cell cycle. Unlike the other cyclins, D-type cyclins do not oscillate during the cell cycle, but instead their levels are controlled by the presence of growth factors.[67-72] All 3 D-type cyclins (cyclin D1, D2 and D3) can activate CDK4 and CDK6, however, CDK4 and CDK6 do not form active complexes with cyclins A, B,C or E. The D-type cyclins execute their function during mid-to-late G1 phase, as the cells cross the G1 restriction point and become independent of mitogens for the completion of cell division.[73] Although the D-type cyclins appear to have overlapping functions, it is thought

that subtle differences between the 3 proteins allow them to function in a highly optimised fashion in specific cell types.[74]

The target substrate for the D-type cyclins is the retinoblastoma protein (pRb). The complexes of cyclin D and CDK4/6 and cyclin E/CDK2 phosphorylate and deactivate the pRb.[64] The complexes of cyclin D/CDK4 and CDK6 have a distinct preference for pRb.[73] This is an important reaction as underphosphorylated pRb binds to the E2F family of transcription factors.[64] Phosphorylation of pRb releases the E2F proteins which in turn activate the transcription of target genes such as DNA polymerases which are critical for the progression to the S phase of the cell cycle.[68,70-72]

Dysregulated activity of cyclins, CDKs and CDKIs results in loss of control of normal cell proliferation. Changes in the expression of cyclin D or CDK4 appear to be commonly found in neoplasia while mutations affecting cyclin A, B and E are much less frequent than those affecting cyclin D.[67] The major checkpoint regulating the cell cycle is in the late G1 phase and cyclin D is strongly implicated in the regulation of the cell cycle at this critical point when the cells are committed to complete cell division.[75] Molecular studies on cyclin D overexpression in human cancers further indicate that cyclin D1 overexpression prevails over that of cyclin D2 and D3.[67,73] It is unclear, however, why cyclin D1 expression is frequently altered in human tumours as oppose to the other D-type cyclins. While cyclin D1 has been found to be amplified, re-arranged or overexpressed in tumours of diverse tissue types,[69,71,72] our knowledge on cyclin D1 expression in odontogenic cysts and

tumours is far less advanced. This is because cyclin D1 has not been fully researched in this group of lesions.

2.3.1. Cyclin D1 expression in odontogenic cysts and tumours

Only 3 studies have evaluated cyclin D1 expression in the OKC,[22,28,29] while thus far no study has evaluated its expression in the OJC. Of those studies that examined cyclin D1 expression in odontogenic cysts and tumours, these included dentigerous cysts, radicular cysts, and ameloblastomas. The findings of these studies are summarised in Table 3.

Table 3. Cyclin D1 expression in odontogenic cysts and tumours

Reference	Study sample	Study findings
Kumamoto *et al.*[4]	AB (n=31)	• The distribution of cyclin D1 was similar to that of cellular proliferation markers (DNA topoisomerase IIα, histone H3). • Cyclin D1 expression was detected in follicular and plexiform AB in the peripheral columnar cells and some of the central polyhedral cells. • Cyclin D1 expression was absent in keratinising cells and granular cells. Basal cell variant of AB showed immunopositive staining for cyclin D1 in most neoplastic cells.
de Vicente *et al.*[22]	OKC (n=11), DC (n=10), RC (n=10), AB (n=10)	• Significant differences in cyclin D1 in expression in OKC and RC (p= 0.001), and AB (p= 0.04).
Lo Muzio *et al.*[28]	OKC with NBCCS (n=14); sporadic OKC (n=16)	• NBCCS associated OKC showed significantly ↑ cyclin D1 expression than sporadic OKC.
Kimi *et al.*[29]	OKC (n=43)	• Cyclin D1 expression was found in 79,2% sporadic OKC, 70% recurrent OKC and 100% syndromic OKC. • Cyclin D1 distribution was similar to DNA topoisomerase IIα; hence was suggested that cyclin D1 reflects the cellular proliferative potential of the OKC lining.

OKC=odontogenic keratocyst; NBCCS=nevoid basal cell carcinoma syndrome; AB=ameloblastoma; DC=dentigerous cyst; RC=radicular cyst

Of the 3 studies on cyclin D1 expression in OKC,[22,28,29] Lo Muzio *et al.*[28] found a significant difference in the expression of cyclin D1 between syndromic and sporadic OKCs. Cyclin D1 overexpression was found in all syndromic OKCs while it was negative in all cases of sporadic OKCs.[28] de Vicente *et al.*[22] excluded syndromic cases from their study but found overexpression of cyclin D1 in 91% of sporadic OKCs. The difference in frequency of cyclin D1 expression in sporadic OKCs in the studies by De Vicente *et al.*[22] and Lo Muzio *et al.*[28] was attributed to varying methodologies and different dilutions of antibodies in these studies. In the third study on this subject, Kimi *et al.*[29] found a higher rate of cyclin D1 positive cells in the syndromic (100%) versus the sporadic (79.2%) and recurrent OKCs (70%). Of note, unlike Lo Muzio *et al.*[61] overexpression of cyclin D1 was also demonstrated in sporadic OKCs in the study by Kimi *et al*.[29] Kimi *et al.*[29] did not, however, demonstrate any significant differences in cyclin D1 expression between sporadic and recurrent OKCs.

Cyclin D1 was found to be expressed in OKCs in the basal and parabasal layers of the lining epithelium.[22,29] It was also found that the distribution of positive cells was similar to that of DNA topoisomerase IIα suggesting that cyclin D1 closely reflects the cellular proliferative potential of the lining epithelium.[29] The other cell cycle-related factors that were analysed immunohistochemically in the former study were p21 and p27 proteins.[29] p21 is an effector of wild-type p53-mediated cell cycle arrest.[64] Positive staining for p21 protein was found in basal and superficial cells in the lining epithelium of OKCs, thereby suggesting that it acts as an inhibitor of cell proliferation in the OKC, as most studies have

observed a reverse pattern of staining with markers of cellular proliferation in the OKC lining.[22,29] The pattern of p27 expression in the OKC lining coincided with those of bcl-2 and TUNEL suggesting that the apoptotic cells are present in the superficial cells of the lining epithelium of OKC but are typically absent in the basal cell layer.[29]

In the study by de Vicente *et al.*[22] immunostaining for cyclin D1 was positive in 91% of OKCs. This was significantly higher than that of the dentigerous cyst (50%), ameloblastoma (40%) and radicular cyst (30%).[22] The cyclin D1 expression was further observed in a focal pattern in OKC, dentigerous cyst and radicular cyst while in all positive ameloblastomas the staining pattern was only diffuse.[22] Taken together, these findings support the neoplastic potential of OKCs, but they also reveal some resemblance of the OKC with odontogenic cysts such as the dentigerous cyst and radicular cyst.[22] In ameloblastomas, cyclin D1 was expressed in the epithelial cells near the basement membrane suggesting that cyclin D1 is involved in cellular proliferation.[4] Follicular and plexiform ameloblastomas showed cyclin D1 expression predominantly in the peripheral columnar cells while the keratinising cells and granular cells in ameloblastoma were cyclin D1 negative.[4] The basal cell variant of ameloblastoma demonstrated a positive reaction for cyclin D1 in most of the neoplastic cells.[4]

In the category of benign non-odontogenic lesions of the jaw, Kauzman *et al.*[76] carried out an immunohistochemical study on cyclin D1 expression in central giant cell granulomas. The distribution of the cyclin D1 positively stained cells in the central giant cell granuloma

was found mainly in the nuclei of THE giant cells with only a few mononuclear cells staining positively for cyclin D1.[76] The presence of cyclin D1 staining in the giant cells of CGCG in the absence of cell proliferation was thought to suggest an aberrant expression of this protein in CGCG.[76] It therefore appears that the presence of cyclin D1 overexpression in odontogenic and non-odontogenic lesions may not always be indicative of increased cellular proliferation and may suggest alternative roles for this protein in the context of different pathologies. This is in keeping with recent findings which show that in addition to its classic function in tumorigenesis cyclin D1 also regulates cellular metabolism, fat differentiation and cellular migration in certain subsets of human tumours.[70]

2.4. Summary of literature review

Ki-67 is a widely used marker of cellular proliferation whose distribution in normal tissues reflects their known cell kinetics.[43] Although its exact function is not yet known Ki-67 identifies cells in all active phases of the cell cycle.[39-41] Thus far only 3 studies have examined Ki-67 expression in the OJC and compared these findings to the OKC.[3,30,59] Although all 3 studies report a significantly greater total number of Ki-67 positive cells in the OKC, [3,30,59] findings regarding the preferential localization of the Ki-67 positive cells in the OJC lining is still not clarified.

The major checkpoint regulating the cell cycle is in the late G1 phase and cyclin D1 is strongly implicated in the regulation of the cell cycle at this critical point when the cell is committed to complete cell division.[73] While cyclin D1 has been extensively analysed in tumours of diverse tissue types, our knowledge on cyclin D1 expression in odontogenic

cysts and tumours is far less advanced. Only 3 studies have evaluated cyclin D1 expression in the OKC,[22,28,29] while no study has as yet evaluated its expression in the OJC.

CHAPTER 3

3.0. AIM AND OBJECTIVES

3.1. Aim

To compare the proliferative activity between the epithelial linings of the OKC and OJC by immunohistochemical staining for Ki-67 and cyclin D1 antigens.

3.2. Objectives

3.2.1. To compare the epithelial linings of the OKC and OJC for any significant difference in the numbers of:

a) Ki-67+ cells

b) Cyclin D1+ cells

3.2.2. To determine whether there is a significant difference between Ki-67 and cyclin D1 expression in the:

a) OKC

b) OJC

3.2.3. To determine if there is a significant difference in Ki-67 expression in the basal versus the suprabasal cells in the:

a) OKC

b) OJC

3.2.4. To determine if there is a significant difference in cyclin D1 expression in the basal versus the suprabasal cells in the:

a) OKC

b) OJC

CHAPTER 4

4.0. MATERIALS AND METHODS

4.1. Tissue samples

From the archived files of the Division of Oral Pathology, Faculty of Health Sciences, University of the Witwatersrand 15 cases of OKC and 15 cases of OJC were selected for this study. Ethics approval has been granted to the Division of Oral Pathology by the Committee for Research on Human Subjects of the University of the Witwatersrand (Ethics Clearance Certificate number: M080850) for the use of stored paraffin wax embedded blocks in histopathology studies (Appendix A).

4.2. Inclusion criteria

The diagnosis of OKC and OJC was verified using haematoxylin and eosin (H&E) stained sections based on the criteria described by Shear.[1] Each OKC histologically demonstrated the cystic lining of uniform thickness usually 5 to 9 cell layers thick, parakeratinised stratified squamous epithelium, basal palisaded columnar or cuboidal cells with hyperchromatic nuclei (Figure 2). One of the cases was a recurrent OKC, while the remaining OKCs represented sporadic cases.

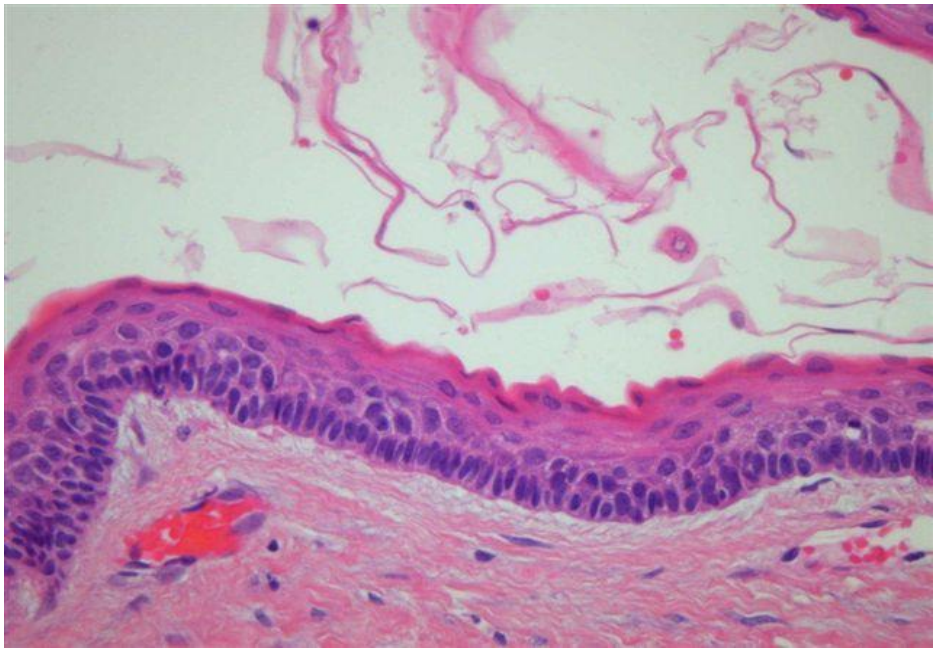


Figure 2. Histological appearance of the OKC demonstrating an epithelial lining of 6-8 cells thick with a parakeratotic corrugated surface layer and prominent palisaded basal cell layer with hyperchromatic nuclei. (H&E, original magnification x400)

The histological criteria for the OJC lesions were cyst lining 6 to 8 cell layers of stratified squamous epithelium with a prominent stratum granulosum and luminal orthokeratin, basal layer of flattened squamous cells with little tendency to polarise (Figure 3).

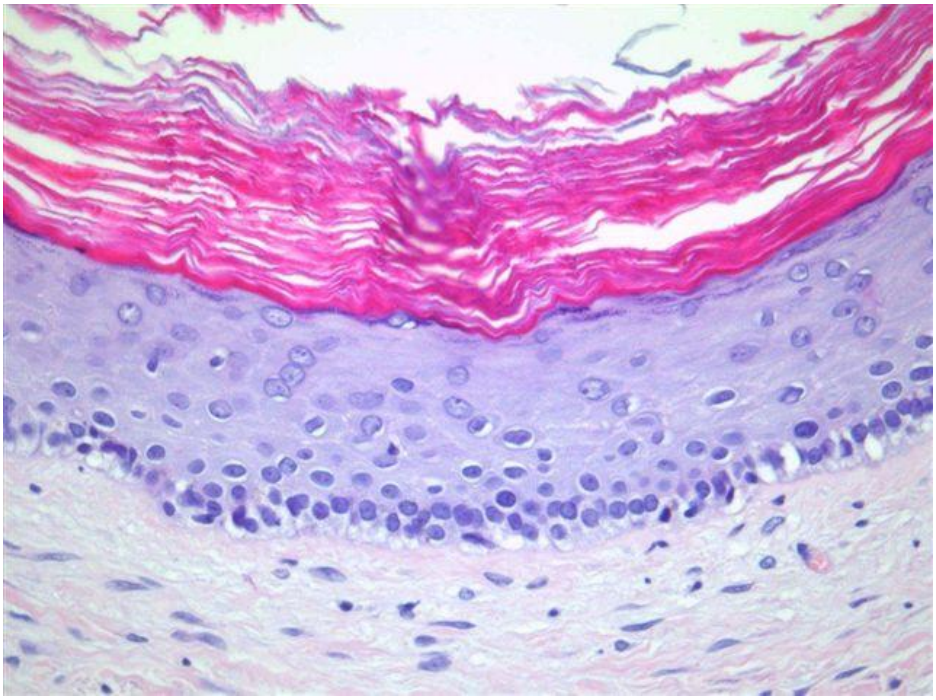


Figure 3. Histological appearance of the OJC demonstrates a squamous epithelial lining, absence of basal cell palisading and prominent keratohyaline granules just beneath the orthokeratotic layers. (H&E, original magnification x400)

The intraosseous location of the OJC was ascertained by the radiographic findings as described in the original histopathology laboratory report. Hard copies of the radiographs were unfortunately not available for review.

4.3. Exclusion criteria

Insufficient cyst lining for histological analysis, inflamed cysts, orthokeratinised cysts where the intra-osseous location was not unequivocally stated in the histopathology report

and biopsy specimens that were subjected to a bone decalcification procedure in view of the potential negative influence of the decalcifying agent on the immunoreactivity of formalin-fixed paraffin embedded tissue (FFPE).[77] None of the OKCs were associated with NBCCS.

4.4. Immunohistochemistry

The FFPE tissue blocks for the 30 selected cases were retrieved for immunohistochemical staining for both Ki-67 and cyclin D1. Immunohistochemistry is a method of detecting the presence of specific proteins in cells or tissues. The key steps in this procedure are:

i) primary antibody binds to the target antigen
ii) antibody-antigen complex is bound by a secondary, enzyme-conjugated, antibody
iii) the enzyme forms a coloured deposit at the sites of antibody-antigen binding in the presence of a chromogen.

Four micron sections were cut from the paraffin blocks and mounted on 3-aminopropyltriethoxy silane (APES) coated slides. Immunohistochemical staining was carried out using the Envision kit (DAKO, ChemMate, Denmark) and visualised with DAB (3,3-diaminobenzidine). Sections were dewaxed, washed in alcohol and antigen was retrieved by microwaving in citrate buffer (pH 6) for 10 min. Sections were then washed in Tris Buffered Saline (TBS) (pH 7.6). Endogenous peroxidase was blocked using 3% hydrogen peroxide in distilled water at room temperature for 20 min. Slides were washed

in TBS briefly and incubated with the primary antibody, Ki-67 (MIB1; DAKO, Glostrup, Denmark) and cyclin D1 (SP4; Thermo Scientific, Fremont, CA), at dilutions of 1:50 for 20 min. Slides were then washed in TBS for 5 min and incubated with Envision (HRP, Rabbit/Mouse) for 30 min. Sections were further washed for another 5 min in TBS and the peroxidase visualised by incubating in DAB for 10 min. Sections were again washed in TBS, counterstained with haematoxylin, washed in water and Scott's solution, dehydrated and then mounted.

Normal tonsil and mantle cell lymphoma were used as positive controls for the Ki-67 and cyclinD1 staining respectively. These tissue types were chosen because of their high antigenic specificity for the respective molecules. Negative controls were prepared by omitting the primary antibody.

4.5. Evaluation of staining

Two investigators simultaneously evaluated the slides with the use of an Eclipse 50*i* double-headed light microscope. This allowed for inter-examiner agreement on the data collected. A light microscope calibration micro-ruler was inserted into the eyepiece of the microscope. The ruler was aligned with the basement membrane of the cyst lining epithelium and an objective of 40X was used. The total numbers of positively stained cells per 10 consecutive lengths of the ruler were counted for each case. This amounted to a total basement membrane (BM) length of 2000μm (2mm) that was evaluated microscopically in each case. The use of the micro-ruler further helped prevent overlap

between consecutive fields. Brown staining of the nuclei was regarded as positive, irrespective of the intensity of the stain, for the Ki-67 and cyclin D1 antigens. In each case counts were recorded separately for the basal layer of cells and for the suprabasal cells, the sum of which constituted the panepithelial (total) cell count. Each alternative specimen was counted by the 2 observers at an interval of about 1-week in order to establish intra-examiner reliability of the data.

4.6. Statistical analysis

Analysis of the data was performed using Stata software, version 11. P-values of equal or less than 0.05 were considered significant.

CHAPTER 5

5.0. RESULTS

5.1. Immunohistochemistry

The epithelial linings of the 15 cases of OKC and 15 cases of OJC that fulfilled the inclusion criteria described on pages 31 and 32, showed immunoreactivity with the antibodies tested. Intra- and inter-observer reliability of the collected data was demonstrated for both OKC and OJC lesions stained with Ki-67 and cyclin D1 (Appendix B1-B4). After reliability of the collected data was established, the data was then subject to further statistical analysis.

5.2. Statistical analysis

Since the data did not follow a Gaussian distribution and the variances were not equal, non-parametric statistical methods were used to analyse the data. An example of one such distribution i.e. Ki-67 expression in OKC and OJC which is depicted in Figure 4. The Kruskal-Wallis test was therefore used to compare the expression of each marker in the 2 cyst types.

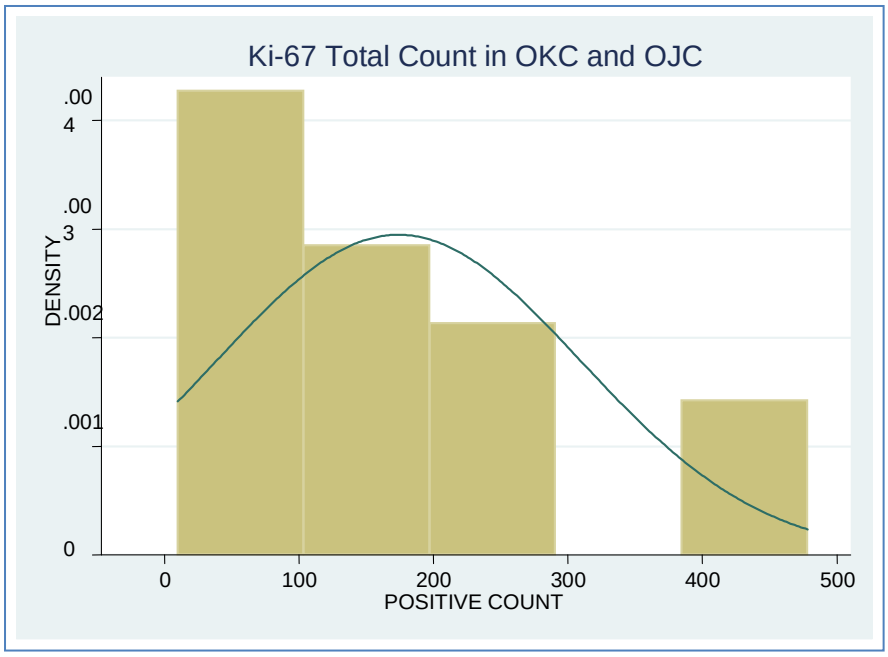


Figure 4. Non-parametric distribution of data for Ki-67+ cell count in the OKC and OJC.

5.3. Ki-67 and cyclin D1 expression in the OKC and OJC lining epithelium

The results of immunohistochemical reactivity for Ki-67 and cyclin D1 in the OKC and OJC are shown in Table 4 and also illustrated in Figure 5-8. The raw data appears in Appendix B1-B4.

Table 4. Ki-67 and cyclin D1 expression in OKC and OJC lining epithelium

Variable	Median positive cells (Inter-quartile range)	*P*-value
Ki-67 Panepithelial		
OKC	215 (147.5 - 414)	0.0005*
OJC	75 (49 - 153)	
Cyclin D1 Panepithelial		
OKC	322 (266 - 490)	0.0003*
OJC	161 (55 - 273)	
OKC		
Ki-67 Panepithelial	215 (147.5 - 414)	0.0095
Cyclin D1 Panepithelial	322 (266 - 490)	
OJC		
Ki-67 Panepithelial	75 (49 - 153)	0.071
Cyclin D1 Panepithelial	161 (55 - 273)	
OKC		
Ki-67 Basal	31 (20 - 70)	0.0001*
Ki-67 Suprabasal	196 (121.5 - 375)	
OJC		
Ki-67 Basal	23 (9 - 62.5)	0.23
Ki-67 Suprabasal	35 (12.5 - 92)	
OKC		
Cyclin D1 Basal	14 (3 - 136)	0.0001*
Cyclin D1 Suprabasal	284 (254 - 381)	
OJC		
Cyclin D1 Basal count	17 (4.5 - 38.5)	0.0007*
Cyclin D1 Suprabasal count	138 (34 - 251)	

5.3.1. Comparison of the panepithelial distribution of Ki-67 and cyclin D1 in the OKC and OJC

When the entire thickness of the epithelial linings (panepithelium/total count; i.e. basal plus suprabasal count) of the OKC and OJC were statistically compared for the presence of Ki-67+ cells, the OJC showed significantly fewer Ki-67+ cells than the OKC (p=0.0005) with a median number of 75 Ki-67+ cells/2mm BM and 215 Ki-67+ cells/2mm BM in the OJC and OKC respectively. The same was true of cyclin D1 in the OJC and OKC (p=0.0003) with the median number of cyclin D1+ cells/2mm BM being 161 and 322 in the OJC and OKC respectively (Table 4).

5.3.2. Comparison of Cyclin D1 and Ki-67 in the OKC and OJC

Cyclin D1 showed significantly greater expression than Ki-67 in the OKC ($p=0.0095$). In the OJC, there was no significant difference in the expression of Ki-67 and cyclin D1 ($p=0.071$) (Table 4).

5.3.3. Distribution of Ki-67 in the OKC and OJC

In the OKC, Ki-67 expression was mainly detected in the suprabasal compartment of the cyst lining ($p=0.0001$) (Figure 5). By contrast, although the expression of Ki-67 also tended to show a predilection for the parabasal cells in the OJC (Figure 6), this did not approach statistical significance ($p=0.23$).

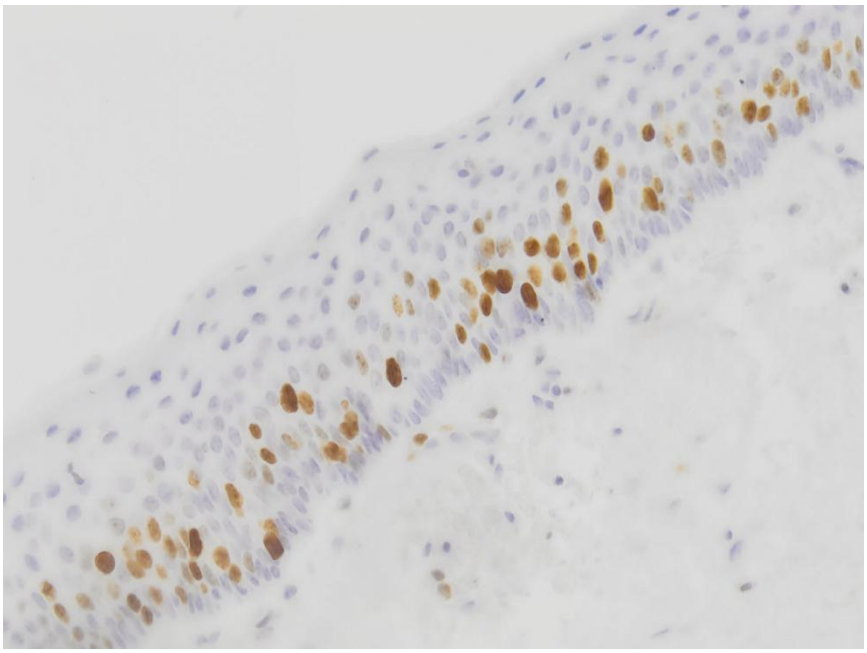


Figure 5. Positive staining for Ki-67 in the nuclei of the suprabasal cells, with occasional basal cells staining positively for the antigen. (Original magnification X400).

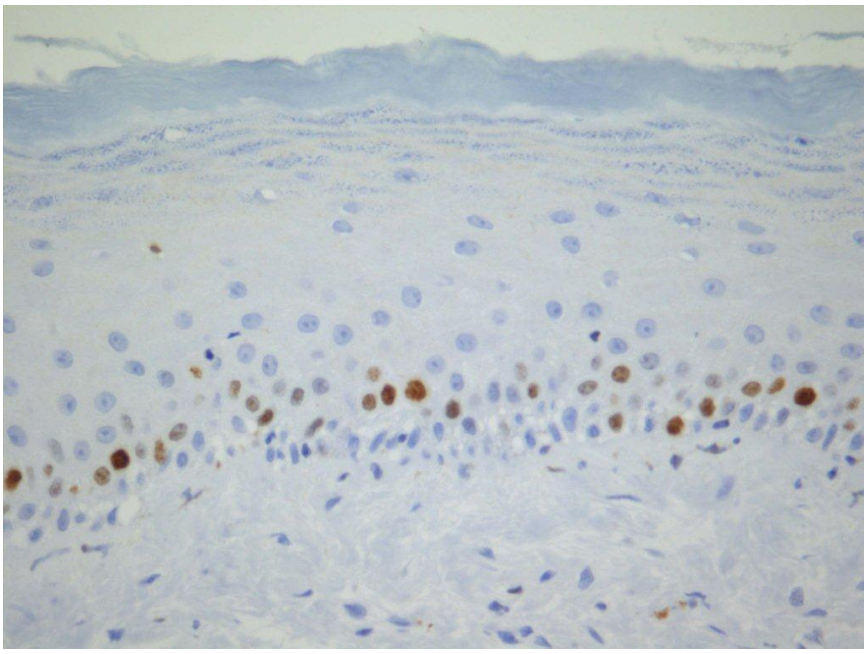


Figure 6. The distribution of cells that are positively stained for the Ki-67 antigen are found in the basal and suprabasal layers of the OJC lining epithelium. (Original magnification, X400)

5.3.4. Distribution of Cyclin D1 in the OKC and OJC

Cyclin D1 showed significantly greater expression suprabasally than in the basal cell layers of both the OKC (p=0.0001) (Figure 7) and OJC (p=0.0007) (Figure 8).

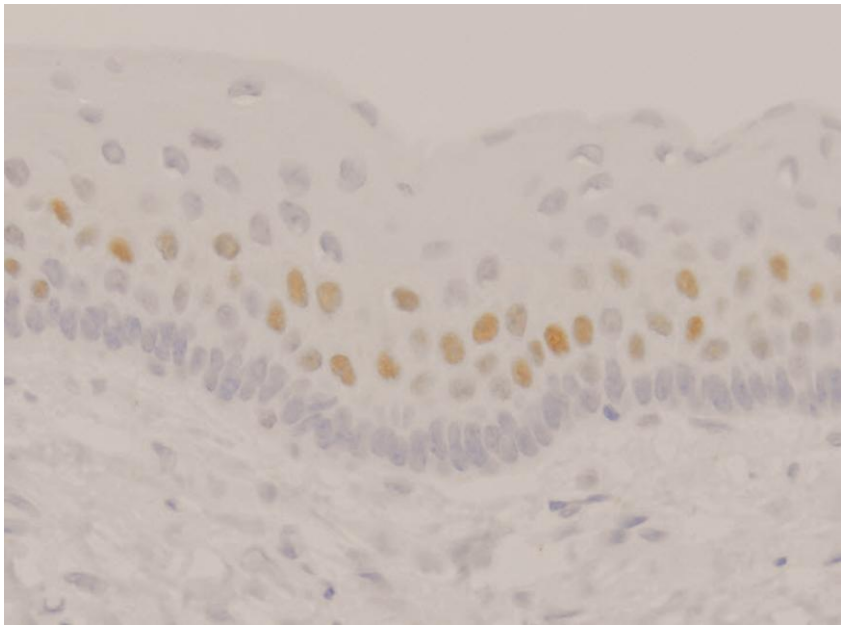


Figure 7. Cyclin D1 staining in the suprabasal compartment of the OKC (Original magnification X400).

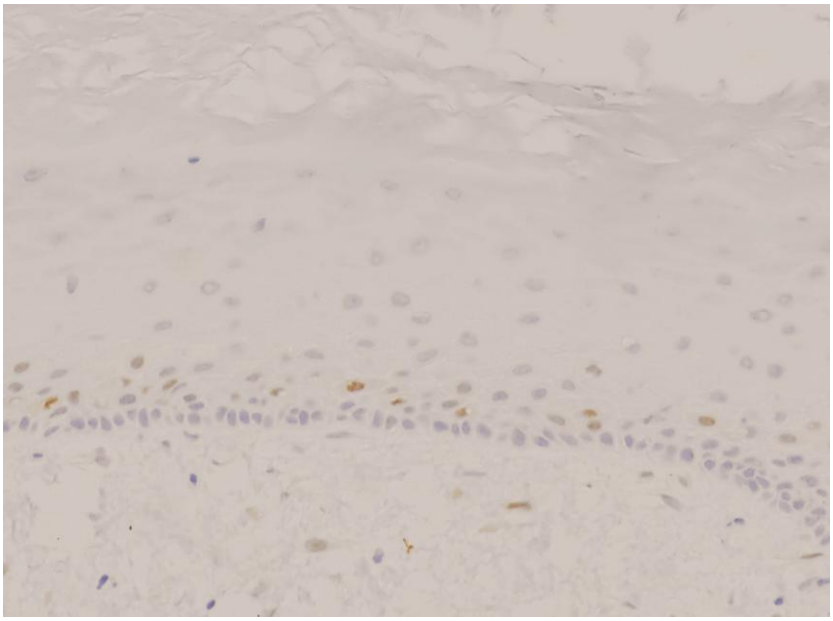


Figure 8. Cyclin D1 staining in the suprabasal layers of the OJC lining epithelium (Original magnification X200).

5.4. Summary of results

The total thickness (panepithelial) of the OJC lining showed significantly fewer Ki-67+ and cyclin D1+ cells than the OKC. In OKC, both Ki-67 and cyclin D1 showed significantly greater expression in the suprabasal compartment of the cyst lining. In OJC, only cyclin D1 showed significantly greater expression suprabasally, while Ki-67 did not show preferential expression for the suprabasal layers of the cyst lining. Furthermore, a quantitative comparison between the total numbers (panepithelial counts) of Ki-67+ and cyclin D1+ cells in the 2 cyst types showed no significant difference in the OJC while there were significantly fewer Ki-67+ cells than cyclin D1+ cells in the OKC.

CHAPTER 6

6.0. DISCUSSION

Among odontogenic cysts the OKC continues to be a subject of great interest by researchers and clinicians alike. This is largely due to its tendency to recur, association with NBCCS and its unusual biological characteristics. While the surgical technique employed in the management of the OKC plays an important role in the risk for recurrence,[78] the epithelial lining of the OKC appears to express an intrinsic growth potential which has not been demonstrated in other jaw cysts.[8] The high proliferative activity of the epithelium of OKC is regarded as its main mechanism of growth.[2,8,10] This is in contrast to OJC which displays a less aggressive clinical pattern of behaviour but which has been less frequently studied. The aim of this study was therefore to compare the proliferative activity of the OKC and OJC as assessed by immunohistochemical expression of Ki-67 and cyclin D1.

In order for cells to proliferate they must enter and complete the cell cycle. Ki-67 is a widely used marker of cellular proliferation whose distribution in normal tissues reflects their known cell kinetics.[43] Although its exact function is not yet known Ki-67 identifies cells in all active phases of the cell cycle.[44] The major checkpoint regulating the cell cycle is in the late G1 phase and cyclin D1 is strongly implicated in the regulation of the cell cycle at this critical point when the cells commits itself to cell division.[75] Few studies have, however, compared the immunohistochemical expression of Ki-67 and cyclin D1 in the OKC.[22] Further, while some studies have compared Ki-67 expression in the OKC and

OJC,[30,59] there is no record of any studies published in English about the expression of cyclin D1 in the OJC. One of the objectives of this study was therefore to examine a series of OJCs for cyclin D1 expression and to compare this with Ki-67.

In odontogenic cysts and tumours, Ki-67 expression has been found to be higher in ameloblastomas and OKCs compared to dentigerous and radicular cysts.[22,25-27] Baghaei *et al.*[30] noted that the average number of Ki-67 positively stained cells was greater in the OKC than the OJC. These authors further attributed the high recurrence rate of OKCs to higher expression of Ki-67.[30] The proliferative and apoptotic indices of the lining epithelium of the OKCs have demonstrated a high rate of cell turnover and apoptotic rate thereby reinforcing the opinion that despite the high cell proliferation index the OKC maintains a cystic configuration by a high turnover of apoptosis in the lining epithelium.[5] The present study using a similar method to Baghaei *et al.*[30] showed similar findings, with the number of Ki-67 positive cells in the OKC being notably higher than in the OJC ($p<0.0005$). The significantly greater expression of Ki-67 in the OKC compared to OJC, as elucidated in the present study, corroborates earlier findings of increased proliferative activity in the cyst lining of the OKC compared to clinically more indolent lesions.

In the present work, the cyst linings of all cases of OKC and OJC showed cyclin D1 expression, with the median number of cyclin D1 positive cells/2mm BM being 322 and 161 in the OKC and OJC respectively. The significantly greater expression of cyclin D1 in the OKC is in keeping with previous studies which showed cyclin D1 expression was

higher in the OKC than in non-neoplastic lesions such as the radicular cyst and dentigerous cyst.[22] Since cyclin D1 has until now not been studied in the OJC and is unchartered territory, the hypothesised cyclin D1 expression in the OJC was shown in this study to be similar to that of more indolent cysts such as the dentigerous and radicular cyst.

The presence of cyclin D1 expression in the OKC and OJC is, however, in contrast to normal oral mucosa where the expression of cyclin D1 is usually not detectable or weak .[79] Increased cell proliferative activity may be considered to account for the overexpression of the cyclin D1 protein in the odontogenic cysts examined, since histopathological studies evaluating cyclin D1 expression have found a positive correlation with other proliferative markers such as PCNA, topoisomerase IIα and histone H3.[4,80] Experimental studies have indicated that cyclin D1 plays a role in cell cycle regulation, in particular progression from the G1 to the S phase of the cell cycle.[81] It would therefore appear that the presence of the cyclin D1 protein would reflect production of the molecule by cycling cells. Cyclin D1 and Ki-67 proteins accumulate during the G- phase of the cell cycle.[40,47,75] Ki-67 protein appears during the transition from G0 to G1 and remains detectable for the remainder of the cell cycle.[40] In cultured cells, cyclin D1 levels rise during mid-G and fall during S phase.[82] It would therefore be anticipated that the fraction of cells positive for Ki-67 should be greater than the proportion containing cyclin D1 when cyclin D1 expression reflects normal cell cycle activity. Our observations, however, deviate from the expected pattern; OKC contained significantly fewer Ki-67 positive cells than cyclin D1 positive cells while there was no significant difference in the numbers of Ki-67 and cyclin D1 positive cells in the OJC. A similar divergence between cyclin D1 and Ki-67 expression,

although examined in different lesions, has been reported by other investigators.[83-85] Taken together, these observations may suggest that the presence of cyclin D1 in pathological tissue probably does not reflect the normal, co-ordinated operation of the cell cycle apparatus.

Another significant feature noted in the present study was the fact that the suprabasal compartment of the OKC epithelium contained significantly more Ki-67 positive cells than the basal cell compartment ($p<0.0001$). This is in agreement with other studies using a variety of different methods of quantifying Ki-67 expression in the OKC,[3,5,12-14,16,18,19,21-25,30,59] as well as those studies using PCNA as a proliferation marker.[26,86,87] All these previous studies have demonstrated that the proliferative centre (intrinsic growth potential) 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, including the OJC as was depicted in this study.[5,17,18] The increased cellular proliferation in the suprabasal layer of the OKC may suggest an abnormal cell cycle control. This unusual pattern of proliferation in the OKC lining is also thought to represent an epithelial disorganisation similar to dysplasia of oral squamous epithelium in premalignant lesions of the oral mucosa, whereby proliferating cells are found in greater numbers in the suprabasal compartment.[12,21,24,25,88] The high proliferative potential of the OKC also reinforces its classification as a benign cystic odontogenic neoplasm.[5]

Additionally, it has been suggested that the expression of Ki-67 is a useful tool in differentiating between the OKC and OJC and possibly may also identify those cysts requiring more radical treatments.[30] Several studies have demonstrated that the OKC tends to grow within the medullary cavity of bone, becoming a large lesion without causing much bone expansion.[23,29,33] Kichi *et al.*[17] suggested that the suprabasal compartment of proliferating cells may allow for lateral (anterior-posterior) spread that is typical of the OKC.

In the present study, unlike the OKC, in the OJC Ki-67 positive cells did not show a predominantly suprabasal localisation and instead were uniformly distributed between the basal and suprabasal compartments of the cyst lining. Of the 3 earlier studies that examined Ki-67 expression in the OJC,[3,30,59] Li *et al.*[3] and Dong *et al.*[59] found Ki-67 positive cells mostly confined to the basal layer while most of the Ki-67 positive cells were found suprabasally in the work by Baghaei *et al*.[30] The notable lack of predilection of the Ki-67 positive cells for the suprabasal compartment that was found in 2 of the 3 studies described above, [3, 59] suggests that once suprabasal most of the cells in the OJC stop dividing and may enter a differentiation programme thereby forming a prominent stratum granulosum and stratum corneum. The molecular mechanisms that underlie this process have not yet been investigated. It remains to be determined whether the process of epithelial stratification and differentiation in the OJC and OKC both resemble that elucidated for mammalian skin wherein two possible mechanisms are described.[89] It seems likely that one of these mechanisms may be operational in the OJC and the other in the OKC. According to these in vitro studies,[89] if the basal cells lack polarity and cuboidal

morphology (as is the case with the OJC), then the proliferating cells in the basal compartment detach periodically from the underlying basal lamina, differentiate into mature keratinocytes as they migrate toward the surface and eventually undergo apoptosis. Alternatively, if the basal cells have a cuboidal morphology and show polarity (as is the case with the OKC), then these basal cells use their polarity to divide asymmetrically in which the mitotic spindle orientates perpendicularly to the basement membrane generating a committed suprabasal cell and a proliferative basal cell.[89] Future studies aimed at exploring these hypotheses may provide a scientific explanation for the molecular biological events that result in the different histomorphological appearances and ultimately biological behaviour of the OKC and OJC.

CHAPTER 7

7.0. CONCLUSIONS

7.1. The significantly greater expression of Ki-67 in the OKC compared to the OJC, as elucidated in the present study, corroborates earlier findings of increased proliferative activity in the cyst lining of the OKC compared to clinically more indolent lesions such as the OJC.

7.2. The accumulation of Ki-67 positive proliferating cells in the suprabasal compartment of the OKC suggests that perhaps a process of asymmetrical cell division is operational in the OKC and warrants further study.

7.3. The results of this study further suggest that the expression of cyclin D1 in the OKC and OJC does not appear to reflect the normal, co-ordinated operation of the cell cycle apparatus. This finding provides motivation for additional studies, using a larger number of cases, to determine the exact role of cyclin D1 in the proliferation, differentiation and clinical behaviour of the OKC and OJC.

7.4. Future prospective studies with long term follow-up (5-10 years) are needed to determine whether cysts lined by both orthokeratinised and parakeratinised epithelium (i.e. cysts with mixed patterns of keratinisation) share the same biological profile as OKCs or not.

APPENDIX A

Ethical clearance form

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

APPENDIX B

APPENDIX B1: Raw data collection of Ki-67 expression in OKC lining epithelium

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 738/05	Field 1	1	7		1	9	
	Field 2	4	9		4	9	
	Field 3	2	3		2	5	
	Field 4	2	4		2	4	
	Field 5	0	4		0	5	
	Field 6	3	6		5	8	
	Field 7	4	21		5	29	
	Field 8	3	12		4	10	
	Field 9	9	12		5	12	
	Field 10	3	6		3	9	
	Total	31	84	115	31	100	131
TJL 155/03	Field 1	7	2		7	3	
	Field 2	2	6		13	8	
	Field 3	7	4		8	4	
	Field 4	6	6		9	6	
	Field 5	7	2		9	2	
	Field 6	13	1		11	3	
	Field 7	14	13		14	15	
	Field 8	10	11		10	11	
	Field 9	12	9		12	9	
	Field 10	11	3		11	3	
	Total	89	57	146	104	64	168
TJL 23/03	Field 1	0	3		1	3	
	Field 2	0	3		1	4	
	Field 3	0	8		0	6	
	Field 4	0	5		1	7	
	Field 5	0	7		1	3	
	Field 6	0	6		0	5	
	Field 7	0	6		1	10	
	Field 8	1	1		0	8	
	Field 9	0	7		0	7	
	Field 10	1	4		1	4	
	Total	2	50	52	6	57	63
TJL1004/03	Field 1	4	19		4	16	
	Field 2	0	22		1	20	
	Field 3	1	18		1	18	
	Field 4	1	13		1	13	
	Field 5	4	29		5	29	
	Field 6	3	28		4	30	
	Field 7	1	19		1	18	
	Field 8	1	42		1	40	
	Field 9	1	25		1	25	
	Field 10	0	18		0	20	
	Total	16	233	249	19	229	248
TJL 469/03	Field 1	6	31		6	30	
	Field 2	3	30		4	27	
	Field 3	1	9		0	16	
	Field 4	0	21		1	24	
	Field 5	4	22		4	17	
	Field 6	1	23		0	25	
	Field 7	1	17		2	17	
	Field 8	1	16		3	19	
	Field 9	0	14		1	14	
	Field 10	0	10		0	10	
	Total	17	193	210	21	199	220

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 41/03	Field 1	1	11		2	17	
	Field 2	2	15		2	18	
	Field 3	3	19		1	19	
	Field 4	1	15		1	11	
	Field 5	1	15		3	15	
	Field 6	3	17		3	17	
	Field 7	0	4		3	5	
	Field 8	4	3		5	8	
	Field 9	4	11		5	6	
	Field 10	6	7		2	10	
	Total	25	117	142	27	126	153
TJL 1283/02	Field 1	1	26				
	Field 2	2	31				
	Field 3	7	27				
	Field 4	8	25				
	Field 5	3	25				
	Field 6	9	16				
	Field 7	1	8				
	Field 8	4	15				
	Field 9	4	28				
	Field 10	5	22				
	Total	44	223	267			
TJL 409/01	Field 1	2	4		2	3	
	Field 2	6	17		5	16	
	Field 3	1	13		1	15	
	Field 4	4	13		4	13	
	Field 5	0	9		0	14	
	Field 6	2	11		1	15	
	Field 7	4	12		1	14	
	Field 8	3	15		1	10	
	Field 9	1	11		2	11	
	Field 10	0	16		0	17	
	Total	23	121	144	17	128	145
TJL 15/00	Field 1	2	11				
	Field 2	3	18				
	Field 3	0	9				
	Field 4	7	15				
	Field 5	5	11				
	Field 6	9	21				
	Field 7	4	16				
	Field 8	3	14				
	Field 9	3	16				
	Field 10	4	19				
	Total	40	150	190			
TJL 82/00	Field 1	15	45				
	Field 2	5	45				
	Field 3	3	27				
	Field 4	9	21				
	Field 5	13	52				
	Field 6	10	55				
	Field 7	8	70				
	Field 8	4	31				
	Field 9	2	40				
	Field 10	1	22				
	Total	70	408	478			

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 857/00	Field 1	7	20				
	Field 2	5	24				
	Field 3	2	15				
	Field 4	2	21				
	Field 5	4	18				
	Field 6	4	22				
	Field 7	7	33				
	Field 8	6	27				
	Field 9	4	28				
	Field 10	7	21				
	Total	48	229	277			
TJL 1095/00	Field 1	3	39				
	Field 2	4	44				
	Field 3	3	52				
	Field 4	3	47				
	Field 5	2	37				
	Field 6	0	29				
	Field 7	3	38				
	Field 8	2	35				
	Field 9	2	42				
	Field 10	1	28				
	Total	23	391	414			
TJL 456/92	Field 1	1	10		1	10	
	Field 2	3	5		3	7	
	Field 3	1	15		1	10	
	Field 4	4	15		4	14	
	Field 5	7	19		6	16	
	Field 6	2	14		2	11	
	Field 7	1	12		0	12	
	Field 8	3	18		2	16	
	Field 9	2	29		1	24	
	Field 10	6	24		4	27	
	Total	30	161	191	24	147	171
34058/09	Field 1	12	31		12	28	
	Field 2	12	45		10	41	
	Field 3	10	34		11	35	
	Field 4	13	35		9	33	
	Field 5	7	24		6	23	
	Field 6	6	53		6	53	
	Field 7	4	49		5	51	
	Field 8	7	39		4	51	
	Field 9	7	35		5	36	
	Field 10	7	27		8	27	
	Total	85	372	457	76	378	454
30636/09	Field 1	5	37		8	38	
	Field 2	5	50		6	41	
	Field 3	6	43		13	33	
	Field 4	5	50		6	39	
	Field 5	8	37		5	54	
	Field 6	10	47		10	47	
	Field 7	5	32		6	46	
	Field 8	6	35		9	43	
	Field 9	10	36		8	32	
	Field 10	3	37		9	33	
	Total	63	404	467	80	406	486

APPENDIX B2: Raw data collection of Ki-67 expression in OJC lining epithelium

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
35511/09	Field 1	11	1		6	0	
	Field 2	9	1		8	2	
	Field 3	7	0		8	0	
	Field 4	5	1		5	1	
	Field 5	6	1		7	1	
	Field 6	12	1		10	2	
	Field 7	13	1		9	2	
	Field 8	16	5		13	3	
	Field 9	4	1		2	5	
	Field 10	3	1		2	3	
	Total	86	13	99	70	19	89
4425/09	Field 1	3	3		3	3	
	Field 2	2	2		2	2	
	Field 3	4	1		3	2	
	Field 4	3	1		2	1	
	Field 5	7	0		6	1	
	Field 6	8	6		8	5	
	Field 7	7	3		7	3	
	Field 8	11	3		11	5	
	Field 9	10	8		10	6	
	Field 10	8	7		10	8	
	Total	63	34	97	62	36	98
2063/09	Field 1	4	11				
	Field 2	5	15				
	Field 3	6	7				
	Field 4	6	10				
	Field 5	7	17				
	Field 6	10	22				
	Field 7	5	15				
	Field 8	6	14				
	Field 9	7	20				
	Field 10	6	19				
	Total	62	150	212			
5008/08	Field 1	0	7		0	7	
	Field 2	0	5		0	13	
	Field 3	0	24		0	31	
	Field 4	0	29		2	30	
	Field 5	2	27		1	20	
	Field 6	1	21		0	27	
	Field 7	0	32		0	29	
	Field 8	0	30		0	26	
	Field 9	0	24		0	24	
	Field 10	0	19		3	23	
	Total	3	218	221	5	230	235
17/06	Field 1	12	4				
	Field 2	6	3				
	Field 3	4	7				
	Field 4	7	11				
	Field 5	13	10				
	Field 6	21	14				
	Field 7	4	9				
	Field 8	4	16				
	Field 9	5	8				
	Field 10	4	10				
	Total	80	92	172			

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
801/03	Field 1	4	0		4	0	
	Field 2	7	0		8	0	
	Field 3	14	0		15	0	
	Field 4	13	1		12	2	
	Field 5	18	4		14	10	
	Field 6	11	0		7	2	
	Field 7	5	1		10	2	
	Field 8	2	1		3	2	
	Field 9	1	0		1	0	
	Field 10	0	0		0	0	
	Total	75	7	82	74	18	92
63/02	Field 1	0	8				
	Field 2	1	4				
	Field 3	1	15				
	Field 4	3	10				
	Field 5	0	9				
	Field 6	1	16				
	Field 7	0	1				
	Field 8	0	3				
	Field 9	0	0				
	Field 10	3	0				
	Total	9	66	75			
243/94	Field 1	1	5				
	Field 2	2	2				
	Field 3	1	5				
	Field 4	3	7				
	Field 5	4	4				
	Field 6	1	10				
	Field 7	0	1				
	Field 8	0	0				
	Field 9	0	2				
	Field 10	0	1				
	Total	12	37	49			
584/92	Field 1	3	0				
	Field 2	0	1				
	Field 3	3	0				
	Field 4	0	0				
	Field 5	0	0				
	Field 6	0	0				
	Field 7	1	0				
	Field 8	0	0				
	Field 9	1	0				
	Field 10	1	0				
	Total	9	1	10			
299/92	Field 1	8	4				
	Field 2	0	2				
	Field 3	0	6				
	Field 4	0	0				
	Field 5	0	0				
	Field 6	0	0				
	Field 7	0	0				
	Field 8	0	0				
	Field 9	0	0				
	Field 10	0	0				
	Total	8	12	20			

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
556/92	Field 1	5	12				
	Field 2	5	17				
	Field 3	5	12				
	Field 4	5	10				
	Field 5	1	10				
	Field 6	3	9				
	Field 7	1	18				
	Field 8	1	9				
	Field 9	0	19				
	Field 10	3	8				
	Total	29	124	153			
641/87	Field 1	4	3		4	1	
	Field 2	14	3		11	3	
	Field 3	2	2		2	2	
	Field 4	5	2		4	3	
	Field 5	6	2		4	2	
	Field 6	1	0		1	0	
	Field 7	5	1		5	1	
	Field 8	5	2		4	2	
	Field 9	7	1		6	1	
	Field 10	5	1		5	2	
	Total	54	17	71	46	17	63
63/02	Field 1	0	8				
	Field 2	1	4				
	Field 3	1	15				
	Field 4	3	10				
	Field 5	0	9				
	Field 6	1	16				
	Field 7	0	1				
	Field 8	0	3				
	Field 9	0	0				
	Field 10	3	0				
	Total	9	66	75			
243/94	Field 1	1	5				
	Field 2	2	2				
	Field 3	1	5				
	Field 4	3	7				
	Field 5	4	4				
	Field 6	1	10				
	Field 7	0	1				
	Field 8	0	0				
	Field 9	0	2				
	Field 10	0	1				
	Total	12	37	49			
584/92	Field 1	3	0				
	Field 2	0	1				
	Field 3	3	0				
	Field 4	0	0				
	Field 5	0	0				
	Field 6	0	0				
	Field 7	1	0				
	Field 8	0	0				
	Field 9	1	0				
	Field 10	1	0				
	Total	9	1	10			

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
299/92	Field 1	8	4				
	Field 2	0	2				
	Field 3	0	6				
	Field 4	0	0				
	Field 5	0	0				
	Field 6	0	0				
	Field 7	0	0				
	Field 8	0	0				
	Field 9	0	0				
	Field 10	0	0				
	Total	8	12	20			
556/92	Field 1	5	12				
	Field 2	5	17				
	Field 3	5	12				
	Field 4	5	10				
	Field 5	1	10				
	Field 6	3	9				
	Field 7	1	18				
	Field 8	1	9				
	Field 9	0	19				
	Field 10	3	8				
	Total	29	124	153			
641/87	Field 1	4	3		4	1	
	Field 2	14	3		11	3	
	Field 3	2	2		2	2	
	Field 4	5	2		4	3	
	Field 5	6	2		4	2	
	Field 6	1	0		1	0	
	Field 7	5	1		5	1	
	Field 8	5	2		4	2	
	Field 9	7	1		6	1	
	Field 10	5	1		5	2	
	Total	54	17	71	46	17	63
269/81	Field 1	1	3				
	Field 2	4	4				
	Field 3	3	1				
	Field 4	1	1				
	Field 5	3	7				
	Field 6	3	4				
	Field 7	3	6				
	Field 8	4	4				
	Field 9	1	0				
	Field 10	0	2				
	Total	23	32	55			
307/78	Field 1	1	1		1	3	
	Field 2	0	0		1	0	
	Field 3	0	4		0	4	
	Field 4	0	1		0	1	
	Field 5	0	1		0	1	
	Field 6	1	0		1	1	
	Field 7	0	0		0	0	
	Field 8	0	1		0	1	
	Field 9	0	0		0	0	
	Field 10	3	1		3	1	
	Total	5	9	14	6	12	18

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
297/75	Field 1	0	10		0	12	
	Field 2	2	8		1	12	
	Field 3	2	6		2	6	
	Field 4	1	5		1	6	
	Field 5	1	0		3	4	
	Field 6	0	4		0	4	
	Field 7	0	1		0	1	
	Field 8	0	1		0	1	
	Field 9	2	2		2	3	
	Field 10	1	1		1	1	
	Total	9	38	47	10	50	60

APPENDIX B3: Raw data collection of cyclin-D1 expression in OKC lining epithelium

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 738/05	Field 1	0	34		0	31	
	Field 2	0	27		0	22	
	Field 3	0	35		0	30	
	Field 4	0	25		0	17	
	Field 5	0	18		0	23	
	Field 6	0	23		0	24	
	Field 7	0	49		0	42	
	Field 8	1	52		1	46	
	Field 9	1	21		1	18	
	Field 10	1	24		1	23	
	Total	3	308	311	3	276	279
TJL 155/03	Field 1	18	16		17	14	
	Field 2	25	34		19	29	
	Field 3	19	23		18	21	
	Field 4	29	17		28	19	
	Field 5	20	23		16	24	
	Field 6	18	17		15	22	
	Field 7	17	23		19	15	
	Field 8	21	16		15	10	
	Field 9	11	4		18	10	
	Field 10	19	11		12	15	
	Total	197	184	381	177	179	356
TJL 23/03	Field 1	0	38		0	34	
	Field 2	0	20		0	20	
	Field 3	0	18		0	17	
	Field 4	0	24		0	21	
	Field 5	0	29		0	25	
	Field 6	0	42		0	43	
	Field 7	0	26		0	18	
	Field 8	0	34		0	25	
	Field 9	0	20		0	17	
	Field 10	0	32		0	25	
	Total	0	283	283	0	245	245
TJL1004/03	Field 1	29	25				
	Field 2	41	46				
	Field 3	23	34				
	Field 4	21	32				
	Field 5	18	45				
	Field 6	23	34				
	Field 7	11	23				
	Field 8	7	44				
	Field 9	2	48				
	Field 10	14	50				
	Total	189	381	570			
TJL 469/03	Field 1	8	29		7	26	
	Field 2	5	22		5	22	
	Field 3	5	24		6	24	
	Field 4	6	20		5	22	
	Field 5	10	27		10	25	
	Field 6	15	14		16	18	
	Field 7	12	18		11	18	
	Field 8	10	22		12	20	
	Field 9	4	19		10	19	
	Field 10	4	27		5	27	
	Total	79	222	301	87	221	308

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 469/03	Field 1	8	29		7	26	
	Field 2	5	22		5	22	
	Field 3	5	24		6	24	
	Field 4	6	20		5	22	
	Field 5	10	27		10	25	
	Field 6	15	14		16	18	
	Field 7	12	18		11	18	
	Field 8	10	22		12	20	
	Field 9	4	19		10	19	
	Field 10	4	27		5	27	
	Total	79	222	301	87	221	308
TJL 41/03	Field 1	13	35				
	Field 2	18	30				
	Field 3	17	33				
	Field 4	27	31				
	Field 5	25	18				
	Field 6	10	31				
	Field 7	26	39				
	Field 8	25	28				
	Field 9	17	24				
	Field 10	20	23				
	Total	198	292	490			
TJL 1283/02	Field 1	1	32				
	Field 2	0	21				
	Field 3	0	18				
	Field 4	1	27				
	Field 5	0	18				
	Field 6	1	38				
	Field 7	2	26				
	Field 8	0	33				
	Field 9	0	25				
	Field 10	0	16				
	Total	5	254	259			
TJL 409/01	Field 1	0	23				
	Field 2	1	38				
	Field 3	1	40				
	Field 4	0	27				
	Field 5	0	24				
	Field 6	0	28				
	Field 7	0	18				
	Field 8	0	24				
	Field 9	0	33				
	Field 10	0	19				
	Total	2	274	276			
TJL 15/00	Field 1	0	29				
	Field 2	0	20				
	Field 3	0	16				
	Field 4	0	29				
	Field 5	0	30				
	Field 6	0	41				
	Field 7	0	34				
	Field 8	0	13				
	Field 9	0	12				
	Field 10	0	10				
	Total	0	234	234			

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 82/00	Field 1	0	15		0	16	
	Field 2	0	10		0	26	
	Field 3	0	18		0	26	
	Field 4	1	40		1	25	
	Field 5	3	54		0	32	
	Field 6	0	41		0	27	
	Field 7	0	22		1	40	
	Field 8	1	38		0	17	
	Field 9	0	18		0	18	
	Field 10	0	20		3	19	
	Total	5	276	281	5	246	251
TJL 857/00	Field 1	11	47				
	Field 2	16	45				
	Field 3	18	45				
	Field 4	19	46				
	Field 5	16	50				
	Field 6	9	58				
	Field 7	12	65				
	Field 8	14	57				
	Field 9	8	63				
	Field 10	13	66				
	Total	136	542	678			
TJL 1095/00	Field 1	1	59				
	Field 2	2	78				
	Field 3	0	86				
	Field 4	3	98				
	Field 5	2	68				
	Field 6	4	65				
	Field 7	0	73				
	Field 8	1	72				
	Field 9	1	69				
	Field 10	0	84				
	Total	14	752	766			
TJL 456/92	Field 1	5	22				
	Field 2	1	31				
	Field 3	5	24				
	Field 4	1	18				
	Field 5	4	26				
	Field 6	4	38				
	Field 7	3	31				
	Field 8	6	32				
	Field 9	1	30				
	Field 10	8	32				
	Total	38	284	322			
34058/09	Field 1	2	41		1	34	
	Field 2	1	41		2	28	
	Field 3	5	47		3	42	
	Field 4	6	28		4	28	
	Field 5	3	12		2	19	
	Field 6	1	36		0	36	
	Field 7	5	26		0	29	
	Field 8	3	26		2	28	
	Field 9	4	33		2	33	
	Field 10	1	22		1	28	
	Total	31	312	343	17	305	322

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
TJL 456/92	Field 1	5	22				
	Field 2	1	31				
	Field 3	5	24				
	Field 4	1	18				
	Field 5	4	26				
	Field 6	4	38				
	Field 7	3	31				
	Field 8	6	32				
	Field 9	1	30				
	Field 10	8	32				
	Total	38	284	322			
34058/09	Field 1	2	41		1	34	
	Field 2	1	41		2	28	
	Field 3	5	47		3	42	
	Field 4	6	28		4	28	
	Field 5	3	12		2	19	
	Field 6	1	36		0	36	
	Field 7	5	26		0	29	
	Field 8	3	26		2	28	
	Field 9	4	33		2	33	
	Field 10	1	22		1	28	
	Total	31	312	343	17	305	322
30636/09	Field 1	1	35		1	41	
	Field 2	1	34		0	33	
	Field 3	0	32		1	46	
	Field 4	1	30		4	39	
	Field 5	0	33		1	45	
	Field 6	1	44		0	52	
	Field 7	0	53		0	62	
	Field 8	1	58		0	56	
	Field 9	4	76		0	60	
	Field 10	2	58		1	52	
	Total	11	453	464	8	486	494

APPENDIX B4: Raw data collection of cyclin D1 expression in OJC lining epithelium

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
35511/09	Field 1	7	5		7	3	
	Field 2	4	15		4	14	
	Field 3	7	15		6	14	
	Field 4	6	10		3	13	
	Field 5	5	16		6	13	
	Field 6	10	18		2	17	
	Field 7	11	23		7	23	
	Field 8	7	27		4	31	
	Field 9	9	14		9	14	
	Field 10	12	27		6	24	
	Total	78	170	248	54	166	220
4425/09	Field 1	2	15		1	13	
	Field 2	2	19		3	24	
	Field 3	2	14		2	14	
	Field 4	2	13		3	13	
	Field 5	2	13		0	13	
	Field 6	6	13		2	14	
	Field 7	2	10		1	7	
	Field 8	2	6		1	7	
	Field 9	0	5		1	8	
	Field 10	0	3		0	4	
	Total	20	111	131	14	117	131
2063/09	Field 1	3	14		0	11	
	Field 2	0	15		1	11	
	Field 3	2	17		1	16	
	Field 4	6	16		6	18	
	Field 5	0	21		0	21	
	Field 6	2	21		1	24	
	Field 7	3	11		2	11	
	Field 8	3	12		3	9	
	Field 9	2	7		3	8	
	Field 10	3	7		2	6	
	Total	24	141	165	19	135	154
5008/08	Field 1	1	28				
	Field 2	3	28				
	Field 3	7	41				
	Field 4	6	26				
	Field 5	0	25				
	Field 6	7	28				
	Field 7	11	19				
	Field 8	8	25				
	Field 9	10	39				
	Field 10	7	43				
	Total	60	302	362			
17/06	Field 1	9	21		8	23	
	Field 2	7	22		5	27	
	Field 3	11	32		8	32	
	Field 4	5	25		4	26	
	Field 5	1	19		4	28	
	Field 6	2	22		2	26	
	Field 7	5	24		3	19	
	Field 8	0	15		0	18	
	Field 9	2	18		0	19	
	Field 10	0	14		1	13	
	Total	42	212	254	35	231	266

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
801/03	Field 1	0	21		0	26	
	Field 2	1	15		0	15	
	Field 3	1	15		1	14	
	Field 4	0	12		2	16	
	Field 5	0	19		0	13	
	Field 6	0	13		1	26	
	Field 7	1	18		0	12	
	Field 8	0	45		1	16	
	Field 9	0	17		1	32	
	Field 10	0	20		0	29	
	Total	3	195	198	6	199	205
63/02	Field 1	0	2		0	3	
	Field 2	0	1		0	0	
	Field 3	1	3		0	3	
	Field 4	0	3		0	4	
	Field 5	0	0		0	1	
	Field 6	0	4		0	2	
	Field 7	0	2		0	1	
	Field 8	0	6		1	3	
	Field 9	0	14		0	4	
	Field 10	0	5		0	0	
	Total	1	40	41	1	21	22
243/94	Field 1	0	2		0	5	
	Field 2	0	3		0	3	
	Field 3	1	1		0	4	
	Field 4	0	1		0	2	
	Field 5	0	0		1	3	
	Field 6	0	2		0	1	
	Field 7	2	0		0	5	
	Field 8	0	0		2	0	
	Field 9	0	0		0	2	
	Field 10	0	0		0	1	
	Total	3	9	12	3	26	29
584/92	Field 1	3	0				
	Field 2	0	1				
	Field 3	3	0				
	Field 4	0	0				
	Field 5	0	0				
	Field 6	0	0				
	Field 7	1	0				
	Field 8	0	0				
	Field 9	1	0				
	Field 10	1	0				
	Total	9	1	10			
299/92	Field 1	0	2				
	Field 2	0	13				
	Field 3	0	0				
	Field 4	0	24				
	Field 5	0	8				
	Field 6	0	0				
	Field 7	0	1				
	Field 8	0	6				
	Field 9	0	1				
	Field 10	0	3				
	Total	0	58	58			

Slide no.	Field Margin	Basal	Suprabasal	Total	Recount Basal	Recount Suprabasal	Recount Total
556/92	Field 1	0	22				
	Field 2	0	27				
	Field 3	3	35				
	Field 4	3	36				
	Field 5	7	39				
	Field 6	2	21				
	Field 7	0	25				
	Field 8	0	34				
	Field 9	0	26				
	Field 10	1	16				
	Total	16	281	297			
641/87	Field 1	10	17				
	Field 2	6	13				
	Field 3	3	8				
	Field 4	1	12				
	Field 5	4	8				
	Field 6	2	14				
	Field 7	4	7				
	Field 8	7	12				
	Field 9	4	19				
	Field 10	0	10				
	Total	41	120	161			
269/81	Field 1	2	5				
	Field 2	2	6				
	Field 3	2	5				
	Field 4	3	4				
	Field 5	2	8				
	Field 6	4	0				
	Field 7	3	4				
	Field 8	2	0				
	Field 9	0	1				
	Field 10	1	1				
	Total	21	34	55			
307/78	Field 1	3	42				
	Field 2	0	28				
	Field 3	0	36				
	Field 4	0	43				
	Field 5	1	25				
	Field 6	0	18				
	Field 7	2	14				
	Field 8	2	21				
	Field 9	4	16				
	Field 10	13	8				
	Total	25	251	276			
297/75	Field 1	0	31				
	Field 2	1	32				
	Field 3	0	24				
	Field 4	1	23				
	Field 5	0	18				
	Field 6	1	29				
	Field 7	0	28				
	Field 8	2	26				
	Field 9	4	25				
	Field 10	3	25				
	Total	12	261	273			

CHAPTER 8

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