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A treatise presented for the degree of Masters of Dental Science (Oral Medicine/Oral Pathology) of the University of Sydney.

2006

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DECLARATION

I certify that this treatise does not incorporate without acknowledgement any material previously submitted for a Degree of Diploma in any University; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference has been made in the text.

Sue-Ching Yeoh
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ABSTRACT
**Purpose:**
Oral squamous cell carcinoma (OSCC) represents the most frequent malignancy of the oral cavity. The overall 5 year survival rate of oral cancer patients has not improved significantly over the past 2 decades, despite the significant advances with multimodality treatment. The identification of genetic alterations associated with OSCC has been the basis of understanding of how normal cells become malignant. The aim of this study was to identify genes associated with oral carcinogenesis by comparing the relative gene expression of OSCC with normal oral epithelium using cDNA microarray.

**Methods:**
Two OSCC cell lines were grown in culture, and a normal oral keratinocyte cell line was established from a primary explant of normal human gingiva. Total RNA was isolated, and converted to cDNA by reverse transcription. Samples were labelled with fluorescent dyes, and hybridised onto an 8K human genome array (AGRF). Arrays were scanned and the data analysed.

**Results:**
Two genes with anti-apoptotic functions were found to be significantly over expressed in both OSCC cell lines. Nine genes were found to be significantly under expressed in the OSCC cell lines. These are known to be active in cell cycle arrest, the negative regulation of cell proliferation, and the negative regulation of cell progression throughout the cell cycle. These findings are consistent with the current model of carcinogenesis.
Conclusion:
cDNA microarray analysis is a useful technique that allows the comparison of a large number of previously identified genes simultaneously, showing the relative over- or under-expression of genes in malignant cells compared with normal cells. Gene expression profiling by microarray shows promise in being able to better predict the prognosis of patients with OSCC, to identify genes that could serve as diagnostic biomarkers and as potential targets for biological therapy.
CHAPTER 1

Oral Squamous Cell Carcinoma
1.1 Oral Squamous Cell Carcinoma (OSCC) – definition

The World Health Organisation defines squamous cell carcinoma (SCC) as “an invasive epithelial neoplasm with varying degrees of squamous differentiation and a propensity to early and extensive lymph node metastases, occurring predominantly in alcohol and tobacco-using adults in the 5th and 6th decades of life” (International Classification of Disease (ICD)-10 code 8070/2) (WHO 2005). According to the World Health Organisation Classification of Tumours Pathology and Genetics, Head and Neck Tumours, oral squamous cell carcinomas (OSCC) are those that arise from within the boundaries of the oral cavity, that is, the mucosa that lies between and including the lips and the palatoglossal folds (WHO 2005).

1.2 Epidemiology

SCC is the most frequent malignancy of the oral cavity, representing over 90% of all oral malignant neoplasms (Franceschi 2000).

Worldwide, 266 672 cases of OSCC were reported (ICD-10) in 2002, representing approximately 5% of all cancers in men and 2% in women (Parkin 2003; Parkin 2003). The region in the world with the highest incidence of OSCC is Melanesia (31.5 per 100,000 per annum (p.a.) in men and 20.2 per 100,000 p.a. in women).
Rates in men are also high in Western Europe (11.3 per 100,000 p.a.), Southern Europe (9.2 per 100,000 p.a.), South-East Asia (12.7 per 100,000 p.a.), Southern Africa (11.1 per 100,000 p.a.), and Australia/New Zealand (10.2 per 100,000 p.a.). In females, incidence is relatively high in Southern Asia (8.3 per 100,000 p.a.) compared with the rest of the world.

These patterns reflect prevalence of specific risk factors, such as tobacco and/or alcohol use in Western Europe, Southern Europe, and Southern Africa, and the chewing of areca nut (areca catechu) products such as betel quid in Melanesia and Southern to Central Asia.

For example, in India and some other Asian countries, oral cancer is the most common malignancy, accounting for more than 50% of all cases of cancer. This increased incidence has been attributed to the widespread use of traditional betel quid or paan, that is, the consumption of products of the areca catechu palm tree often in combination with tobacco (Sankaranarayanan 1990; Franceschi 2000).

In general, the worldwide incidence is higher in males compared with females (ratio of 2:1). This ratio was previously 3:1 in the 1980s, with the change attributed to the increased proportion of women smoking (Regezi 2003). In contrast, in certain countries, in particular, India, the highest rates of OSCC are found in women who are frequent users of chewing tobacco (Franceschi 2000).
OSCC has traditionally been a disease affecting the over fifty age group, however, in recent years, there has been an increased incidence in younger patients, with seemingly none of the previously identified or traditional risk factors (Koch 1999; Mackenzie 2000; Iype 2001; Annertz 2002; Llewellyn 2004).

Oral cancer accounted for 145 500 deaths annually in the world in 2002, two-thirds of which occurred in developing countries. (IARC 2003) The mortality rate of oral cancer is related to the stage of disease at the time of diagnosis. The five-year survival rate by stage is approximately 77% for localised disease (Stages I and II), 41% for disease with regional lymph node metastases (Stage III), and 9% if distant metastases (below the clavicles) are present (Stage IV) (Silverman 1984).

1.3 Aetiology/risk factors

The aetiology of OSCC is complex and incompletely understood. A number of exogenous risk factors have been identified, but globally, the most important are tobacco and/or alcohol consumption. Betel quid/areca nut use is also a significant risk factor for OSCC especially in many Asian and Indian Subcontinent communities. However, dietary factors and infection by specific microorganisms such as Candida albicans, Human Papilloma Virus (HPV) and Herpes Simplex Virus (HSV) have also been implicated in OSCC (Daftary 1991). Endogenous risk factors include genetic factors and increasing age.
1.3.1 Tobacco:

Tobacco is defined as the products made from the leaves of various plants of the genus Nicotiana, and is in all forms, able to initiate as well as promote OSCC formation (Farlax 2006).

All forms of tobacco use have been strongly implicated in causing oral cancer, and have also been strongly linked to other malignancies of the upper digestive and respiratory tract (IARC 2004). Pipe and cigar smoking are seemingly more carcinogenic than cigarette smoking. Reverse smoking, when the lighted end of the cigarette is held inside the mouth, is also associated with a significantly higher risk relative to conventional smoking techniques, for the development of OSCC (IARC 2003).

Approximately 300 carcinogens and pro-carcinogens are found in tobacco smoke and its derivatives, water soluble compounds, which contaminates the saliva. The major carcinogens in cigarette smoke are the polycyclic aromatic hydrocarbons, nitrosamines, aldehydes and aromatic amines.

The chronic use of smokeless tobacco, in the form of either inhaled or chewed snuff (non-flue cured ground and finely cut tobacco) or chewing tobacco (loose leaf tobacco), mainly used in Scandinavian countries and North America, is also believed to increase the risk of oral cancer, but these forms of tobacco use are thought to be less carcinogenic than if the tobacco is smoked (Johnson 2001).
The traditional tobacco products used in the Middle East and Sudan are powdered, fermented and then mixed with sodium bicarbonate. These contain a very high level of tobacco-specific nitrosamines and so considered highly carcinogenic (Idris 1995).

1.3.2 Alcohol:

Alcohol is defined as a colourless volatile flammable liquid, (chemical formula C₂H₅OH), synthesized or obtained by the fermentation of sugars and starches and widely used, either pure or denatured, as a solvent and in drugs, cleaning solutions, explosives, and intoxicating beverages (Farlax 2006).

Alcoholic beverages contain both carcinogens and procarcinogens including ethanol, nitrosamines and urethane. These agents are also known to increase the risk of developing OSCC (Harty 1997).

Alcohol has both an independent (La Vecchia 1999) and a synergistic role with tobacco in oral carcinogenesis (Rothman 1972; Blot 1988). Approximately 75% of the disease burden of oral cancer in Europe, the Americas and Japan can be attributed to the combination of alcohol and tobacco usage (Blot 1988; Blot 1996).

Alcohol is thought to increase the risk of OSCC via several mechanisms. Firstly, ethanol acts as a solvent, helping other carcinogens (in particular, those present in tobacco) to pass through the cell membranes of the lining mucosa of the oral cavity and upper aerodigestive tract. Secondly, liver metabolism is enhanced by ethanol and therefore
increases the action of xenobiotic metabolising enzymes which can activate carcinogens. Thirdly, in addition to causing local irritation, ethanol may interfere with the epithelial cell’s ability to repair alkylated DNA (Ogden 1998; Wright 1998).

Molecular studies suggest that the carcinogenic risks associated with alcohol may also be related to the effects of a specific alcohol metabolite, acetaldehyde, through the alteration of keratinocyte gene expression (Timmons 2002).

1.3.3 Betel quid/areca nut:
Betel quid or paan usage (tobacco mixed with areca nut, slaked lime (calcium hydroxide) and spices) in the Indian subcontinent, parts of South-East Asia, China and Taiwan and in their emigrant communities, is a major cause of oral cancer. This has been especially noticeable in Australia, where emigrants from these countries import and continue to use areca nut products (Cox 2000). Areca nut has been declared a known human carcinogen (IARC 2003). The use of betel quid in India accounts for approximately 50% of oral and oropharyngeal cancers in men, and over 90% in women (Balaram 2002).

The use of betel quid containing areca nut and tobacco increases the relative risk of oral cancer by 8-15 times compared with a 1 to 4 fold increase in relative risk associated with the use of quid without tobacco (Murti 1995).

Areca nut chewing is also the most important aetiological factor in oral submucous fibrosis, a proven potentially malignant condition (Murti 1985).
1.3.4 Dietary factors:

Oral cancer has been associated with an inadequate intake of key micronutrients, such as iron (Notani 1987). This is especially evident in patients affected by Plummer-Vinson syndrome (also known as Patterson-Kelly syndrome or sideropenic dysphagia) which typically affects middle-aged women, manifesting as iron-deficiency anaemia, a painful red tongue, mucosal atrophy, and oesophageal webbing causing dysphagia (Rashid 1999). These patients are at a higher risk of developing oral cancer (Watts 1961).

Fresh vegetables and fruits rich in Vitamin A, C, and E have been described as "protective" dietary agents, while meat and red chili powder may be risk factors (De Stefani 1998; De Stefani 1998; Negri 2000; Tavani 2001; Marchioni 2002).

1.3.5 Human Papilloma Virus (HPV):

A number of studies have implicated viruses in oral carcinogenesis (Gillison 2001). HPV Types 16 and 18 DNA have been shown to be present in some tumours, (Niv 2000) particularly basaloid SCC tumours of the palatine tonsils and base of tongue, and up to 50% of tonsillar and oropharyngeal SCC (Watts 1991; Scully 1992; Ostwald 1994). HPV E6 and E7 oncoproteins bind to and induce degradation of the tumour suppressor protein p53, leading to acceleration of the cell cycle and compromised DNA repair (Gopalakrishnan 1997).
1.3.6 Human Herpes Viruses (HHVs):
Members of the human herpes virus family may also be involved in oral carcinogenesis. Herpes simplex virus (HSV) may interact synergistically with other risk factors to cause malignant transformation (Scully 1993). HSV 2 interacts in vitro with oncogenic types of HPV to induce tumours. HSV 1 DNA has been detected in biopsy specimens of oral carcinoma (Eglin 1983). However, HSV DNA can also be found in normal oral mucosa, making it difficult to establish a causal relationship between the virus and oral cancer (Cox 1993). HHV 6 DNA also has been detected in some OSCC, however the relevance of this is still unclear (Scully 1993; Flaitz 1998).

1.3.7 Candida:
Chronic hyperplastic candidiasis is classified as a potentially malignant lesion (Gupta 1989; Sitheeque 2003). Candida albicans has also been shown to produce a carcinogen, N-nitrosobenzylmethyamine (Krogh 1987). However, there is a lack of reliable large scale data to indicate the true rate of malignant transformation of lesions infected with Candida. Coinfection of a previously altered oral mucosal surface may explain this observation (Sitheeque 2003).

1.3.8 Syphilis:
Syphilitic leukoplakia, developing in late-stage disease (tertiary syphilis), also has a markedly increased malignant potential, but is no longer a significant risk factor in Western countries due to the decreased incidence of syphilis with the advent of antibiotics (Dickenson 1995).
1.3.9 Immunosuppression:
Patients who have a compromised immune system appear to be at a higher risk of developing OSCC. This increased risk has been documented for haematopoietic stem cell transplant as well as solid organ transplant recipients, who are on long term immunosuppressant medication (Hernandez 2003; Demarosi 2005).

1.3.10 Genetic factors:
Not all tobacco and alcohol consumers develop OSCC, and so the question arises of the role of inherited or genetic factors in the development of oral cancer. Genetic differences in the ability of an individual to negate the effects of carcinogenic agents may, or may not predispose an individual towards the development of cancers. There is conflicting evidence about the relative importance of genetic factors. Some epidemiological studies show that a positive family history is a risk factor in oral cancer (Copper 1995; Foulkes 1995; Foulkes 1996) with 3.5 to 3.79 increase in the relative risk of individuals with a positive family history for head and neck cancer developing oral cancer themselves. However, in a study of 44 000 pairs of concordant twins, no significant correlation for cancer of the lip, oral cavity or pharynx was found (Lichtenstein 2000).

At least one investigation has shown positive p53 (tumour suppressor gene) reactivity for all synchronous (occurring within 6 months of the primary OSCC) tumours of the mouth, oesophagus, and stomach, indicating a common predisposition toward malignant development (Partridge 1999).
1.4 Potentially malignant lesions/conditions:

A potentially malignant lesion is defined as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart (WHO 2005).

A potentially malignant condition is defined as a generalised clinical state associated with a significantly increased risk of developing cancer (WHO 2005). (Table 1.1)

Approximately 6% of oral leukoplakias progress to carcinoma (Silverman 1984).

Some people have a tendency toward the development of multiple mucosal cancers, sometimes called “field cancerisation”. This may reflect diffuse exposure to local carcinogens, a process that increases the malignant transformation potential of all exposed epithelial cells (Cianfriglia 1999; Partridge 1999, Ha 2003; Whipple 2004).
TABLE 1.1

Potentially malignant lesions and potentially malignant condition (WHO 2005).

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<thead>
<tr>
<th>Potentially Malignant Lesions</th>
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1.5 Second tumours

Second tumours develop in 10-35% of patients with a first primary OSCC (Black 1983; Cianfriglia 1999; Fukuzawa 1999; van Oilen 2000). The risk of a second primary lesion in the head and neck region or upper airways is approximately 5% per year for the first 7 years following the initial tumour (Regezi 2003).

In patients with more than one upper aerodigestive tract malignancy, approximately one third of the tumours arise simultaneously. Of the rest, the second lesion usually develops within 3 years after the initial cancer (Black 1983).

1.6 Pathogenesis

Oral carcinogenesis is associated with cumulative gene alterations. It involves progressive genetic damage and alteration of normal cell regulation, loss of genetic heterogeneity, activation of oncogenes and the inactivation of tumour suppressor genes (Hanahan 2000; Partridge 2000; Williams 2000). These alterations influence basic cell regulatory pathways such as cell division, cellular differentiation and apoptosis (Partridge. M. 1998; Partridge. M. 1999). In neoplasia, cell proliferation is excessive and autonomous, uncoordinated with the normal tissues with ongoing cell division, despite DNA damage, due to loss of cell cycle check-points (Nagpal 2003).
1.6.1 Genomic instability:
Genomic instability creates the tendency and vulnerability of the genome to attain alterations, including loss of heterozygosity and microsatellite instability. Cytogenetic and molecular studies in OSCC have shown genetic alterations in the key chromosomes responsible for repairing DNA damage caused by carcinogens, and other mutagens.

Progression to malignant transformation has been associated with loss of heterozygosity in chromosomes 3p, 9p, 13p, and 17p and microsatellite instability, that is the presence of additional repeated segments, microsatellite alleles in tumor cells. This results in inherent susceptibility of these affected parts of the cell genome to alteration, a susceptibility that is increased by mutations in the genes that code for DNA repair mechanisms, that normally operated to correct these errors (3p, 6p, 7q, 11p and 11q) (Scully 2000; Friedlander 2001).

Chromosomal structural alterations are associated with dysplasia specifically: (9q21, 3p21, 17p13), carcinoma in situ (11q13, 13q21, 14q31) and invasive carcinoma (4q26-28, 6p, 8p, 8q) (Califano 1996, Gollin 2001).

1.6.2 Proto-oncogenes and oncogenes:
A proto-oncogene is a normal gene that codes for proteins that regulates cell growth, regulation and differentiation. Modification, for example, by mutation of a proto-oncogene, results in an oncogene. Expression or mutation of these genes will result in uncontrolled cell growth and tumourigenesis. Oncogenes which may be involved in
cancer development include the genes that code for epidermal growth factor receptor (EGFR) and tumour growth factor alpha (TGF-α), (Todd 1989; Todd 1999; Grandis 2000), and specifically c-myc/N-myc, (Spandidos 1985; Facchini 1998) the K-ras/H-ras gene family, (Todd 1997) PRAD-1/cyclin D1, (Jares 1995) hst-1/int-2, (Somers 1992) and Stat-3 (Nagpal 2002).

1.6.3 Tumour suppressor genes:

Tumour suppressor genes negatively control cell growth, determine cell cycle arrest, and induce apoptosis (Lee 1992). They are responsible for maintaining genetic fidelity. Inactivation of tumour suppressor genes can occur by point mutations, deletions and rearrangement or methylation in both alleles, and is considered to be a seminal event in the development of malignancy (Weinberg 1991; Yokota 1993). The most widely studied tumour suppressor gene is p53, known to be involved in the maintenance of genetic stability and has a role in the control of cell cycle progression, differentiation, DNA repair and apoptosis. Other important tumour suppressor genes are pRb and p16. Lack of pRb and p16 expression has been observed in 66% and 63% of OSCC respectively (Pande 1998).

1.6.4 Telomerase activity:

Telomeres, located at the tips of chromosomes, function to protect the chromosome against degradation. The telomeres normally become progressively shorter with each cell division, a process which ultimately will lead normal cells to cell death. Telomere length is monitored by p53 which induces cell-cycle arrest or apoptosis when a certain amount
of shortening has occurred. The ribonucleoprotein enzyme telomerase maintains the activity of the telomeres by extending the telomeric sequences at the chromosome ends. Mutations in the gene expressing this enzyme may result in the increased or persisting telomerase activity which is a common feature of neoplasia (Pardue 1999). In a study by Mao et al, altered telomerase activity was found in 100% of OSCC cell lines, 90% of invasive cancers and 100% of dysplastic lesions (Mao 1996).

1.7 Clinical features

SCC can develop in any part of the oral cavity. The sites most commonly affected vary depending on which aetiological factors are present. The most common site for intra-oral SCC occurring in Western countries is the posterior and latero-ventral surfaces of the tongue, accounting for over 50% of OSCC (Anneroth 1986; Atula 1996; Friedlander 1998; Hart 1999; Franceschi 2000; Annertz 2002). The floor of the mouth is the second most affected site (35%), followed in descending order of frequency by the retromolar trigone, soft palate, gingiva, buccal mucosa, labial mucosa, and the hard palate (Regezi 2003).

The clinical appearance of OSCC can take many forms. Early lesions are usually asymptomatic, with variable clinical features, ranging from leukoplakia, a small exophytic growth which in the early stages may show no ulceration or erythema, to a small indolent ulcer, or an area of erythroplakia. Suspicious features are persistent
ulceration, induration, and fixation of affected tissue to underlying structures, and destruction of adjacent bony structures. Lymph node involvement may occur early, but enlarged regional nodes may be reactive and do not necessarily indicate metastatic spread.

An advanced lesion may present as a broad based, exophytic mass with a rough, nodular, verruciform, haemorrhagic or necrotic surface, or as a deeply destructive and crater-like ulcer with raised, rolled everted edges due to invasion of the tumour downwards and laterally under the adjacent mucosa. Infiltration of muscle may result in impaired function. Bone invasion may be detected on radiographs and may be noted clinically by mobility of the teeth. Perineural invasion may result in sensory or motor deficit in the areas supplied by the affected nerves.

1.8 Patterns of spread

There are several potential patterns of spread of OSCC, namely local invasion of adjacent structures; metastasis via lymphatic drainage to regional lymph nodes; perineural invasion; and less commonly, haematogenous spread to distant sites.
1.8.1 Direct local spread:

Local invasion is the most common pattern of spread from OSCC, with invasion of adjacent tissues including muscle and bone, by direct extension and growth, with infiltration and destruction of the surrounding tissues.

The local spread of OSCC is predicted by local anatomical features, and follows the “path of least resistance”. SCC of the floor of mouth and palate tend to initially spread superficially and then invade the deeper tissues. Tumour involving the lateral margin of tongue generally tends to invade deeply, as opposed to lateral spread (WHO 2005).

Adjacent bone involvement is more common in the mandible than the maxilla, usually occurring via the periodontal ligament if the patient is dentate. In edentulous areas the tumour invades directly through the crest of the alveolar ridge progressing through the marrow spaces between the trabeculae of cancellous bone (McGregor 1988, McGregor 1989).

Extension into the mandible through foramina (for example, the mental foramen from lip cancer), can occur, but is very uncommon (McGregor 1988).

1.8.2 Lymphatic spread:

Regional/local lymph node involvement confers a worse prognosis. Spread from the primary site to adjacent lymph nodes is most commonly caused by embolism, rather than by direct permeation of tumour cells via the lymphatic vessels from the adjacent lesions.
Once tumour is present in the neck, further spread between nodes can be embolic or by permeation (WHO 2005).

The lymphatic spread from different sites of head and neck SCC are relatively predictable (Mukherji 2001). Cervical lymph nodes may be anatomically divided into 7 groups: submental and submandibular nodes (Level I), upper jugular nodes (Level II), middle jugular nodes (Level III), lower jugular nodes (Level IV), posterior triangle nodes (Level V), upper visceral nodes (Level VI), and superior mediastinal nodes (Level VII). Other groups of lymph nodes are the supraclavicular and retropharyngeal nodes. (Figure 1.1)

Levels I, II and III, are considered at highest risk for metastasis from OSCC and to a lesser extent Level IV. Level II lymph nodes are most frequently involved, however tumours can involve the Level III or IV lymph nodes, with or without involvement of Level I, giving rise to the concept of skip metastases (Woolgar 1997; Woolgar 2003). This pattern of predictable spread is useful in treatment planning for both surgery, including neck dissection, and determining the extent of the field for radiotherapy.

Commonly, tumours arising left or right of the midline will involve ipsilateral lymph nodes. Those tumours that involve the midline (most commonly the posterior tongue or soft palate) may affect bilateral cervical lymph nodes. Extracapsular spread of tumour involving lymph nodes is associated with a poor prognosis (Woolgar 2003), and can only be determined histopathologically, subsequent to neck dissection.
FIGURE 1.1

Cervical lymph node groups. Taken from http://www.thymic.org (accessed 10th September 2006)
Several studies have attempted to predict lymphatic spread from features of the primary tumour (Woolgar 1995; Genden 2003). Primary tumour size and site have been found to be important (Platz 1985), whereas tumour differentiation has not been shown to be a reliable predictor (Kearsley 1993). A non-cohesive invasive front has been associated with an increased likelihood of metastasis (Kirita 1994). Perineural spread at the invasive front, lympho-vascular invasion and tumour thickness are other factors which are associated with an increased risk of metastasis (Ravasz 1993).

A tumour thickness of 5mm or greater when measured from the deepest point of invasion to the presumed original surface level, ignoring exophytic growths, on assessing the original surface level in ulcerated tumours, has been associated with an increased risk of nodal involvement (Charoenrat 2003).

1.8.4 Haematogenous spread:

Distant metastasis as a result of haematogenous spread is uncommon and relatively late occurrence in OSCC. The lung is the most common organ of involvement, and less frequently the liver (Sosaki 2000). The likelihood of haematogenous spread is increased in patients who have cervical lymph node involvement at multiple levels, which suggests that the route of entry of tumours into the circulation is most often via the large veins in the neck (WHO 2005).
1.8.5 Perineural spread:
Perineural invasion tends to involve branches of either the Trigeminal or Facial Cranial Nerves. The likelihood of this occurring is dependent on the original site and extent of the primary tumour and its proximity to the nerves.

1.9 Investigations

Physical examination, including visual inspection and manual palpation of both the oral cavity primary tumour site and neck to identify lymph node involvement is critical. Direct pharyngolaryngoscopy may be performed. Cranial nerve examination may be indicated if perineural involvement is suspected.

1.9.1 Diagnostic tests
The diagnosis is confirmed by biopsy, taken from the clinically most suspicious area. This procedure should provide material for routine histopathological examination. At this time, part of the fresh tissue specimen may be used for cDNA microarray experiments. This technique can provide further information of the tumour characteristics and susceptibility to the different treatment modalities. Fine needle aspirate biopsy of enlarged cervical lymph nodes may be helpful if no obvious primary tumour is observed. The search for an unknown primary tumour may include direct pharyngolaryngoscopy with biopsy of high-risk sites such as the base of tongue, nasopharynx, and usually a tonsillectomy, in combination with various imaging modalities.
1.9.2 Histopathology

OSCC is an epithelial neoplasm generally beginning as a focal clonal overgrowth of altered stem cells near the basement membrane, expanding upward and laterally, replacing the normal epithelium (Regezi 2003). In many cases, the neoplastic process begins with normal epithelium progressing through hyperplasia to dysplasia, to carcinoma in situ and invasive carcinoma (Nagpal 2003). However, some OSCC’s arise without a precursor lesion.

Epithelial dysplasia (dys- meaning “bad or abnormal”, plasia – meaning “growth”) is a term is used to describe a variety of architectural and cytological changes in the epithelium that may indicate an increased risk of malignant transformation (Crissman 1989).

The development of OSCC may be preceded by other lesions in the oral cavity. These lesions may show cellular changes that indicate the possible subsequent development of malignancy. The individual cellular changes are often termed “atypia”, and the general disturbance of the epithelium termed “dysplasia” (Kramer 1978). The diagnosis of epithelial dysplasia is, in itself subjective. Studies have shown poor intra- and inter-examiner reliability when grading dysplastic lesions (Pindborg 1985; Abbey 1995; Karabulut 1995). Furthermore, dysplasia is an unreliable predictor of malignant transformation, as some mildly dysplastic lesions will progress, and some severely dysplastic lesions appear not to progress to OSCC. In addition, OSCC has been reported to arise de novo, with no precursor dysplastic lesion (Lumerman 1995).
The grading of dysplasia does not yield information about the aggressiveness or the susceptibility to certain modalities of treatment of the lesion. Techniques such as cDNA microarray may be able to provide such information, contributing to more comprehensive and predictable patient care.

In 1997, the World Health Organisation defined squamous epithelial dysplasia as “a precancerous lesion of stratified squamous epithelium characterized by cellular atypia and loss of normal maturation and stratification short of carcinoma in situ” (WHO 1997). In broad terms, there is a disturbance in the normal growth, proliferation, maturation and organisation of the cells. The more prominent or numerous these changes are, the more severe the grade of dysplasia. (Table 1.2)
TABLE 1.2

Criteria used for diagnosing dysplasia (WHO 2005):

<table>
<thead>
<tr>
<th>Architectural Features</th>
<th>Cytological Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irregular epithelial stratification</td>
<td>Abnormal variation in nuclear size (anisonucleosis)</td>
</tr>
<tr>
<td>Loss of basal cell polarity</td>
<td>Abnormal variation in nuclear shape (nuclear pleomorphism)</td>
</tr>
<tr>
<td>Drop shaped rete ridges</td>
<td>Abnormal variation in cell size (anisocytosis)</td>
</tr>
<tr>
<td>Increased numbers of mitotic figures</td>
<td>Abnormal variation in cell shape (cellular pleomorphism)</td>
</tr>
<tr>
<td>Abnormally superficial mitotic figures</td>
<td>Increased nuclear-cytoplasm ratio</td>
</tr>
<tr>
<td>Premature keratinisation in single cells</td>
<td>Increased nuclear size</td>
</tr>
<tr>
<td>Keratin pearls in rete ridges</td>
<td>Atypical mitotic figures</td>
</tr>
<tr>
<td></td>
<td>Increased number and size of nucleoli</td>
</tr>
<tr>
<td></td>
<td>Nuclear hyperchromatism</td>
</tr>
</tbody>
</table>
SCC arises from dysplastic surface epithelium and is characterized by invasive islands and cords of malignant squamous epithelial cells. Invasion is represented by irregular extension of lesional epithelium through the basement membrane and into subepithelial connective tissue. Individual squamous cells and sheets or islands of cells are seen to be existing and persisting independently, within the connective tissues, without attachment to the surface epithelium. Invading cells and cell masses may extend deeply into underlying adipose tissue, muscle or bone, destroying the original tissue as they progress. Lesional cells may surround and destroy blood vessels, and may invade into the lumina of veins or lymphatic vessels (Neville 2002; WHO 2005).

A strong inflammatory or immune response to invading epithelium, and focal areas of necrosis may be present. The lesional epithelium may induce the formation of new small blood vessels (angiogenesis) and, occasionally, dense fibrosis (desmoplasia) (Neville 2002).

Grading involves the histopathologic evaluation of the degree to which the tumour resembles its parent tissue, in both morphology as well as function. Lesions are usually graded on a three-point (Grade I-III) scale. The less differentiated tumours receive the higher numerals. The tumour grade is often related to its biological behaviour. A tumour that is mature enough to closely resemble its tissue of origin seems to grow at a slightly slower pace and to metastasise later in its course. Such a tumour is called low-grade, Grade I, or well-differentiated squamous cell carcinoma. In contrast, a tumour with much cellular and nuclear pleomorphism and with little or no keratin production, and the cells
may appear so immature that it becomes difficult to identify the tissue of origin. These tumours often enlarge rapidly, metastasise early, and are termed high-grade, Grade III, poorly differentiated, or anaplastic. A tumour with a microscopic appearance somewhere between these two extremes is labelled "moderately differentiated" carcinoma (Anneroth 1986).

There are 4 characteristics of the deep invasive tumour margin used to formally assess histological grading: (1) degree of keratinisation (graded as highly, moderately, minimally keratinised, or no keratinisation); (2) degree of nuclear polymorphism (assessed as mild, moderate, abundant or extreme); (3) pattern of invasion (ie. pushing, well-defined infiltration borders, infiltrating solid cords and/or strands, small groups of cords of infiltrating cells, marked and widespread cellular dissociation in small groups and/or by a single cell) and; (4) degree of lymphoplasmacytic infiltration (graded as marked, moderate, slight or none) (Byrne 1992).

The grading of SCC is a subjective process, depending on the area of the tumour sampled and the individual pathologist’s criteria for evaluation. Clinical staging seems to correlate much better with the prognosis than microscopic grading (Anneroth 1986).

The diagnosis of SCC almost always is made with routine light microscopy. Special studies that use monoclonal antibodies directed against cytokeratins may be used to distinguish high-grade or poorly differentiated SCC from other malignancies, as well as identify the cell of origin in lymph node metastases.
1.9.3 Imaging

Intra-oral plain radiographs, as well as an orthopantomogram (OPG), may be used to identify underlying bone involvement. Three-dimensional imaging with computer tomography (CT) and magnetic resonance imaging (MRI) is used to supplement the clinical evaluation and staging of the primary tumour and regional lymph nodes. CT scan or MRI give a clearer indication about the local extent of the disease and also help to identify lymph node metastases. CT scanning is useful in evaluating cortical bone involvement. MRI is more informative for the evaluation of soft tissue and neurovascular bundle involvement. Distant metastasis from oral and oropharyngeal cancer is uncommon at presentation. Positron emission tomography (PET) is a relatively recently developed imaging modality that allows for the improved detection of metastases. In addition, routine radiograph of the chest will screen for lung metastases, as well as possible synchronous lung carcinoma, for which the typical OSCC patient demographic group, that is middle aged and elderly men who are long term smokers and drinkers, are also at risk of developing (Dombi 2001; Merkx 2002).

1.10 Staging – TNM classification

Tumour size and the extent of metastatic spread of cancer are the best indicators of the patient’s prognosis. These parameters can be quantified to “stage” the disease. The most popular staging protocol is the tumour-node-metastasis (TNM) system (UICC 2002).
TNM classifications are used to stage most cancers, the protocol individualized to each specific anatomical site as well as tumour type. This classification is based on three main clinical features:

1. $T =$ size of the primary tumour, in centimeters
2. $N =$ involvement of local lymph nodes
3. $M =$ distant metastasis

Once the three parameters are determined, they are tallied together to determine the appropriate stage (Table 1.3). A higher stage usually indicates a worse prognosis.
TABLE 1.3

TNM staging classification for lip and oral cavity tumours

T- Primary Tumour

<table>
<thead>
<tr>
<th>TX</th>
<th>Primary tumour cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour 2cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour more than 2 cm but not more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T4a</td>
<td>Lip – Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (chin or nose)</td>
</tr>
<tr>
<td>T4a</td>
<td>Oral cavity – Tumour invades though cortical bone, into deep/extrinsic muscle of tongue, maxillary sinus, or skin of face</td>
</tr>
<tr>
<td>T4b</td>
<td>Lip and oral cavity – Tumour invades masticator space, pterygoid plates, or skull base, or encases internal carotid artery</td>
</tr>
</tbody>
</table>

Note – Superficial erosion alone of bone/tooth socket by gingival primary is not sufficient to classify a tumour as T4.
N – Regional Lymph Nodes

<table>
<thead>
<tr>
<th>NX</th>
<th>Regional lymph nodes cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest diameter</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2c</td>
<td>Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in a lymph node more than 6 cm in greatest dimension</td>
</tr>
</tbody>
</table>

Note – midline nodes are considered ipsilateral nodes.

M – Distant Metastasis

<table>
<thead>
<tr>
<th>MX</th>
<th>Distant metastasis cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
1.11 Management

There are many treatment options for OSCC. Unfortunately, at present, there is very little way of predicting which tumours will respond best to which specific treatment modality. The use of microarray technology may improve this aspect of treatment planning by identifying the best therapeutic approach for each individual case.

Currently, treatment consisting of surgery alone is usually reserved for very small or early cancers. Surgery may involve resection of the primary tumour with a margin of clinically normal tissue and/or neck dissection of regional lymph nodes, if they are suspected to be involved. The type of neck dissection required depends on the number and location of the suspected metastatic nodes (Medina 1989; Shah 1995).

At the time of surgery, frozen tissue sections may be examined to check for oncologically clear margins as well as assess for perineural spread.

In most cases, surgery is followed by adjuvant radiotherapy to both the primary site as well as the involved neck. This serves to “sterilise” the tissue of any residual micrometastases when the surgical margins have been involved or close, or their presence in regional lymph nodes is suspected or confirmed by neck dissection. Surgery with adjuvant chemoradiation may also be an option in these cases (Fu 2000; Ghali 2000).
Radiotherapy as a stand-alone treatment is rare and usually reserved for those cases where surgery is unfeasible. This may be due to site factors (for example, when oncologically clear margins are difficult to obtain or surgery is too destructive and reconstruction too difficult), or tumour factors (for example, extent and size). In cases where the patient is deemed unfit for surgery due to concurrent medical comorbidities, radiotherapy alone may be considered (Horiot 1997; Fu 2000).

In recent years, concurrent chemoradiotherapy with platinum-based agents has been used in the treatment of advanced or inoperable cancers, with increasing success. Chemotherapy is useful, especially when the metastases are widespread, as it exerts its effects systemically and not just locally (Bourhis 1999; Cooper 2004; Altundag 2005).

In palliative cases, where the prognosis is poor due to the extent of the tumour and its metastases, minor surgery or low-dose radiotherapy/chemotherapy may be given to debulk the tumour and so to improve the quality of life.

1.12 Prognosis and predictive factors

The 5 year disease-free survival rate for intra-oral cancer is approximately 76% if there are no metastases present at the time of diagnosis (Stages I/II). Once metastases to the cervical lymph nodes occurs (Stage III), this rate drops to 41%, and to 9% when metastases are present below the level of the clavicles (Stage IV). In general, the greatest
incidence of disease-related mortality occurs within the first 5 years following diagnosis (Shiboski 2000; Silverman 2001).

Conventional treatment is associated with significant morbidity affecting speech, swallowing, and overall quality of life. Despite these interventions, recurrence of the disease is observed in approximately 50% of patients, either locally, regionally, or at a distant site with high rates of associated mortality (Belbin 2002). Tumour size and nodal status are the most significant prognostic factors (Platz 1985).

Histological grade correlates poorly with patient outcome, (Roland 1992; Kearsley 1993) with the value of grading improving when only the deeply invasive margins of the tumour are evaluated (Bryne 1989; Bryne 1992; Odell 1994). Tumours invading with a broad front or pushing borders have been found to be less aggressive than tumours that spread diffusely with strands or single cells (Shingaki 1988; Umeda 1992; Kirita 1994).

The main factors that adversely influence prognosis are two or more positive regional lymph nodes, extracapsular spread of nodal disease, or tumour involving the surgical resection margins (Laramore 1992). Increased tumour thickness and vascular invasion also suggest a worse prognosis. Increased density of leukocytes and blood vessels has been associated with a higher risk of progressive tumour growth, metastasis, and death in patients (Shintani 2004).
Despite recent advances in surgery, radiotherapy and chemotherapy for the treatment of OSCC, there has been very little improvement in the prognosis and disease free survival rates over the past 40 years (Todd 2002).

Molecular markers with unequivocal prognostic and/or predictive significance are starting to emerge. Studies using flow cytometry to assess DNA ploidy have demonstrated that this parameter is a significant predictor of survival for patients with OSCC (Hemmer 2006). DNA ploidy identified by flow cytometry has also been used to successfully monitor the cytotoxic effects of chemotherapy in vivo (Hemmer 1995). In addition, experimentation with other molecular biology techniques, such as cDNA microarray, will add to our understanding of the process of carcinogenesis, with the potential to develop more specific and effective therapeutic strategies.
CHAPTER 2

cDNA microarray
2.1 cDNA microarray – a brief overview

Utilisation of DNA microarrays has increased, mainly for research to identify genes related to disease and targets for clinical intervention. It has also been used as a tool for characterising gene expression patterns (Kuo 2004).

Complementary DNA (cDNA) microarray is a technique that allows the comparison of a large number of previously identified genes simultaneously, showing the relative over and/or under expression of genes in normal and diseased cells (Todd 2002). Microarray analysis is a powerful tool for extensively studying the molecular basis of interactions on a scale previously unattainable with conventional analysis methods (Tomioka 2006). In traditional biological research, genes were studied one at a time through a series of laborious and time-consuming experimental techniques. In contrast, cDNA microarray experiments are relatively straightforward, and results may be obtained within a few days (Kuo 2003). Studies demonstrate that gene detection by microarrays correlate with standard methods such as Northern blot hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR), and real-time polymerase chain reaction (real-time PCR) (Alevizos 2001; Sok 2003). These quantitative and qualitative aspects of microarray analysis can be exploited and applied to OSCC research.

The human genome is believed to contain some 40,000 genes. DNA sequences vary by approximately 0.1% between individuals and this variation is termed the polymorphism rate. When a gene is expressed, messenger RNA (mRNA) is transcribed from the gene’s
DNA sequence and serves as a template to guide the synthesis of a protein. As all cells in the body contain identical sets of genes, various tissues are differentiated based largely upon which genes are expressed. The mRNA transcript “mirrors” (biochemically) the corresponding DNA coding region, therefore the amount of mRNA in a cell or tissue is a direct measure of gene expression. With microarrays, the over and under expression of tens of thousands of genes can be measured simultaneously, and this measurement that can aid in the understanding of cellular genetic processes and how they malfunction in disease (Kuo 2004).

DNA microarrays consist of distinct DNA sequences called “probes”, which are tethered on immobile surface and then exposed to a “target”. The target is the pooled mRNA from a tissue sample of interest. Microarrays detect gene expression levels in parallel by measuring the hybridization of mRNA to several thousand probes immobilized on a glass surface, and this constitutes a reasonably sensitive, high-throughput method for detecting expression patterns (Kuo 2002).

The two most commonly used types of DNA microarrays are: (1) the photolithographic oligonucleotide microarrays and (2) the robotically spotted cDNA microarrays. Both use hybridization of labeled nucleic acid transcripts to measure gene expression. The two differ in their manufacturing, and methods of measuring gene expression.

Oligonucleotide microarrays were developed by Affymetrix (Santa Clara,CA; http://www.Affymetrix.com) utilizing a photolithographic technique to create high
density arrays of probes of single stranded DNA sequences of 25-base pair length (Lipshutz 1999). Each genechip contains 6 to 12,000 probe sets; each probe set represents a gene or expressed sequence tag. There are 16–20 pairs of probes for each gene. The probes are parts of sequences found in publicly available databases such as GenBank (http://www.ncbi.nlm.nih.gov) (Benson 2000) and Unigene (http://www.ncbi.nlm.nih.gov/UniGene) which are complementary to gene sequences of interest. Control probes are used to minimise possible problems with cross-hybridization and do not bind to the gene sequence of interest. The exact probe sequences are proprietary to Affymetrix.

The second approach, pioneered at the Brown Lab at Stanford University (http://cmgm.stanford.edu/pbrown/mguide/index.html), is created by robotically localizing individual samples of purified cDNA clones (double stranded polymerase chain reaction products amplified from expression sequence tag clones) onto a glass slide (Bassett 2000) and provides a greater degree of flexibility. These spotted cDNA microarrays allow for the preparation of smaller customized arrays for specific investigations. In contrast to oligonucleotide probes, DNA probes on spotted microarrays are transferred from wells to the substrate as intact DNA strands and require the use of two target samples: a test and a control. The test sample is obtained from the tissue of interest, while the control sample is independent from the tissue of interest. Up to 10,000 cDNA probes can be placed on the substrate.
RNA can be extracted from either a cell sample grown in culture (e.g. cell lines) or the tissue of interest. Cell lines are excellent for obtaining a potentially unlimited supply of pure cell populations and for single-gene manipulation; however, gene expression profiles in culture may have little resemblance to in vivo human gene expression due to the lack of stromal interactions within the cell line. The use of human tissue, however, has many technical obstacles (Emmert-Buck 2000). To date, the effects from trauma associated with surgical excision and resultant ischaemia of the sample, on gene expression are not known. Human biopsy specimens can "dilute" the cancer cells of interest with normal neighbouring tissue and inflammatory infiltrate. While techniques like laser capture microdissection allow for 93-100% purity of cell populations, the efficiency of recovery of a diverse and complex transcriptome has not yet been established (Fend 2000). The large amount of target sample needed for array hybridization often requires an amplification step that may distort relative gene levels (Todd 2002).

Experimental and control mRNA samples are hybridised with separate fluorescent tags (cyanine 3-dCTP (Cy 3) and cyanine 5-dCTP (Cy 5)), allowing for detection of both samples in the same experiment by means of a confocal laser scanner (Kuo 2002). For example, if a control sample is labeled with a green dye and an experimental sample is labeled with a red dye, the relative amounts of each sample that hybridise to the array can determine the relative amounts of each sequence within both samples (Todd 2002). The greater the number of copies of a particular RNA transcript in a tissue, the greater the binding to the matched probe on the microarray.
In general, there are advantages and disadvantages to each microarray technology; the crucial difference between them is that cDNA microarrays provide the amount of each transcript relative to another sample, whereas oligonucleotide arrays provide an absolute amount for each transcript (Kuo 2004).

Once genes are identified as differentially expressed, they require biological validation. A microarray experiment is a multistep process, which is prone to errors, biases, and sometimes overinterpretation (Brown 2000). In addition, quality issues in the data will significantly affect the final results. Validation is generally carried out at the RNA level by one of three methods: Northern blot, real-time PCR, or in situ hybridization on tissue sections (Kuo 2003).
FIGURE 2.1

This diagram gives a brief overview of the microarray experimental process.
FIGURE 2.2

Diagram explaining the significance of the coloured “spots” visualized on a hybridized microarray. The intensity of colour directly correlates with the amount of cDNA bound to the gene represented by that spot.
2.2 Gene expression profiling in OSCC

A number of studies have investigated the application of microarrays in OSCC.

The first study using cDNA microarray technology to characterize transformation-related genes in oral cancer was undertaken by Chang et al. (Chang 1998) mRNA samples in this experiment were derived from cultured oral keratinocyte cell lines.

cDNA microarrays to evaluate gene over-expression in cultured head and neck SCC cell lines identified over-expression of 13 genes when comparing average expression between the SCC group and normal group. Four novel sequences were found to be over-expressed (Villaret 2000).

cDNA microarrays have also been used to identify several differentiation and growth-related genes in head and neck cancers. A study by Leethanakul et al used normal and malignant keratinocytes obtained from biopsy specimens by means of laser capture microdissection. A commercially available cDNA array, containing 588 cancer-related genes, was used. Several expected and novel gene expression patterns were noted, with a 2 to 20 fold reduction in the expression of cytokeratins (2E, 2P, 6A-F, 7, 13-15, 17-19) in tumour tissue with respect to normal tissue, as well as overexpression of 58 genes belonging to diverse functional groups. Cyclin D1, MMP-7, -10, -14, TGF-α/β, PDGF, HGF, and VEGF-C were all elevated. Wnt and MAP kinase signal pathway genes, including ERK1, JNK isoforms, p38, and ERK6 were all elevated. Apoptosis inhibitors
IAP and Akt 2 were also overexpressed in the malignant keratinocytes (Leethanakul 2000).

A similar study used Affymetrix oligonucleotide microarrays to analyse gene expression in OSCC (Alevizos 2001) with paired samples of neoplastic and normal tissue. Approximately 600 genes were noted to be differentially expressed in OSCC, including oncogenes, tumour suppressor genes, transcription factors, xenobiotic enzymes, metastatic proteins, and other genes not previously reported to be associated with OSCC.

In 2002, Al Moustafa et al reported changes in the expression of 213 genes in head and neck SCC, with 91 genes overexpressed in tumoural tissue and 122 underexpressed (Al Moustafa 2002).

Another study identified 210 genes associated with OSCC; of these, some (CKS1, TSPY, CBK, TLE-4, and BCHE) had previously been associated with other types of cancer but not oral cancer (Kuo 2002).

Mendez et al investigated gene expression in 26 OSCCs, 2 premalignant lesions, and 18 samples of normal tissue form the oral cavity; finding 314 genes with differential expression between normal and tumoural tissue, 239 being overexpressed and 75 being underexpressed. However, these 314 genes did not show differences in expression among tumours at different developmental stages, or between metastatic and non-metastatic tumours, except for the ribosomal protein S13. The authors suggested that
these results indicate that most of the changes in gene expression occur during the initial stages of tumour development (Mendez 2002).

In 2003, Ibrahim et al studied gene expression in tumoural and normal tissues of 11 black patients from Sudan and 11 white patients from Norway. There were marked differences in gene expression between tumoural and normal tissue in both populations: 181 genes (31% of the total analysed) in the Sudanese patients, and 195 (33% of the total analysed) in the Norwegian patients. A total of 123 common genes showed altered expression in both groups, despite the ethnic and environmental differences between the 2 populations (Ibrahim 2003).

Gonzales et al studied gene expression in head and neck SCC patients, finding 9 genes with marked expression differences between tumoural and normal tissues: CAGB, CD24, LEKT1, ZNF-185, TGM3, EHF, headpin, ABCG1, and perostin (Gonzales 2003).

The expression of 57 genes previously related with OSCC was studied in 15 patients by Nagata et al (Nagata 2003); 7 genes (MMP-1, MMP-3, upA, integrin-alpha3, paxillin, tenascin C, and IL-6) were found to be overexpressed in metastatic tumours (Somozac-Martin 2005).

In 2004, a study into the differential gene expression in OSCC patients who chewed betel quid using cDNA microarray identified 84 deregulated genes involved in cell adhesion, cell shape, growth, apoptosis, angiogenesis, metastasis, and metabolism (Tsai 2004).
cDNA microarray has also been used to identify overexpressed genes which correlated with nodal metastasis from oral cancer (Irie 2004; Warner 2004). This may aid in the development of more tailored therapies for these patients.

Microarray technology has also been used to differentiate gene expression profiles of primary and metastatic tongue squamous cell carcinoma, in an attempt to identify metastasis-related genes (Vigneswaran 2005).

2.3 Applications of cDNA microarray to OSCC

OSCC diagnosis has shown little improvement in sensitivity and specificity, and despite advances in surgery, radiation, and chemotherapy, 5-year cancer survival rates have changed little over the past 40 years, and remain among the worst of cancers of all anatomic sites. It is hoped that a better understanding of the molecular determinants of oral carcinogenesis will improve diagnosis and disease classification/tumour sub-typing, treatment, and monitoring of the disease (Todd 2002). DNA microarrays can be applied to predict major clinical outcomes, such as metastasis, recurrence, response to therapy and cancer related mortality (Ntzani 2003).
2.3.1 Diagnosis:
DNA microarray techniques can offer molecular screening for specific cancers and the possibility of identifying candidate genes that are predictors of tumor subtypes. Microarrays can aid in the identification of malignancy in an equivocal biopsy sample, and the sub-classification of histologically identical phenotypes. The results may be useful for improved cancer classification and tumour sub-typing.

2.3.2 Treatment planning:
In individual patients, the identification of certain expression profiles can lead to a more accurate diagnosis (Hwang 2003). Tailored treatment plans may be made, with microarrays potentially able to identify chemo- or radiotherapy sensitive or resistant tumours (Cree 2003; Mercer 2003). Aggressive tumours may also be identified, which has implications for surgical planning.

2.3.3 Progression and monitoring of disease:
Microarrays can also be used to delineate the progression of cancers based on their gene expression patterns. The technique can be applied to monitoring of disease, and prediction of tumour recurrence (Todd 2002).

Genetic aberrations in OSCC have been investigated as markers of disease progression including amplifications of 3q, 8q, 9q, 5p, 7p, and 11q13 (Kuo 2004).

Furthermore, even though tissue margins close to resected tumours may look microscopically normal, there is increasing evidence that genetic damage may be
widespread in the adjacent oral epithelial cells, which may explain synchronous tumours (Scholes 1998). DNA microarray technology will be useful in assessing the degree of genetic damage in both the primary tumour and the surrounding mucosa, which could alert clinicians to the possibility of tumour recurrence. Gene expression profiling using microarray technology may, in the future also aid in prognostication.

2.3.4 Prediction of metastases:
Tumours that have a greater risk of metastasis can be identified, with cDNA microarray already used to identify overexpressed genes which correlated with nodal metastasis from oral cancer (Warner 2004). This information has important implications for treatment planning.

2.3.5 Prediction of malignant transformation:
Premalignant and malignant oral lesions have been documented to contain genetic changes that are present prior to phenotypic and morphologic changes (Scully 2000; Schliephake 2003).

In cases of “field cancerisation”, relatively large areas of mucosa have a high likelihood of eventually developing carcinoma, yet may not exhibit all the classic histological or clinical markers of malignancy. The use of DNA microarray technology may lead to earlier diagnosis and/or better prediction of premalignant lesions transformation, indicating which suspected lesions require treatment, and with what degree of aggressiveness (Todd 2002). Given the functional and cosmetic importance of the oral cavity, predicting which areas of mucosa require aggressive treatment and which can be
observed would improve outcomes and decrease treatment related morbidity (Whipple 2004).

Microarray technology could also be applied to predict which cases of isolated leukoplakia and associated epithelial dysplasia will undergo malignant transformation (Kuo 2002). Some leukoplakias can transform and eventually progress to oral cancer. Currently, microscopic examination fails to identify the small subset of these leukoplakias that progress to oral cancer. Obtaining gene expression profiles or genomic "fingerprints" of these lesions and comparing them to normal and cancer expression profiles may allow the differentiation of benign leukoplakias from potentially malignant lesions.

2.3.6 Identification of drug and gene therapeutic interventions:
Gene expression data can provide information about specific genes that are assumed to play a role in cancer formation. This may lead to the identification of genes that can be used as targets for drug or gene therapy (Aitman 2001).
CHAPTER 3

Project Description
3.1 Project background

The identification of genetic alterations associated with OSCC has been the basis of understanding of how normal cells become malignant. Many of these alterations have been found by comparing the differences between the profile and relative gene expression of normal, non-malignant cells and tumour cells.

The most recent work has proposed gene profiling by microarray for the prediction of patient outcome, more comprehensive and accurate tumour classification, as well as to search for genes that could serve as biomarkers and as targets for therapeutic intervention.

3.2 Research description

This research project looked at the difference in gene expression profiles between oral squamous cell carcinoma and normal non-malignant oral epithelium using cDNA microarray, thus identifying genes that are relatively over- and under-expressed.
3.3 Hypothesis

There is a difference in the gene expression profile of OSCC cells compared to normal oral squamous keratinocytes.

3.4 Aims

The aims of this project were to:

1. Establish OSCC cell lines to serve as the study tissue.

2. Establish a normal non-malignant oral keratinocyte cell line to serve as an experimental control.

3. Use cDNA microarray to compare the relative gene expression between these two populations of cells.
CHAPTER 4

Materials and methods
4.1 Ethical approval, patient consent and scientific approval

The Human Research Ethics Committee, Western Sydney Area Health Service and the University of Sydney, Human Ethics Committee approved this study. Participants were provided with information regarding the study and informed written consent was obtained from each of the patients who agreed to participate in the study.

The Scientific Committee of the Western Sydney Area Health Service, based at Westmead Hospital approved this study.

4.2 Grant application

This study was supported by a grant from the Australian Dental Research Foundation.

4.3 Subject tissue

Patients were supplied with an information sheet outlining the purpose of the study, procedure and risks involved. Informed consent was obtained from all patients involved, to allow the obtaining of a small sample of tissue taken by surgical resection, in the course of treatment for their OSCC, to be used in this study. A “Consent to Participate in Research” form was completed and signed (Appendix1).
4.3.1 OSCC tissue:
Tissue obtained from 12 patients diagnosed with OSCC that had been referred to the Head and Neck Cancer Service, Westmead Hospital, NSW, Australia for surgical resection (with the addition of adjuvant radiotherapy in select cases) of their primary tumours were included in this study.

4.3.2 Control tissue:
Normal oral mucosa obtained from 2 patients, who had undergone minor oral surgery involving removal of some gingival tissue as part of the treatment in the Oral Surgery clinics at Westmead Centre for Oral Health served as controls. Patients were selected randomly. A “Consent to Participate in Research” form was completed and signed by these participants (Appendix 2).
### TABLE 4.1 OSCC patient population demographics

<table>
<thead>
<tr>
<th>Patient number</th>
<th>ID</th>
<th>Age</th>
<th>Gender</th>
<th>Smoking history (pack years)</th>
<th>Alcohol Consumption</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JP</td>
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<td>Female</td>
<td>30</td>
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<td>Left lateral tongue</td>
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<td>2</td>
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### TABLE 4.2 Control patient population demographics

<table>
<thead>
<tr>
<th>Patient number</th>
<th>ID</th>
<th>Age</th>
<th>Gender</th>
<th>Smoking history (pack years)</th>
<th>Alcohol Consumption</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>45</td>
<td>Male</td>
<td>Yes</td>
<td>Yes</td>
<td>Gingiva adjacent to left maxillary second molar</td>
</tr>
<tr>
<td>2</td>
<td>IB</td>
<td>53</td>
<td>Male</td>
<td>No</td>
<td>Yes</td>
<td>Maxillary anterior labial gingiva</td>
</tr>
</tbody>
</table>
4.4 Tissue handling

4.4.1 Primary tumour specimens from OSCC patients

All unfixed surgical tissue (OSCC) was transported immediately from the operating theatres to the Department of Anatomical Pathology, Westmead Hospital.

Unfixed primary tumour specimens were received from only 9 of the 12 patients undergoing primary tumour excised at the time of the operation. The ischaemic time (time between excision or interruption of blood supply of the tissue and the start of cell culture) was not documented for individual cases but was usually approximately 20 minutes and did not exceed 60 minutes. Three primary tumour specimens could not be used because the fresh specimens were mistakenly fixed in formol saline before sample tissue could be obtained.

A sample of the primary tumour, not involving the surgical margin, was taken from each unfixed excision specimen. This tissue sample was then placed in a tube containing Hank's balanced salt solution (HBSS) (Sigma, St Louis, USA), for cell culture. The remainder of the specimen was routinely fixed in formol saline and processed for the undertaking of diagnostic histopathology, by members of the Department of Anatomical Pathology, at Westmead Hospital.
4.4.2 Oral mucosa from patients without malignancy

Two patients without OSCC or other malignancy, who were undergoing minor oral surgery procedures in the Department of Oral and Maxillofacial Surgery, Westmead Centre for Oral Health, gave consent for the obtaining of their tissues for this study. The control tissue obtained was from the gingiva / oral mucosa adjacent to the site of the removal of teeth. The unfixed tissue was placed in a tube containing HBSS for cell culture.

4.5 OSCC cell lines

The cell lines H413, derived from a moderately differentiated OSCC obtained from the buccal mucosa of a 53 year old female (Prime 1990), and SCC-25, derived from an OSCC involving the tongue of a 70 year old male were used. The method to establish cell lines from the primary tumour tissue and normal human gingiva is described in the next section.

4.6 Establishment of cultured cell lines (OSCC)

4.6.1 Aim

To establish a series of OSCC cell lines in order to facilitate in vitro investigations of the behaviour and properties of these tumours.
4.6.2 Cell culture:

The tissue sample was immediately soaked in sterile HBSS supplemented with 100 iu/ml penicillin (CSL Biosciences, Parkville, Vic, Australia), 100 µg/ml, streptomycin (JRH Biosciences, Lenexa, USA) and 2.5 µg/ml amphotericin B (Sigma, St Louis, USA). Obviously necrotic or haemorrhagic areas were removed under a dissecting microscope and then discarded. The tissue was washed a minimum of 3 times with the buffer, then placed into a sterile Petri disk where it was finely minced, using a scalpel with a size 15 sterile blade. The resulting fragments were then transferred into a 25 cm² plastic culture flask with sufficient conditioning medium added to cover the fragments in order to facilitate attachment to the flask floor.

Conditioning medium for early culture of these fragments was prepared as follows: discarded human umbilical endothelial cell (HUVEC) culture medium which had been centrifuged and filtered to remove cell debris and fresh Dulbecco’s Modified Eagle Medium (DMEM, Joklik modification) (Sigma Cat # M 0518) containing 10% bovine calf serum (BCS) (JRH Biosciences) supplemented with 100 iu/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B, and L-glutamine were mixed in 1:2 (v/v) proportions. Each flask was incubated at 37°C and in a 5% CO₂ atmosphere, for not more than 48 hours. Fresh conditioned medium sufficient to cover all the tissue fragments was added to the flask. The medium was changed twice weekly and the growth of cell from the fragments was monitored using phase contrast microscopy.
The contaminating fibroblasts were removed enzymatically using 0.1% trypsin (JRH Biosciences). Two ml of 0.1% trypsin were added to the flask and then the flask was examined by phase contrast microscopy for a maximum of 3 minutes. Fibroblasts detached from the flask floor and the epithelial cells, before the epithelial cells themselves became dislodged. The trypsin solution was decanted from the flask, once a sufficient number of fibroblasts had become detached. The culture was washed with HBSS and new conditioned medium was used to grow the remaining cells. Subculture was only performed when contamination by proliferating fibroblasts had been minimized.

The percentage of recycled HUVEC culture medium in the conditioned medium was reduced sequentially, with continuing growth of the explanted epithelial cells.

4.6.3 Results:

From the 9 primary tumour samples with which epithelial cell culture was attempted, 4 (40%) samples proved to be contaminated during culture and 3 (30%) samples were excluded because fibroblast growth dominated the culture. The remaining 2 (20%) samples exhibited no growth.

Although antibiotics and antifungal agents were added to the washing buffer as well as the culture medium, 4 samples became infected. Contamination of the mucosal tissue explants by salivary or dental plaque microorganisms in the culture is thought to have been responsible. Infected flasks were identified both macroscopically by cloudy
conditioned medium and microscopically by the presence of filamentous organisms floating in the media.

Although no cell lines were successfully established from these samples, many of the explants grew well in the initial stages. At approximately 4 days after incubation, the growth of cells from the edge of the tissue fragments could be seen under a phase contrast microscope. (Figures 4.1, 4.2, 4.3, 4.4 and 4.5)

As a result, no OSCC cell lines were established from the 9 primary tumour samples, so, the donated cell lines (H413 and SCC-25) were used for the subsequent microarray experiments. (Figures 4.6 and 4.7)

4.6.4 Culture conditions:
All the OSCC cell lines used in this study were kept frozen in liquid nitrogen in the Department of Oral Pathology at Westmead Hospital until needed for culture and experiments. After slow thawing at approximately 37°C for 5 minutes, the cells were cultured in a humidified atmosphere of 95% air with 5% CO₂ at 37°C.

The media used for the H413 cell line consisted of DMEM (Joklik modification) supplemented with 10% BCS, 100 iu/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B.
The media used for the SCC-25 cell line followed the ATCC guidelines (ATCC 2005) consisting of DMEM and Ham’s Nutrient Mixture F12 (1:1), 1.2g/L sodium bicarbonate, 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate supplemented with 400 ng/ml hydrocortisone, 10% BCS and 100 iu/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B.

Cells were harvested for experiments with 0.02% trypsin/0.25% ethylene diamine tetraacetic acid (EDTA) (JRH Biosciences).
FIGURE 4.1

A phase contrast photomicrograph showing early growth of epithelial cells (---) from the edge of the OSCC tissue fragment within 2 days of incubation.

( x 10 magnification)
FIGURE 4.2

Phase contrast photomicrograph taken after 5 days of incubation. Spindle-shaped cells resembling fibroblasts (➡️) are seen surrounding the polyhedral epithelial cells. (⬅️) (x 10 magnification)
FIGURE 4.3

Phase contrast photomicrograph taken after 10 days of incubation. Polyhedral epithelial cells (→) have grown to confluence. (x 10 magnification)
FIGURE 4.4

A phase contrast photomicrograph taken after 2 weeks of incubation. This shows the growth of two different populations of cells; spindle shaped cells resembling fibroblasts, ( ) and polyhedral epithelial-like cells. ( ) (x 10 magnification)
FIGURE 4.5

A phase contrast photomicrograph showing nests of polyhedral epithelial cells (←) surrounded by spindle shaped fibroblasts (→). The epithelial cell population was unable to expand even after the fibroblasts were removed using 0.02% trypsin. (x 10 magnification)
FIGURE 4.6

Phase contrast photomicrograph showing the H413 OSCC cell line. ( ) (x 10 magnification)
FIGURE 4.7

Phase contrast photomicrograph of the SCC-25 OSCC cell line. ( ) (x 10 magnification)
4.7 Establishment of cultured cell lines (non-malignant keratinocytes)

4.7.1 Aim:
To establish a normal, non-malignant oral keratinocyte cell line to serve as a normal control for the microarray experiments.

4.7.2 Tissues:
As described, tissue samples were obtained from 2 dental patients without OSCC or other malignancy, with their informed witnessed consent, undergoing minor oral surgery in the Department of Oral and Maxillofacial Surgery, Westmead Centre for Oral Health, NSW Australia. Control tissue was obtained from the gingival or adjacent oral mucosa involved in the removal of teeth.

4.7.3 Cell culture:
The unfixed tissue was placed in a tube containing HBSS for cell culture. The tissue sample was immediately soaked in sterile HBSS supplemented with 100 iu/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml, amphotericin B and L-glutamine. The tissue was washed several times with the buffer, then placed into a sterile Petri dish where it was finely minced, using a scalpel with a size 15 sterile blade. The resulting fragments were then transferred into a 25 cm² plastic culture flask and sufficient conditioned medium to cover the fragments was added to facilitate attachment to the flask.
Conditioned medium for culture of these fragments was prepared as follows: fresh DMEM and Ham’s Nutrient Mixture F12 (3:1) containing 20% BCS supplemented with 100 iu/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B, L-glutamine and 2.57 IU/ml insulin.

Each flask was incubated at 37°C and in a 5% CO₂ atmosphere, for not more than 48 hours. Fresh conditioned medium sufficient to cover all the fragments was added to the flask. The medium was changed twice weekly and the growth of cell from the fragments was monitored using a phase contrast microscope.

The contaminating fibroblasts were removed enzymatically using 0.1% trypsin. 2 ml of 0.1% trypsin were added to the flask and the flask was examined by phase contrast microscopy for approximately 3 minutes. Fibroblasts became detached from the flask and the epithelial cells before the epithelial cells themselves became dislodged. The trypsin solution was decanted from the flask, once an optimal number of fibroblasts had become detached. The culture was washed with HBSS and the conditioned medium was used again to grow the remaining cells. Subculture was performed when contamination by proliferating fibroblasts had been minimized.

The resulting normal non-malignant oral keratinocyte cell line was frozen in liquid nitrogen for later use as the control tissue in the microarray experiments (Figure 4.8).
4.7.4 Results:
From the 2 samples of normal gingival tissue with which epithelial cell culture was attempted, 1 (50%) cell line was established. The unsuccessful sample was discarded due to contamination during culture.

Contaminating fibroblasts were removed as described above, until the culture consisted of only keratinocytes. These cells did not show any sign of senescence after several passages in culture medium, and finally the cells could be frozen in liquid nitrogen for later use.

4.7.5 Culture conditions:
The normal oral keratinocyte cell lines used in this study was kept frozen in liquid nitrogen in the Department of Oral Pathology at Westmead Hospital until needed for culture and experiments. After slow thawing, the cells were cultured in humidified atmosphere of 95% air and 5% CO₂ at 37° C.

The medium used consisted of DMEM and Ham’s Nutrient Mixture F12 (3:1) containing 20% BCS supplemented with 100 iu/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B, L-glutamine and 2.5IU/ml insulin. Cells were harvested for experiments with 0.02% trypsin/0.25% EDTA.
FIGURE 4.8

Phase contrast photomicrograph of the non-malignant oral keratinocytes (polyhedral cells) (←) as well as some spindle-shaped fibroblast (→) (x 10 magnification)
4.8 Sample preparation and RNA extraction method for cultured cell lines

The cultured cell lines used in this study were harvested for extraction of their RNA only when the cell layer was confluent and covered the floor of a 75cm² flask for extraction of RNA. 10 ml of HBSS were used to wash the cells after the removal of the culture medium. The cells were removed from the flasks enzymatically, by incubating them for 5 minutes with 5 ml of 0.25% trypsin. The cell suspension was collected and diluted with 5 ml of HBSS, before centrifugation for 5 minutes at 1000 rpm and 4° C to pellet the cells. The supernatant was discarded, and cells were washed with 1 ml PBS, centrifuged again, and the supernatant discarded.

The cells were now ready to be lysed for RNA extraction using RNAqueous isolation kit (Ambion Cat#1912, Austin, Texas, USA) according to the manufacturer’s instructions.

All solutions applied to isolated RNA and not obtained from the RNAqueous kits were treated for 24 hours by addition of 1μl of diethylpyrocarbonate (DEPC) per litre of solution to inactivate any contaminant RNAs before autoclaving.
4.9 Quantification of isolated RNA

Quantification of the concentration of the isolated RNA for each specimen was performed using a spectrophotometer (Beckman, Model # DU 640). The light absorbance was measured at 260nm and 280nm. The concentration of isolated RNA was determined using the formula developed by Warburg and Christian (1942):

\[ \text{Concentration of RNA (µg /µl) = } \text{Abs}_{260\text{ nm}} \times 0.04 \times \text{dilution factor} \]

The yield of RNA from each specimen needed to be a minimum of 50µg before proceeding to the next step.

4.10 Bioanalysis of the RNA samples

The integrity of the RNA samples was checked using the Agilent 2100 BioAnalyser (Agilent Technologies, Palo Alto, California, USA) and RNA Lab Chip™ to confirm that RNA used for microarray experiments was not degraded.

A total of 2 µl of each samples dilution (150 µg/µl RNA in DEPC H₂O) were prepared, and run through this process, which involved the electrophoretic separation of the sample RNA on the RNA Lab Chip™, and detection by laser induced fluorescence.
An electropherogram and gel-like image was generated for each sample, providing a detailed visual assessment of the quality of each RNA sample.

An RNA integrity number (RIN) based on a numbering system from 1 to 10, was generated, with 1 being the most degraded profile and 10 being the most intact.

All RNA samples used in these experiments had an RIN of 10 (Figure 4.9).
FIGURE 4.9

BioAnalyser results: (A) electropherogram and (B) “gel-like” image of RNA isolated from SCC-25 OSCC cell line.

(A) The electropherogram indicated that the total RNA separated into two sharp peaks of distinct fluorescence intensity corresponding to 18S and 28S ribosomal RNA was readily apparent. (B) The BioAnalyser software coverts these peaks into bands in a “gel-like” image. Both the electropherogram and “gel-like” image indicate the isolated RNA was intact. Similar results were seen for all 22 RNA samples used for microarray analysis.
Subsequently, 5.7 μl of sodium acetate (3M, pH 5.2) and 114 μl of 100% ethanol were mixed with the remaining undiluted samples, and these were stored overnight in a -80°C refrigerator for the RNA to precipitate.

4.11 Reverse transcription, cDNA synthesis

Precipitated RNA was collected by centrifugation at 13 000 rpm at 4°C for 5 minutes. Supernatants were discarded and the pellets washed with 100 μl of 70% ethanol/DEPC H₂O. Samples were centrifugated under the same conditions, and the supernatant was again discarded. The samples were then left to air dry for approximately 60 minutes until the pellet appeared translucent. Once this occurred, the pellets were resuspended in 10.5 μl of DEPC H₂O. Following this, 0.5 μl of 0.5 μg/μl poly dT₁₅ primer (Roche Molecular Biochemicals, New Jersey, USA) was added to each sample. Samples were incubated at 70°C for 10 minutes, then snap frozen in ice.

Master mixes were prepared: sufficient for two samples, 12 μl of 5X first strand buffer supplied with the Superscript III reverse transcriptase (Invitrogen, Carlbad, California, USA) was added to 6 μl of 0.1M dithiothreitol which was also supplied with the enzyme before addition of 8.3 μl DEPC H₂O and 1.2 μl of dNTP mix (25mM each of dATP, dCTP and dGTP, 15mM aminoallyl-dUTP and 10mM dTTP) (Sigma, St Louis, USA). 14.5 μl of this master mix was added to each sample and these were incubated at 42°C for 3 hours.
After this time, 10 µl each of 1M sodium hydroxide and 0.5M EDTA was added to each sample, which were subsequently incubated for 15 minutes at 65° C to allow the RNA templates to hydrolyse before the addition of 25 µl of 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0. Samples were stored overnight at -20° C.

4.12 cDNA purification and labelling with Cy dyes

cDNA preparations were purified using the Qiaquick® PCR Purification kit (Qiagen, Hilden Germany) according to manufacturer’s instructions, except that purification columns were washed with a solution of potassium phosphate (5mM) pH 8.0 and 80% ethanol. cDNA was then eluted using 2 volumes of 55 µl milli Q H₂O and quantified using absorbance spectrophotometry at 260nm and 280 nm and the formula specified above for RNA quantification.

A minimum of 0.75µg of cDNA was labeled and hybridized as detailed below.

cDNA was then concentrated using a SC110A Speed Vac Concentrator (Savant Instruments, Holbrook, New York) to 5 µl.

3 µl of 0.1M sodium bicarbonate was added to each 5 µl sample, as well as 2 µl of appropriate Cyanine (Cy) dye (Amersham Biosciences, Buckinghamshire, UK). The Cy3
and Cy5 dyes were prepared by resuspending aliquots in 2 μl dimethyl sulfoxide (DMSO). Samples were then left to couple in the dark for 2 hours.

Labeled cDNA was purified using PCR Product Purification kits according to the manufacturer’s instructions, except that labeled cDNA was eluted using 2 volumes of 55μl milli Q H₂O. Labeled cDNA was quantified by absorbance spectrophotometry at 260nm, 550nm and 650nm. To determine if the Cy dye coupling reaction was successful, the amount of dye coupled to the cDNA was calculated using the following formulae:

\[
\text{pmol nucleotide} = \left[ A_{260} \times \text{vol (μl)} \times 37 \text{ng/μl} \times 1000 \text{ pg/mg} \right] 324.5 \text{ pg/pmole}
\]

\[
\text{pmol Cy3} = \left[ A_{550} \times \text{vol (μl)} \right] / 0.15
\]

\[
\text{pmol Cy5} = \left[ A_{650} \times \text{vol (μl)} \right] / 0.25
\]

\[
\text{nucleotides/dye} = \text{pmol nucleotide/pmole Cy dye}
\]

Using these formulae, the minimum values for a successful coupling reaction were considered to be >25 pmol of dye incorporation and a nucleotide to dye ratio of <50.

The second formula used was:

\[
\text{No. dye molecules/1000 nucleotides} = \frac{A_{\text{dye}}}{A_{260}} \times \frac{9010 \text{ M}^{-1} \text{ cm}^{-1}}{\text{dye extinction coefficient}} \times 1000
\]

Where extinction coefficient of Cy3 = 150000 and Cy5 = 250000

94
The minimum values for a successful reaction were considered to be 20-50 dye molecules/1000 nucleotides. Coupling reactions were considered successful if the results of both sets of calculations were greater than or equal to the minimum values described.

Cy3 and Cy5 samples were then combined, and volume reduced to 13.25 µl using the Speed Vac Concentrator.

4.13 Hybridisation

Microarrays (Australian Genome Research Foundation (AGRF) 8K human genome array) were added to a pre-warmed (42° C) coplin jar containing the prehybridisation solution (0.6g bovine serum albumin (BSA) dissolved in 30ml DEPC H2O, 15 ml formamide, 15ml 20x sodium chloride sodium citrate (SSC), 0.6ml 10% sodium dodecyl sulphate (SDS)) and incubated for 45 minutes. Arrays were then rinsed in DEPC H2O in a 50ml centrifuge tube and spun at 750 rpm for 5 minutes.

To the 13.5 µl cDNA, 25µl of 2x hybridization buffer (100 µl formamide, 50 µl of 20x SSC, 4 µl of 10% SDS, and 50µl DEPC H2O), 4.25 µl Cot1 DNA, 3.25 µl PolyA and 4.25 µl salmon sperm DNA was added. This was heated to 100° C for 2 minutes, then cooled to laboratory temperature.
The sample was then applied to a clean coverslip. The array was inverted onto the coverslip and air bubbles were gently removed. The array was placed in the hybridization chamber with the remainder of the 180 μl of 2x hybridisation buffer, and left to hybridise at 42° C overnight.

Wash solutions were prepared:

<table>
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<th>1x SSC/0.2% SDS</th>
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</tr>
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<td>20x SSC</td>
<td>1ml</td>
<td>0.1ml</td>
<td>0.25ml</td>
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<tr>
<td>10% SDS</td>
<td>0.4ml</td>
<td>0.4ml</td>
<td>-</td>
</tr>
<tr>
<td>DEPC H₂O</td>
<td>18.6ml</td>
<td>19.5ml</td>
<td>49.75ml</td>
</tr>
</tbody>
</table>

The array was removed from the hybridization chamber and agitated in 1x SSC/0.2% SDS until the coverslip was detached and discarded. The array was then washed gently in the same solution for 5 minutes, subsequently washed in 0.1xSSC/0.2%SDS for 5 minutes, before two final washes in 0.1x SSC for 2 minutes. The microarray was centrifugated in a 50ml tube at 750rpm for 3 minutes until all moisture was removed from the slide.

### 4.14 Scanning and data analysis

Microarrays were scanned (Axon Instruments model GenePix 4000B) to produce a digital record of the red and green fluorescence at each point on the array (Figure 4.10). Data was collected using GenePixPro v6.0 software.
FIGURE 4.10

Scanned image of an experimental microarray. The Cy3 and Cy5 channels are coloured green and red respectively, with yellow representing an equal balance of the two. Overlay of the two channels reveals information on colour balance, uniformity of hybridization, pot uniformity, background and artifacts. This also provides a rough impression of the number of genes that are differentially expressed between the two samples tested.
CHAPTER 5

Results
5.1 Results

In the initial arm of the experiment, tissue samples were obtained from 12 patients with OSCC. The mean age of these patients was 61 years of age, with a male to female ratio of 2:1. This was in keeping with the reported population demographic of OSCC patients in Western countries. In addition, 11 out of 12 of patients from whom tissue samples were smokers (average of 27.5 pack years), and 7 out of 12 patients reported a history of alcohol consumption. Again, this is consistent with the reported OSCC patient demographics.

From the 9 primary tumour samples with which epithelial cell culture was attempted, 4 (40%) samples proved to be contaminated during culture and 3 (30%) samples were excluded because fibroblast growth dominated the culture. The remaining 2 (20%) samples exhibited no growth.

From the 2 samples of normal gingival tissue with which epithelial cell culture was attempted, 1 (50%) cell line was established. The unsuccessful sample was discarded due to contamination during culture.

The non-malignant keratinocyte cell line used as the control sample was derived from the patient who was a non-smoker and non-drinker.
The number of microarrays analysed were as follows:

1. Seven arrays for SCC-25 compared with the control tissue.
2. Four arrays for H413 compared with the control tissue.

Each experiment involved two separate total RNA samples so that in total 22 samples of RNA were isolated, subjected to BioAnalyser analysis and reverse transcription before labelling and hybridising as described above.

Once the arrays were scanned, the photomultiplier tube (PMT) voltage was adjusted so that the brightest pixels fall just short of the level of saturation thus increasing the sensitivity of the image analysis for the less bright pixels. Individual spots were located on the slide automatically by the GenePix program which separated the pixels into those in the spot itself (foreground) and those in the background by fitting a circle of constant diameter to all spots on the image. Individual circle diameters were manually adjusted to cover irregularly sized/shaped spots.

Spots for which the intensity of fluorescence on both channels was not greater than twice the background fluorescence were excluded from further analysis. Data was then entered into the Bioconductor™ program (Bioconductor 2006) for scaling.

Plots were formed of the whole array dataset for each individual array to check that there was no obvious skewing of the data and to monitor the overall spread of data after normalisation. Since the majority of genes in this set were not expected to be differentially expressed, and the genes of interest that are over or under expressed would
balance out, the median value on a box plot of the log 2 ratios would be approximately zero (i.e. red:green 1:1). In addition, the expected range of data from the different arrays showed similar patterns (Figures 5.1, 5.2, 5.3).

The log-differential expression ratio (M) was calculated using:

\[ M = \log_2 \frac{R}{G} \]

where R is the background corrected red intensity, and G is the background corrected green intensity.

The log-intensity of a spot (A) was calculated using:

\[ A = \frac{1}{2} \log_2 RG \]

MA plots were also charted, to confirm that the changes in red:green ratios are independent of intensity of detection. The line of best fit was fairly straight, with a gradient of approximately zero, confirming this parameter. (Figure 5.4)

Data was normalized using the Print-tip Loess method (Yang 2001) which assumes that the average red:green ratio is one at any given intensity but takes into account the fact that there may be some influence of intensity (particularly at the low or high intensities). So rather than normalising the median or mean ratio across the entire dataset, it adjusts each datapoint to the local intensity-weighted ratio (Loess Line on the MA plot). Normalisation allows for the adjustment for any bias which arises from variation in the microarray technology rather than from biological differences between the RNA samples.
of the printed probes. Most commonly, this red-green bias is due to differences between the labeling efficiencies and scanning properties of the two dyes, further complicated by the use of different scanner settings. Other biases may arise from variation between spatial positions of the spots on a slide or between slides. Positions on a slide may vary because of differences between the print-tips on the array printer, variation over the course of the print-run or non-uniformity in the hybridization. Differences between arrays may arise from differences in print quality or from differences in ambient conditions when the arrays were processed. Therefore it is necessary to normalize the intensities before any subsequent analysis is carried out (Smyth 2002).
FIGURE 5.1

Box plot – raw data

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Cell lines used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H413 vs normal</td>
</tr>
<tr>
<td>2</td>
<td>H413 vs normal</td>
</tr>
<tr>
<td>3</td>
<td>H413 vs normal</td>
</tr>
<tr>
<td>4</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>5</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>6</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>7</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>8</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>9</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>10</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>11</td>
<td>H413 vs normal</td>
</tr>
</tbody>
</table>
FIGURE 5.2

Box plot – normalised scaled data

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Cell lines used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>2</td>
<td>H413 vs normal</td>
</tr>
<tr>
<td>3</td>
<td>H413 vs normal</td>
</tr>
<tr>
<td>4</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>5</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>6</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>7</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>8</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>9</td>
<td>SCC25 vs normal</td>
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<tr>
<td>10</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>11</td>
<td>H413 vs normal</td>
</tr>
</tbody>
</table>
FIGURE 5.3

Box plot – Loess normalised data

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Cell lines used</th>
</tr>
</thead>
<tbody>
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<tr>
<td>4</td>
<td>SCC25 vs normal</td>
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<tr>
<td>5</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>6</td>
<td>SCC25 vs normal</td>
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<tr>
<td>7</td>
<td>SCC25 vs normal</td>
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<tr>
<td>8</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>9</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>10</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>11</td>
<td>H413 vs normal</td>
</tr>
</tbody>
</table>
FIGURE 5.4

MA plots from all microarray experiments.

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Cell lines used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<tr>
<td>3</td>
<td>H413 vs normal</td>
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<tr>
<td>4</td>
<td>SCC25 vs normal</td>
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<td>SCC25 vs normal</td>
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<td>SCC25 vs normal</td>
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<tr>
<td>7</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>8</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>9</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>10</td>
<td>SCC25 vs normal</td>
</tr>
<tr>
<td>11</td>
<td>H413 vs normal</td>
</tr>
</tbody>
</table>
Genes were ranked in order of evidence for differential expression, from strongest to weakest evidence, using the penalized t-statistic (Efron 2001).

\[
t = \frac{\bar{M}}{(a+s)/\sqrt{n}}
\]

\(\bar{M}\) is the mean of M-values for any particular gene across the replicate arrays.

a is the 90th percentile of the s values

s is the standard deviation

n is the number of samples

The critical value for the ranking statistic above which any value was considered to be significant was \(t > 2\) or \(t < -2\).

This short-list of genes was then entered in the GoMiner™ software program available at http://discover.nci.nih.gov/gominer/ (Zeeberg 2003) which sorted them into functional ontologies. Genes with a p-value of <0.05 were deemed to be statistically significantly differentially expressed.
In total, 11 microarray experiments were completed:

SCC-25 OSCC cell line compared with the non-malignant oral keratinocyte cell line (7 experiments)

H413 OSCC cell line compared with the non-malignant oral keratinocyte cell line (4 experiments)

Genes that were differentially expressed common to all microarray experiments conducted were analysed for overall trends rather than differentially expressed genes unique to each particular cell line.

Only 11 genes were found to be significantly differentially expressed ($p \leq 0.05$) in the OSCC cell lines on comparison with the normal non-malignant oral keratinocyte cell line. Two of these genes were over-expressed, and the remaining nine genes were found to under-expressed. The differentially expressed genes were classified, based on gene ontology, so as to specify their biological activity.

The genes found to be differentially expressed in the OSCC cultured cell lines were involved in four biological processes namely (1) “regulation of apoptosis”, (2) “cell cycle arrest”, (3) “negative regulation of cell proliferation”, and (4) “negative regulation of cell progression through cell cycle” (Table 5.1).

In addition, there was a trend towards over expression of angiogenesis related genes and growth factors in the OSCC cell lines, although this was not found to be statistically significant.
A comprehensive list of all genes that were found to be commonly differentially expressed in all microarray experiments is included in Table 5.2.
Table 5.1 Significantly differentially expressed genes in OSCC cell lines compared with a normal oral keratinocyte cell line.

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Gene Name</th>
<th>Function(s)</th>
<th>Over/Under Expression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIRC2</td>
<td>Tumour necrosis factor receptor type 2</td>
<td>• Regulation of apoptosis/ anti-apoptosis</td>
<td>Over</td>
<td>0.04</td>
</tr>
<tr>
<td>BIRC3</td>
<td>Tumour necrosis factor receptor type 3</td>
<td>• Regulation of apoptosis/ anti-apoptosis</td>
<td>Over</td>
<td>0.04</td>
</tr>
<tr>
<td>CDN1b</td>
<td>Cyclin dependent kinase inhibitor 1b</td>
<td>• Cell cycle arrest</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Negative regulation of cell proliferation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDN1c</td>
<td>Cyclin dependent kinase inhibitor 1c</td>
<td>• Cell cycle arrest</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Negative regulation of cell proliferation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAS1</td>
<td>Glycolipid anchor surface protein 1</td>
<td>• Cell cycle arrest</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Negative regulation of cell proliferation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAS7</td>
<td>Glycolipid anchor surface protein 7</td>
<td>• Cell cycle arrest</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td>BTG3</td>
<td>B cell translocation gene 3</td>
<td>• Negative regulation of cell proliferation</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td>IBP6</td>
<td>Insulin-like growth factor 6</td>
<td>• Negative regulation of cell proliferation</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td>IBP7</td>
<td>Insulin-like growth factor 7</td>
<td>• Negative regulation of cell proliferation</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td>IFM</td>
<td>Mitochondrial translation initiation factor</td>
<td>• Negative regulation of cell proliferation</td>
<td>Under</td>
<td>0.01</td>
</tr>
<tr>
<td>RHOB</td>
<td>ras homolog gene family, member B</td>
<td>• Negative regulation of progression through cell cycle</td>
<td>Under</td>
<td>0.03</td>
</tr>
<tr>
<td>Name</td>
<td>M</td>
<td>s</td>
<td>n</td>
<td>t</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-----</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Uteroglobin</td>
<td>-3.45</td>
<td>2.43</td>
<td>22.00</td>
<td>-4.43</td>
</tr>
<tr>
<td>aniloride binding protein 1 (amine oxidase (copper-containing))</td>
<td>-2.96</td>
<td>1.95</td>
<td>21.00</td>
<td>-4.28</td>
</tr>
<tr>
<td>S100 calcium-binding protein A8 (calgranulin A)</td>
<td>-3.91</td>
<td>2.97</td>
<td>21.00</td>
<td>-4.28</td>
</tr>
<tr>
<td>S100 calcium-binding protein A9 (calgranulin B)</td>
<td>-3.88</td>
<td>3.18</td>
<td>22.00</td>
<td>-4.14</td>
</tr>
<tr>
<td>amphiregulin (schwannoma-derived growth factor)</td>
<td>-3.08</td>
<td>2.08</td>
<td>18.00</td>
<td>-3.96</td>
</tr>
<tr>
<td>carbonic anhydrase II</td>
<td>-2.93</td>
<td>2.26</td>
<td>22.00</td>
<td>-3.95</td>
</tr>
<tr>
<td>laminin, beta 2 (laminin S)</td>
<td>-3.48</td>
<td>2.85</td>
<td>21.00</td>
<td>-3.93</td>
</tr>
<tr>
<td>Granulysin</td>
<td>-2.55</td>
<td>1.76</td>
<td>21.00</td>
<td>-3.92</td>
</tr>
<tr>
<td>matrix metalloproteinase 7 (matrilysin, uterine)</td>
<td>-2.61</td>
<td>1.80</td>
<td>20.00</td>
<td>-3.87</td>
</tr>
<tr>
<td>phosphatase and tensin homolog (mutated in multiple advanced cancers 1)</td>
<td>-2.73</td>
<td>2.12</td>
<td>22.00</td>
<td>-3.84</td>
</tr>
<tr>
<td>deformed epidermal autoregulatory factor 1 (Drosophila)</td>
<td>-2.75</td>
<td>2.10</td>
<td>21.00</td>
<td>-3.79</td>
</tr>
<tr>
<td>serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3</td>
<td>-2.99</td>
<td>2.06</td>
<td>17.00</td>
<td>-3.77</td>
</tr>
<tr>
<td>transmembrane 4 superfamily member 1</td>
<td>-2.95</td>
<td>2.38</td>
<td>21.00</td>
<td>-3.76</td>
</tr>
<tr>
<td>Homo sapiens cDNA: FLJ23538 fis, clone LNG08010, highly similar to BETα2</td>
<td>-2.22</td>
<td>1.57</td>
<td>22.00</td>
<td>-3.73</td>
</tr>
<tr>
<td>Human MEN1 region clone epsilon/beta mRNA</td>
<td>-2.53</td>
<td>2.10</td>
<td>22.00</td>
<td>-3.58</td>
</tr>
<tr>
<td>baculoviral IAP repeat-containing 3</td>
<td>-2.33</td>
<td>1.66</td>
<td>18.00</td>
<td>-3.43</td>
</tr>
<tr>
<td>protease, serine, 4 (trypsin 4, brain)</td>
<td>-2.41</td>
<td>1.54</td>
<td>15.00</td>
<td>-3.39</td>
</tr>
<tr>
<td>interleukin 1, beta</td>
<td>-2.41</td>
<td>1.72</td>
<td>18.00</td>
<td>-3.36</td>
</tr>
<tr>
<td>sema domain, seven thrombospondin repeats (type 1 and type 1-like),</td>
<td>-2.32</td>
<td>1.72</td>
<td>18.00</td>
<td>-3.36</td>
</tr>
<tr>
<td>transmembrane domain (TM) and short cytoplasmic domain, (sephorin) 5A</td>
<td>-2.63</td>
<td>2.49</td>
<td>22.00</td>
<td>-3.33</td>
</tr>
<tr>
<td>ribosomal protein S4, Y-linked</td>
<td>-2.47</td>
<td>2.16</td>
<td>19.00</td>
<td>-3.19</td>
</tr>
<tr>
<td>Small proline-rich protein SPRK [human, odontogenic keratocysts, mRNA Partial, 317 nt]</td>
<td>-1.92</td>
<td>1.42</td>
<td>19.00</td>
<td>-3.18</td>
</tr>
<tr>
<td>cathepsin L2</td>
<td>-1.58</td>
<td>1.07</td>
<td>21.00</td>
<td>-3.17</td>
</tr>
<tr>
<td>solute carrier family 27 (fatty acid transporter), member 4</td>
<td>-1.78</td>
<td>1.25</td>
<td>19.00</td>
<td>-3.15</td>
</tr>
<tr>
<td>cathepsin C</td>
<td>-2.66</td>
<td>0.01</td>
<td>2.00</td>
<td>-3.08</td>
</tr>
</tbody>
</table>

Table 5.2 Genes found to be differentially expressed in OSCC cell lines compared with a normal oral keratinocyte cell line using the penalised t-statistic, with t > 2 or t < -2.
<table>
<thead>
<tr>
<th>Name</th>
<th>M</th>
<th>s</th>
<th>n</th>
<th>t</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>chaperonin containing TCP1, subunit 6A (zeta 1)</td>
<td>-1.61</td>
<td>1.13</td>
<td>20.00</td>
<td>-3.08</td>
<td>↑</td>
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<tr>
<td>ESTs</td>
<td>-2.10</td>
<td>1.80</td>
<td>19.00</td>
<td>-3.04</td>
<td>↑</td>
</tr>
<tr>
<td>fatty acid binding protein 4, adipocyte</td>
<td>-2.15</td>
<td>2.16</td>
<td>22.00</td>
<td>-2.99</td>
<td>↑</td>
</tr>
<tr>
<td>small inducible cytokine subfamily B (Cys-X-Cys), member 14 (BRAK)</td>
<td>-2.01</td>
<td>1.84</td>
<td>19.00</td>
<td>-2.87</td>
<td>↑</td>
</tr>
<tr>
<td>tumor necrosis factor, alpha-induced protein 3</td>
<td>-1.44</td>
<td>1.09</td>
<td>21.00</td>
<td>-2.85</td>
<td>↑</td>
</tr>
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<td>uridine phosphorylase</td>
<td>-1.63</td>
<td>1.30</td>
<td>19.00</td>
<td>-2.82</td>
<td>↑</td>
</tr>
<tr>
<td>lysyl oxidase-like 2</td>
<td>-1.73</td>
<td>1.39</td>
<td>18.00</td>
<td>-2.82</td>
<td>↑</td>
</tr>
<tr>
<td>FAT tumor suppressor (Drosophila) homolog</td>
<td>-2.08</td>
<td>1.52</td>
<td>13.00</td>
<td>-2.74</td>
<td>↑</td>
</tr>
<tr>
<td>phosphofructokinase, platelet</td>
<td>-1.48</td>
<td>1.31</td>
<td>22.00</td>
<td>-2.74</td>
<td>↑</td>
</tr>
<tr>
<td>fatty acid binding protein 5 (psoriasis-associated)</td>
<td>-1.72</td>
<td>1.73</td>
<td>22.00</td>
<td>-2.74</td>
<td>↑</td>
</tr>
<tr>
<td>myxovirus (influenza) resistance 1, homolog of murine (interferon-inducible protein p78)</td>
<td>-1.97</td>
<td>1.94</td>
<td>19.00</td>
<td>-2.72</td>
<td>↑</td>
</tr>
<tr>
<td>bone morphogenetic protein 2</td>
<td>-1.39</td>
<td>1.03</td>
<td>19.00</td>
<td>-2.70</td>
<td>↑</td>
</tr>
<tr>
<td>Involutrin</td>
<td>-1.55</td>
<td>1.44</td>
<td>21.00</td>
<td>-2.67</td>
<td>↑</td>
</tr>
<tr>
<td>ribosomal protein L44</td>
<td>-1.39</td>
<td>1.23</td>
<td>22.00</td>
<td>-2.67</td>
<td>↑</td>
</tr>
<tr>
<td>POM (POM121 rat homolog) and ZP3 fusion protein</td>
<td>-1.25</td>
<td>0.69</td>
<td>16.00</td>
<td>-2.62</td>
<td>↑</td>
</tr>
<tr>
<td>adenosine deaminase</td>
<td>-1.23</td>
<td>0.94</td>
<td>21.00</td>
<td>-2.61</td>
<td>↑</td>
</tr>
<tr>
<td>protein C receptor, endothelial (EPCR)</td>
<td>-1.47</td>
<td>0.98</td>
<td>15.00</td>
<td>-2.59</td>
<td>↑</td>
</tr>
<tr>
<td>inhibin, beta A (activin A, activin AB alpha polypeptide)</td>
<td>-1.59</td>
<td>1.32</td>
<td>17.00</td>
<td>-2.58</td>
<td>↑</td>
</tr>
<tr>
<td>aldo-keto reductase family 1, member B11 (aldo reductase-like)</td>
<td>-1.53</td>
<td>1.09</td>
<td>15.00</td>
<td>-2.57</td>
<td>↑</td>
</tr>
<tr>
<td>solute carrier family 25 (mitochondrial carrier adenine nucleotide translocator), member 5</td>
<td>-1.30</td>
<td>1.17</td>
<td>22.00</td>
<td>-2.56</td>
<td>↑</td>
</tr>
<tr>
<td>POM (POM121 rat homolog) and ZP3 fusion protein</td>
<td>-1.25</td>
<td>0.75</td>
<td>16.00</td>
<td>-2.53</td>
<td>↑</td>
</tr>
<tr>
<td>Diubiquitin</td>
<td>-1.97</td>
<td>1.29</td>
<td>10.00</td>
<td>-2.49</td>
<td>↑</td>
</tr>
<tr>
<td>synaptonemal complex protein 2</td>
<td>-2.54</td>
<td>0.26</td>
<td>2.00</td>
<td>-2.43</td>
<td>↑</td>
</tr>
<tr>
<td>400000000000000</td>
<td>-1.22</td>
<td>1.11</td>
<td>21.00</td>
<td>-2.39</td>
<td>↑</td>
</tr>
<tr>
<td>Nebulette</td>
<td>-1.23</td>
<td>1.10</td>
<td>20.00</td>
<td>-2.38</td>
<td>↑</td>
</tr>
<tr>
<td>lamin B2</td>
<td>-1.19</td>
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<td>kallikrein 8 (neuropsin/oasain)</td>
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<td>dystroglycan 1 (dystrophin-associated glycoprotein 1)</td>
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<td>0.74</td>
<td>19.00</td>
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<td>↑</td>
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<td>proline-rich protein with nuclear targeting signal</td>
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<td>0.67</td>
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<td>bone gamma-carboxyglutamate (gla) protein (osteocalcin)</td>
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<td>0.16</td>
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<td>↓</td>
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<td>NADH dehydrogenase (ubiquinone) Fe-S protein 8 (23kD) (NADH-coenzyme Q reductase)</td>
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<td>0.62</td>
<td>22.00</td>
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<td>↓</td>
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<td>glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)</td>
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<td>0.92</td>
<td>19.00</td>
<td>2.00</td>
<td>↓</td>
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<td>UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)</td>
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<td>1.02</td>
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<td>SMC4 (structural maintenance of chromosomes 4, yeast)-like 1</td>
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<td>0.86</td>
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<td>non-metastatic cells 3, protein expressed in</td>
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<td>1.27</td>
<td>20.00</td>
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<td>0.79</td>
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<td>1.66</td>
<td>17.00</td>
<td>2.05</td>
<td>↓</td>
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<td>Name</td>
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<td>MAD (mothers against decapentaplegic, Drosophila) homolog 1</td>
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<td>0.14</td>
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<td>1.87</td>
<td>3.00</td>
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<td>sulfotransferase family, cytosolic, 1A, phenol-prefering, member 2</td>
<td>1.28</td>
<td>1.01</td>
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<td>Name</td>
<td>M</td>
<td>s</td>
<td>n</td>
<td>t</td>
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<td>1.42</td>
<td>12.00</td>
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<td>0.57</td>
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<td>0.95</td>
<td>22.00</td>
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<td>20.00</td>
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<td>↓</td>
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<td>1.36</td>
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<td>thymosin, beta 4, X chromosome</td>
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<td>hyothetical protein</td>
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<td>interferon, alpha-inducible protein (clone IFI-6-16)</td>
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<td>1.25</td>
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<td>calmodulin-like 3</td>
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<td>myo-inositol 1-phosphate synthase A1</td>
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<td>1.77</td>
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<tr>
<td>Name</td>
<td>M</td>
<td>s</td>
<td>n</td>
<td>t</td>
<td>Δ</td>
</tr>
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<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
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<tr>
<td>apical protein, Xenopus laevis-like</td>
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<td>1.15</td>
<td>17.00</td>
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<td>dickkopf (Xenopus laevis) homolog 1</td>
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<td>similar to rat myomegalin</td>
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<td>1.31</td>
<td>21.00</td>
<td>2.70</td>
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<td>paired box gene 6 (aniridia, keratitis)</td>
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<td>1.24</td>
<td>19.00</td>
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<td>↓</td>
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<td>2.74</td>
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<td>glutaredoxin (thioltransferase)</td>
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<td>1.09</td>
<td>19.00</td>
<td>2.76</td>
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<td>22.00</td>
<td>2.77</td>
<td>↓</td>
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<td>MHC class I region ORF</td>
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<td>15.00</td>
<td>2.77</td>
<td>↓</td>
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<td>hexabrachion (tenascin C, cytotactin)</td>
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<td>1.28</td>
<td>18.00</td>
<td>2.77</td>
<td>↓</td>
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<td>0.44</td>
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<td>1.51</td>
<td>0.99</td>
<td>18.00</td>
<td>2.90</td>
<td>↓</td>
</tr>
<tr>
<td>Name</td>
<td>M</td>
<td>s</td>
<td>n</td>
<td>t</td>
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<td>p8 protein (candidate of metastasis 1)</td>
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<td>EGF-like-domain, multiple 3</td>
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<td>1.47</td>
<td>17.00</td>
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<td>0.45</td>
<td>22.00</td>
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<td>1.94</td>
<td>21.00</td>
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<td>16.00</td>
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<td>1.75</td>
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<td>lectin, galactoside-binding, soluble, 7 (galectin 7)</td>
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<td>2.22</td>
<td>15.00</td>
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<td>1.66</td>
<td>17.00</td>
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<td>1.52</td>
<td>21.00</td>
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<td>1.17</td>
<td>22.00</td>
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<td>H64138No Annotation</td>
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<td>0.81</td>
<td>12.00</td>
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<td>↓</td>
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<td>1.70</td>
<td>0.96</td>
<td>21.00</td>
<td>3.56</td>
<td>↓</td>
</tr>
<tr>
<td>folate receptor 1 (adult)</td>
<td>2.99</td>
<td>2.58</td>
<td>21.00</td>
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<td>2.27</td>
<td>21.00</td>
<td>3.90</td>
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CHAPTER 6

Discussion
6.1 Discussion

Although, due to a variety of technical difficulties, the overall number of experiments completed was small, the results of the cDNA microarray analysis obtained, were consistent with those of similar studies described in the literature. (Villaret 2000; Nagata 2003; Tsai 2004)

Two genes (BIRC 2 and BIRC 3) with anti-apoptotic functions were found to be significantly over expressed in both the OSCC cell lines. This finding is in keeping with the current model of carcinogenesis, in which the genetic and cellular mechanisms that induce “cell suicide”, that is apoptosis, are impaired, thereby allowing inaccuracies in the cell genome to accumulate, and so results in oncogenesis.

Similarly, the genes which were found to be significantly under expressed in the OSCC cell lines (CDN 1b, CDN 1c, GAS1, GAS7, BTG 3, IBP 6, IBP 7, IFM, RHOB) are known to be active in cell cycle arrest, the negative regulation of cell proliferation, and the negative regulation of cell progression through the cell cycle. This is again consistent with the current model of carcinogenesis in which the cell proliferation is unchecked, and therefore both excessive and autonomous (Nagpal 2003). Cell proliferation, and tissue growth is uncoordinated with the normal tissues with cell division continuing, despite DNA damage due to loss of cell cycle check-points (Hanahan 2000; Nagpal 2003).

One of the main reasons that only a small number of genes were found to be significantly differentially expressed in the OSCC cell lines researched in this series of experiments, compared with the non-malignant oral keratinocyte cell line, was because only genes that
were commonly differentially expressed across all 11 OSCC cell lines were “flagged” for analysis. In this way, genes were identified that are thought to be implicated in the overall process of carcinogenesis, rather than unique to a particular OSCC cell line.

As previously discussed, microarray experiments can be performed using either oligonucleotide arrays or cDNA arrays. In the case of the current study, cDNA microarrays were chosen, as these provide information on the amount of each gene present in a study sample relative to that of the control sample. This enabled qualitative analysis and identification of those genes that were relatively over or under expressed in the OSCC cell lines. In contrast, oligonucleotide arrays provide a quantitative analysis on the absolute amount of each gene present (Kuo 2004) whereas cDNA microarrays investigate the components of a cells’ genes and their relationship and proportion to the whole cellular genome.

cDNA microarray experiments can be performed using two main sources of tissue RNA, namely RNA derived from cultured cell lines, and that extracted from whole unfixed tissue.

In this set of experiments, the RNA was extracted from previously long-established OSCC cell lines, and the successfully established, new, non-malignant oral keratinocyte cell line. The main advantage of this method is that cells cultured in-vitro, are a pure population from a single clone, and yield a potentially unlimited supply of consistent RNA for analysis.
Disadvantages of this method include the difficulty in establishing pure cell lines from primary tissue explants, as occurred in these experiments, as well as the risk that cells grown in culture differ in behaviour, and therefore express different genes, compared to cells in-vivo, due to the lack of stromal interactions associated with pure cultured cell lines (Fend 2000).

The difficulties encountered growing OSCC cell lines from primary surgical specimen explants are well reported in the literature. Prime et al (Prime 1990) found of a total of 48 specimens collected, 18 samples (38%) becoming infected with bacteria or yeast; 13 (27%) showing no evidence of cell outgrowth from the explants; 7 (15%) with limited epithelial growth and overgrowth by host fibroblasts; and only 10 (20%) resulted in a viable and continuous cell growth (a success rate of only one in ten). In a similar study (Easty 1981) 7 out of 36 explants (19%) becoming contaminated; and only 10 (27%) showing continuous cell growth. Of these 10, only 2 grew withough contamination by fibroblasts. The remaining 8 explants produced rapidly proliferating fibroblast-like cells which, if not repeatedly reduced in number, either detached colonies of epithelial cells from the culture surface or grew over the epithelial cells and so inhibited their proliferation. These problems that arise during cell line establishment further confirms the concern that laboratory-cultured cell lines are not necessarily an accurate reflection of in-vivo cell behaviour. Clearly, extensive resources, as available in commercial laboratories, will allow the further development and provision of OSCC cell culture lines for further genetic analysis.
The alternative source of RNA for use in microarray experiments is from direct extraction from whole unfixed tissue. While this method does not have the problems of cell culture, several other disadvantages exist. Most surgical specimens would be a mixture of cancer cells and normal and inflammatory cells from the neighbouring tissue and inflammatory infiltrate, thus yielding a “diluted” cancer cell population. Another major disadvantage, especially in the case of OSCC is the need for large surgical specimens to facilitate the extraction of an adequate amount of RNA, which may require an additional amplification step that may distort relative gene levels (Emmert-Buck 2000; Todd 2002). Many large or advanced OSCCs are not amenable to surgical resection, and are instead treated with concurrent chemo-radiotherapy, and so the likelihood of obtaining an adequately sized specimen for cell culture experiments is rare. In addition, another important consideration is the risk of compromising the oncological margins of the resection specimen when removing an adequate amount of tissue to generate the minimum amount of RNA for microarray experiments. This has implications for the histopathological examination, in which visualisation of the tumour margins is of paramount to identify whether the tumour extends beyond the margins of the specimen, indicative that the tumour has been incompletely removed. Residual tumour left in situ consequently requires further treatment and may adversely affect the prognosis for the patient.

The rate of degradation of RNA in unfixed surgical specimens also needs to be considered. Successful microarray experiments rely on extracting an adequate amount of intact RNA. RNAse present in unfixed tissue naturally degrades cellular RNA, and so
RNA extraction from fresh, unfixed tissues, must be performed quickly in order to yield both relatively intact and sufficient RNA for analysis and/or experimentation (Todd 2002). For these reason this method of RNA extraction was not used in these experiments.

One of the most significant advances in medical research has been the completion of the human genome sequence. This has provided the basis of knowledge that will allow further insight into the human genetic code. However, in order to understand the genetic determinants of both health and disease processes, the entire set of genes that are expressed by a distinct cell population at a particular time need to be investigated.

Traditionally, several techniques have been used to detect the expression of genes in a tissue or cell specific manner. These techniques include Northern blotting, in situ hybridization, and the polymerase chain reaction. The limitation of all these techniques is that they are only able to analyse one specific gene or region of a chromosome at a time, thus failing to provide insight into the global gene expression profile of a particular cell or tissue type.

One of the main issues with cDNA microarray is the requirement for the analysis of the enormous amount of data, generated by only a single experiment. The difficulty lies in finding meaningful results from this mass of raw data on gene expression, and this has required advances in the field of bioinformatics, the discipline of piecing together biological information by way of computational methods. Theoretically, microarray
technology can be used to determine and analyse complex biological systems involved in gene and cellular function; however, efficient computerised tools are required to sort through the enormous information that characterises RNA expression so as to find functional relationships.

Increasingly, statistical software programs are becoming available to analyse the data from such microarray experiments. These programs are designed to be used in accordance with the overall aim of the experiment. For example, as used in these experiments, the GoMiner™ software program is specifically designed to look for functional ontologies that are differentially expressed as a group, rather than isolating single genes whose differential expression may not necessarily be significant.

Microarray technology has the potential to greatly enhance knowledge about gene expression, but certain drawbacks exist. Errors in the gene sequences could be incorporated during the manufacture of the arrays, and consequently compromising the fidelity of the DNA fragments immobilized to the array surface have been noted (Knight 2001; Kothapalli 2002). However, one study by Ross et al, the majority of gene sequences spotted onto the microarray were verified as being correct and accurate (Ross 2000).

However, worryingly, Kuo et al compared the data obtained from cDNA microarray and oligonucleotide microarray experiments and found very little correlation between these two different platforms (Kuo 2002). In addition, many investigators have reported
microarray data without confirming the results by other traditional gene expression techniques such as PCR and Northern blot analysis. A study by Kothapalli et al observed variable reliability of differentially expressed data. In this study, cDNA fragments corresponding to differentially expressed genes spotted on the microarrays were excised from the plasmid DNA and used as probes in Northern blots. Only 47% provided positive results as indicated by microarray. Kothapalli et al found that although all the sequences for the down-regulated genes were correct, Northern blot analysis did not confirm differential expression of these genes, in contrast to the microarray data (Kothapalli 2002).

Difficulty in distinguishing between two genes that share a high degree of sequence similarity has also been noted. The low specificity of probes is also a frequently encountered problem. This is especially noticeable when DNA sequences are nearly identical between two genes and the probes are generated from the 3' end of the genes. If the different nucleotide sequence occurs at the 5' end of the gene, there is a possibility that this will not be recognized during hybridization, leading to inaccurate binding (Kothapalli 2002).

Much of the current literature on cDNA microarray has only investigated its use in-vitro; however, this technology may also be applied to other areas of the clinical practice of oncology. The use of cDNA microarray is a relatively new technique, which has the advantage of being able to compare a large number of genes simultaneously in a single experiment. Microarrays allow convenient access to large amounts of genetic
information, so this technique is useful both in research, and may have some clinical applications. Its use in cancer research is promising, with the ability to analyse cancer at the genetic level. Potential uses include the more accurate and comprehensive diagnosis and tumour classification, prognostication, as well as identification of genes that may serve as biomarkers and targets for biological therapy. Pharmacogenomics is the study of how an individual's genetic inheritance affects the body's and individual cells response to drugs, which microarray technology could be used to identify and so exploit. Functional genomics and molecular toxicology could be correlated to the genetic profiles of patients and assess their therapeutic response to certain drugs. This implies that it may be possible to tailor a patient's treatment regime, particularly the chemotherapy arm, depending on their individual genetic profile, and that of their OSCC, for improved clinical outcomes.

In the discipline of Oral Medicine/Oral Pathology, microarray technology is being utilized to assess the malignant potential of oral lichen planus (Bowden 2004). Oral lesions of lichen planus have been studied and their molecular profile, in most cases, has been shown to be unlike the changes seen in dysplastic lesions and OSCC (Mendez 2002). Initial studies have shown that in some cases of dysplasia seen within lichenoid lesions, the molecular profiles share the same genetic pattern as in premalignant and malignant lesions (Kuo 2003). It is expected that gene expression profiles will allow determination of which lesions are at highest risk of malignant progression.

Despite the present complexity in elucidating meaningful information from microarray experiments, as well as issues regarding the accuracy of the data obtained, this technique
is continually being refined. Microarray technology has already started to, and will continue to evolve into a valuable tool for clinical practice.

6.2 Future studies

Further research is needed to validate the results of this present study. Ideally, additional experiments would be performed using more, either commercially available or self-developed OSCC cell lines to increase the study sample size.

The technique for culturing OSCC cells in vitro needs to be further refined, to decrease the rate of contamination and subsequent infection of the explants. New and more efficient methods in controlling the fibroblast overgrowth also need to be developed.

Results obtained regarding differentially expressed genes from the microarray experiments need to be validated using PCR or Northern blot technology.
APPENDICES
Appendix 1:

PARTICIPANT INFORMATION
Head & Neck Cancer Patients

Title of Project: Culture of normal and malignant mouth cells
Name of Investigators: Dr SC Yeoh
                            Dr G Morgan
                            Dr I Kalmins
                            Dr YS Wimardhani
                            Dr S Chan
                            Professor DM Walker

What is the purpose of the study?
This research project aims to establish continuous cultures of cancer cells in our
laboratory in order to examine the behaviour of these cells and their expression of cancer-
related genes and to compare their interactions with that of cells grown from normal
mouth tissues. We would like to ask you for some cancer and, where appropriate, normal-
appearing adjacent tissues derived from your surgical specimen so that we might attempt
to grow a cell line to facilitate the studies related to mouth cancer.

This research project aims to grow cancer cells in the laboratory to examine their
behaviour and the genetic information they contain which controls their interaction with
normal cells from the inside of the mouth.

Who will be invited to enter the study?
You have been invited to join this study because you are undergoing a surgical procedure
as part of the treatment for your cancer.

What will happen in the study?
This study involves the use of a limited amount of tissues taken from your cancer as well
as adjacent normal tissue that are removed as part of the planned treatment for your
cancer. The fresh tissue samples will be used, where possible, in the establishment of
respective cell lines in our laboratory. The cancer cells will then be examined for their
behaviour and their expression of cancer-related genes in our studies related to mouth
cancer.
Are there any risks?
This will place no extra demands on you, will not affect your operation in any way and will not put you at any additional risk.

Confidentiality
All aspects of this study, including the results, will be strictly confidential and only the researchers will have access to your personal information. Original data will be stored in a locked office and entered into a database that will be password-protected. Any publication of the results from this study will only use unidentifiable information.

Do you have a choice?
Participation in this study is voluntary and you may withdraw from the project at any time. If you do not wish to participate in this study, your decision will not affect your treatment.

Complaints
If you have any concerns regarding the conduct of the study, please contact the Westmead Hospital Patient Representative, Ms Jillian Gwynne Lewis, Telephone No 9845 7014, Email address: jillian_lewis@wsahs.nsw.gov.au

Contact Details
If you have any problems while on the study, please contact Dr SC Yeoh or Dr Sheena Chan

Telephone No (working hours) 9845 7892 or 9845 7879
Telephone No (after hours) 9498 6082

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CONSENT TO PARTICIPATE IN RESEARCH

Title of Research Project: Culture of normal and malignant mouth cells

Name of Researchers: Dr S-C Yeoh  
Dr G Morgan  
Dr I Kalnins  
Dr YS Wimardhani  
Dr S Chan  
Professor DM Walker

1. I understand that the researcher will conduct this study in a manner conforming with ethical and scientific principles set out by the National Health and Medical Research Council of Australia and the Good Clinical Research Practice Guidelines of the Therapeutic Goods Administration.

2. I acknowledge that I have read, or have had read to me the Participant Information Sheet relating to this study. I acknowledge that I understand the Participant Information Sheet. I acknowledge that the general purposes, methods, demands and possible risks and inconveniences which may occur to me during the study have been explained to me by ______________________ (“the researcher”) and I, being over the age of 16 years or over the age of 14 years but under the age of 16 years (delete as applicable), acknowledge that I understand the general purposes, methods, demands and possible risks and inconveniences which may occur during the study.

3. I acknowledge that I have been given time to consider the information and to seek other advice.

4. I acknowledge that refusal to take part in this study will not affect the usual treatment of my condition.

5. I acknowledge that I am volunteering to take part in this study and I may withdraw at any time.

6. I acknowledge that this research has been approved by the Western Sydney Area Health Service Human Research Ethics Committee.

7. I acknowledge that I have received a copy of this form and the Participant Information Sheet, which I have signed.

8. I acknowledge that sponsoring pharmaceutical companies and any regulatory authorities may have access to my medical records to monitor the research in which I
am agreeing to participate. However, my identity will not be disclosed to anyone else. *(Please delete this paragraph if not applicable)*

*Before signing, please read ‘IMPORTANT NOTE’ following.*

Name of participant ___________________________ Date of Birth __________________

Address of participant ____________________________

Name of parent or guardian (where applicable) ____________________________

Address of parent or guardian (where applicable) ____________________________

Signature of participant ___________________________ Date: __________________

Signature of parent or guardian (where applicable) ___________________________ Date: __________________

Signature of researcher ___________________________ Date: __________________

Signature of witness ___________________________ Date: __________________
IMPORTANT NOTE

This consent should only be signed as follows:
1. Where a participant is over the age of 16 years, then by the participant personally.
2. Where the participant is between the age of 14 and 16 years, it should be signed by the participant and by a parent or guardian.
3. Where the participant is under the age of 14 years, then the parent or guardian only should sign the consent form.
4. Where a participant is under a legal or intellectual disability, e.g., unconscious, then particular consent should be sought from the Human Research Ethics Committee as to whether the person should take part in the research.

INDEPENDENT WITNESS:

I, __________________________________________ (name of independent witness)

of __________________________________________ hereby certify as follows:

1. I was present when __________________________________________ (“the participant”) appeared to read or had read to him/her a document entitled Participant Information Sheet; or
   I was told by __________________________________________ (“the participant”) that he/she had read a document entitled Participant Information Sheet
   (*Delete as applicable)

2. I was present when __________________________________________ (“the researcher”) explained the general purposes, methods, demands and the possible risks and inconveniences of participating in the study to the participant. I asked the participant whether he/she had understood the Participant Information Sheet and understood what he/she had been told and he/she told me that he/she did understand.

3. I observed the participant sign the consent to participate in research and he/she appeared to me to be signing the document freely and without duress.

4. The participant showed me a form of identification which satisfied me as to his/her identity.

5. I am not involved in any way as a researcher in this project.

6. (Delete this clause if not applicable) I was present when
_____________ ("the interpreter") read the Participant Information sheet to the participant in the _______________ (here insert appropriate language) language. I certify that when the researcher explained the general purposes, methods, demands and possible risks and inconveniences of participating in the study that what was said by both the researcher and the participant was translated by the interpreter from the English language into the _______________ language and vice versa. When I spoke to the participant what I said and what the participant said was translated by the interpreter from the English language into the _______________ language and vice versa.

Name of independent witness

________________________________________

Address

________________________________________

Signature of independent witness ___________________________ Date: __________________

Relationship to participant of independent witness

________________________________________
CONSENT TO PARTICIPATE IN RESEARCH

INTERPRETER:
If an interpreter is used, the following addition is necessary –

I ____________________________________________ (name of interpreter)

of __________________________________________ certify
as follows:

1. I am qualified to translate speech and writing from the English language into the
   __________________ language and vice versa.

2. I read the Participant Information Sheet to the participant in the
   __________________ language and he/she appeared to understand it.

3. I was present when the researcher explained the general purposes, methods, demands
   and possible risks and inconveniences of participating in the study to the participant
   and I translated all that was said by the researcher and by the participant from the
   English language into the __________________ language and vice versa.

4. I was present when the independent witness spoke to the participant and I translated
   all that was said by the independent witness and by the participant from the English
   language into the __________________ language and vice versa.

Signature of Interpreter ___________________ Date ___________________
Appendix 2:

Western Sydney HEALTH
Area Health Service

PARTICIPANT INFORMATION
DENTAL PATIENTS WITHOUT CANCER HAVING TEETH REMOVED OR OTHER MOUTH OPERATION

Title of Project: Culture of normal and malignant mouth cells
Name of Investigators: Dr SC Yeoh
Dr G Morgan
Dr I Kalnins
Dr YS Wimardhani
Dr S Chan
Professor DM Walker

What is the purpose of the study?
This research project aims to establish continuous cultures of cancer cells in our laboratory in order to examine the behaviour of these cells and their expression of cancer-related genes and to compare their interactions with that of epithelial cells grown from normal oral tissues. We would emphasise that you do not have a diagnosis of oral cancer, but we would like to ask you for some normal oral tissue to enable us to grow normal oral epithelial cell lines to facilitate the studies related to oral cancer.

Who will be invited to enter the study?
You have been invited to join this study because you are undergoing removal of teeth, gum or other oral surgery or periodontal procedures and do not have any sign of mouth cancer.

What will happen in the study?
This study involves the use of a small part of normal oral tissue such as gum, which is often removed routinely during the removal of teeth. The fresh tissue sample will be used, where possible, in the establishment of a normal oral epithelial cell line in our laboratory. The normal oral epithelial cells will then be examined for their behaviour and used as normal control tissue for comparison in our studies related to oral cancer. The cells grown from your oral tissue sample will be grown for approximately 2 subcultures before they will be discarded.

Are there any risks?
This will place no extra demands on you, will not affect your operation in any way and will not put you at any risk.
Confidentiality
All aspects of this study, including the results, will be strictly confidential and only the researchers will have access to your personal information. Original data will be stored in a locked office and entered into a database that will be password-protected. Any publication of the results from this study will only use unidentifiable information.

Do you have a choice?
Participation in this study is voluntary and you may withdraw from the project at any time. If you do not wish to participate in this study, your decision will not affect your treatment.

Complaints
If you have any concerns regarding the conduct of the study, please contact the Westmead Hospital Patient Representative, Ms Jillian Gwynne Lewis, Telephone No 9845 7014, Email address: jillian_lewis@wsahs.nsw.gov.au

Contact Details
If you have any problems while on the study, please contact Dr SC Yeoh or Dr Sheena Chan

Telephone No (working hours) 9845 7892 or 9845 7879
Telephone No (after hours) 9498 6082
CONSENT TO PARTICIPATE IN RESEARCH

Title of Research Project: Culture of normal and malignant mouth cells

Name of Researchers: Dr S-C Yeoh
                        Dr G Morgan
                        Dr I Kalmins
                        Dr YS Wimardhani
                        Dr S Chan
                        Professor DM Walker

9. I understand that the researcher will conduct this study in a manner conforming with ethical and scientific principles set out by the National Health and Medical Research Council of Australia and the Good Clinical Research Practice Guidelines of the Therapeutic Goods Administration.

10. I acknowledge that I have read, or have had read to me the Participant Information Sheet relating to this study. I acknowledge that I understand the Participant Information Sheet. I acknowledge that the general purposes, methods, demands and possible risks and inconveniences which may occur to me during the study have been explained to me by _____________________ ("the researcher") and I, being over the age of 16 years or over the age of 14 years but under the age of 16 years (delete as applicable), acknowledge that I understand the general purposes, methods, demands and possible risks and inconveniences which may occur during the study.

11. I acknowledge that I have been given time to consider the information and to seek other advice.

12. I acknowledge that refusal to take part in this study will not affect the usual treatment of my condition.

13. I acknowledge that I am volunteering to take part in this study and I may withdraw at any time.

14. I acknowledge that this research has been approved by the Western Sydney Area Health Service Human Research Ethics Committee.

15. I acknowledge that I have received a copy of this form and the Participant Information Sheet, which I have signed.
16. I acknowledge that sponsoring pharmaceutical companies and any regulatory authorities may have access to my medical records to monitor the research in which I am agreeing to participate. However, my identity will not be disclosed to anyone else. *(Please delete this paragraph if not applicable)*

*Before signing, please read ‘IMPORTANT NOTE’ following.*

Name of participant ___________________________ Date of Birth ________________

Address of participant
_________________________________________

Name of parent or guardian (where applicable)
_________________________________________

Address of parent or guardian (where applicable)
_________________________________________

Signature of participant ___________________________ Date: ________________

Signature of parent or guardian (where applicable) ___________________________ Date: ________________

Signature of researcher ___________________________ Date: ________________

Signature of witness ___________________________ Date: ________________
IMPORTANT NOTE

This consent should only be signed as follows:
5. Where a participant is over the age of 16 years, then by the participant personally.
6. Where the participant is between the age of 14 and 16 years, it should be signed by the participant and by a parent or guardian.
7. Where the participant is under the age of 14 years, then the parent or guardian only should sign the consent form.
8. Where a participant is under a legal or intellectual disability, eg unconscious, then particular consent should be sought from the Human Research Ethics Committee as to whether the person should take part in the research.

INDEPENDENT WITNESS:

I, ________________________________ (name of independent witness)
of ________________________________ hereby certify as follows:

7. I was present when ________________________________ ("the participant") appeared to read or had read to him/her a document entitled Participant Information Sheet; or
   I was told by ________________________________ ("the participant") that he/she had read a document entitled Participant Information Sheet
   (*Delete as applicable)

8. I was present when ________________________________ ("the researcher") explained the general purposes, methods, demands and the possible risks and inconveniences of participating in the study to the participant. I asked the participant whether he/she had understood the Participant Information Sheet and understood what he/she had been told and he/she told me that he/she did understand.

9. I observed the participant sign the consent to participate in research and he/she appeared to me to be signing the document freely and without duress.

10. The participant showed me a form of identification which satisfied me as to his/her identity.

11. I am not involved in any way as a researcher in this project.

12. (Delete this clause if not applicable) I was present when
("the interpreter") read the Participant Information sheet to the participant in the ____________ (here insert appropriate language) language. I certify that when the researcher explained the general purposes, methods, demands and possible risks and inconveniences of participating in the study that what was said by both the researcher and the participant was translated by the interpreter from the English language into the ____________ language and vice versa. When I spoke to the participant what I said and what the participant said was translated by the interpreter from the English language into the ____________ language and vice versa.

Name of independent witness
_________________________________________________________

Address
_________________________________________________________

Signature of independent witness __________________________ Date: ____________

Relationship to participant of independent witness
_________________________________________________________
CONSENT TO PARTICIPATE IN RESEARCH

INTERPRETER:

If an interpreter is used, the following addition is necessary –

I ____________________________________________ (name of interpreter)

of ____________________________________________ certify as follows:

5. I am qualified to translate speech and writing from the English language into the _____________ language and vice versa.

6. I read the Participant Information Sheet to the participant in the _____________ language and he/she appeared to understand it.

7. I was present when the researcher explained the general purposes, methods, demands and possible risks and inconveniences of participating in the study to the participant and I translated all that was said by the researcher and by the participant from the English language into the _____________ language and vice versa.

8. I was present when the independent witness spoke to the participant and I translated all that was said by the independent witness and by the participant from the English language into the _____________ language and vice versa.

Signature of Interpreter ___________________ Date ___________________
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ESSAYS

The following two essays were submitted in November 2004 (MDSc Program Year 1).

In retrospect, the structure, style and content reflect an early and immature understanding of each topic, compared with the current depth of knowledge that has been obtained as a result of my progression through, and near completion of, the MDSc program.
MDSc Oral Medicine/Oral Pathology

Year 1, Essay 1

By Sue-Ching Yeoh

2004

What is meant by the term epithelial dysplasia? Describe the histopathological/cellular features and critically review the various grading systems and their prognostic significance.
Introduction:

Epithelial dysplasia (dys- meaning “bad or abnormal”, plasia – meaning “growth”) is a term which is used widely in oral pathology to describe a variety of architectural and cytological changes in the epithelium that may indicate an increased risk of malignant transformation. Other commonly used terminology includes – oral intraepithelial neoplasia (OIN), and squamous intraepithelial neoplasia (SIN). There are several grading systems currently in use to describe and attempt to categorise these changes in order to predict prognosis, however, many studies have made note of the limitations of these systems. Many difficulties are related to the lack of inter- and intra-observer reliability, with the main problem being the categorising of features that are part of a continuum of epithelial change.

The development of oral cancer is often preceded by other lesions in the oral cavity. These lesions may show cellular changes that indicate the possible subsequent development of malignancy. The individual cellular changes are often termed “atypia”, and the general disturbance of the epithelium termed “dysplasia”.¹

In 1997, the World Health Organisation defined squamous epithelial dysplasia as “a precancerous lesion of stratified squamous epithelium characterized by cellular atypia and loss of normal maturation and stratification short of carcinoma in situ”.² In broad terms, there is a disturbance in the normal growth, proliferation, maturation and organisation of the cells.
Features:

The WHO also specifies the nature of the histological changes that one should expect to observe in epithelial dysplasia:

1. Loss of polarity of the basal cells
2. More than one layer of cells of basaloïd appearance
3. Increase nuclear – cytoplasm ratio
4. Drop-shaped rete ridges – the rete ridges become bulbous in shape
5. Irregular epithelial stratification – the organisation of the individual cell layers becomes disturbed, with no clearly differentiated basal and spinous layers.
6. Increased number of mitotic figures
7. Mitotic figures that are abnormal in form
8. The presence of mitotic figures in the superficial half of the epithelium
9. Cellular and nuclear pleomorphism
10. Nuclear hyperchromatism
11. Enlarged nucleoli
12. Loss of intercellular adherence
13. Keratinisation of single cells or cell groups in the prickle cell layer.

Importantly, it must be noted that not all these changes are necessarily observed in any one case. However, the more prominent or numerous these changes are, the more severe the grade of dysplasia. It is also important to realise that some cellular atypia may be present in other settings, such as in inflammatory conditions, lichen planus, candidiasis and in regenerating epithelium.
Figure 1 - Dysplasia: hyperkeratosis, loss of basal cell polarity, abnormal mitoses in the spinous layer. Taken from “Oral Diseases” 3rd Ed. page 14.22

Figure 2 – Dysplasia: drop-shaped rete ridges with patchy areas of hyperkeratosis. Taken from “Oral Diseases” 3rd Ed. page 14.22
Figure 3 – Dysplasia: widespread nuclear hyperchromatism. Taken from “Oral Diseases” 3rd Ed. page 14.22³

Figure 4 – Dysplasia: loss of intercellular adherence. Taken from “Oral Diseases” 3rd ed. page 14.23³
Figure 5 – Dysplasia low power: deep cell keratinisation extending almost to the basal cell layer and associated with other features of dysplasia, notably a giant hyperchromatic nucleus. Taken from “Oral Diseases” 3rd Ed. page 14.23

Figure 6 – Dysplasia higher power: individual keratinize cells are scattered at varying depths on the spinous layer, which shows other features of dysplasia, notably increased nuclear to cytoplasm ratio, hyperchromatism and loss of basal cell polarity. Taken from “Oral Diseases” 3rd Ed. page 14.23
Historical Grading Systems:

In an effort to quantify the histological changes observed in epithelial dysplasia, and in order to extrapolate what is seen microscopically, to some sort of indication of the risk of malignant transformation and prognosis, several grading systems have been developed. Most journal papers identify the same list of features of dysplasia, however, this is not accompanied by any explanation of how the severity of the disturbances should be recorded. In the literature, there is also very little mention of the weighting of individual characteristics, or if they should all be of equal importance. Although the criteria for the recognition of these individual dysplastic features have been described in considerable detail, the final assessment is essentially subjective.

Historically, Smith and Pindborg 1969 attempted to address the issue of the lack of inter- and intra-observer reliability by developing a standardized system of grading oral epithelial dysplasia on an objective and semi-quantitative level. The observer first concentrated on one photographically standardized microscopic feature at a time, and then allocated a weighted score to each. A grading of “absent” was given a score of 0, whilst a grading of “slight” or “marked” was scored between 1 and 10. Some features were given a higher score than others, for example, marked pleomorphism of cells and nuclei were given a score of 6, whilst marked basal cell hyperplasia received a score of 4. This implies that the authors felt that marked pleomorphism had more serious implications that basal cell hyperplasia, which in turn suggests a certain subjectivity. The final score was the summation of individual feature scores, and was expressed as a score out of a possible total of 75. A lesion which scored between 11 and 25 was considered
mildly dysplastic, 26 and 45 as moderately dysplastic, and higher than 45 were considered severely dysplastic. Originally intended for research purposes, this system has not been used widely for the practical grading of lesions.⁴ Katz et al 1985⁵ found this method of grading valuable for the purposes of standardization. However, the question of the accuracy of the weighting given to each characteristic remains, with the suggestion that further investigations were needed to assess which histological features are most valuable in predicting the potential for the development of malignancy. Over time, this method for scoring dysplasia has proven too cumbersome to use on a daily basis. It has also proven relatively insensitive, with some hyperplastic lesions attaining high scores.⁶

Other methods have been trialed in the past. Kramer et al⁷ used computer-aided analysis of histological features of oral epithelial dysplasia. 1974. Bancoczy and Csiba⁸ diagnosed epithelial lesions as dysplastic when two or more of the above listed features were observed.

**Current Grading Systems:**

The system that is currently used most frequently by pathologists is based on the WHO list of histological changes. This scheme divides the changes seen in epithelial dysplasia into the categories of “mild”, “moderate”, and “severe”.

1. Mild epithelial dysplasia – “minimal” dysplastic alterations confined to the lower third of the epithelium. There may be slight nuclear abnormalities, mostly marked in the basal third of the epithelial thickness and minimal in the upper layers, where the cells show maturation and stratification. A few, but no
abnormal mitoses may be present, usually accompanied by keratosis and chronic inflammation.

2. Moderate epithelial dysplasia – dysplastic changes seen in up to two thirds of the thickness of the epithelium. The lesion displays more marked nuclear abnormalities and nucleoli tend to be observed, with changes most marked in the basal two thirds of the epithelium. Nuclear abnormalities may persist up to the surface, but cell maturation and stratification are evident in the upper layers.

3. Severe epithelial dysplasia – dysplastic cells fill more that two thirds but less than the entire thickness of the epithelium. Lesions show marked nuclear abnormalities and loss of maturation involving more that two thirds of the epithelium, with some stratification of the most superficial layers. Mitoses, some of which are abnormal, may be present in the upper layers. The presence of some maturation and stratification of the cells in the most superficial parts of the lesion distinguishes it from carcinoma in situ (CIS).

Sometimes, the additional categories of “carcinoma in situ” and “verrucous hyperplasia with dysplasia” are also used.

4. Carcinoma in situ (CIS) – the entire thickness of the epithelium contains less differentiated basaloid or squamous epithelial cells with enlarged, hyperchromatic nuclei and a variable number of typical and atypical mitotic figures; no invasion of the submucosa is to be noted in numerous sections.

5. Verrucous hyperplasia with dysplasia – when the epithelium exhibits considerable thickening with surface papillations, hyperparakeratosis and parakeratin plugging, with the occasional dysplastic cells confined to the lower third of the epithelium.
Figure 7 – Mild epithelial dysplasia: hyperchromatic and slightly pleomorphic nuclei in the basal and parabasal cell layers. Taken from “Oral and Maxillofacial Pathology” 2nd Ed. page 343

Figure 8 – Moderate epithelial dysplasia: changes extend to the midpoint of the epithelium and are characterized by nuclear hyperchromatism, pleomorphism, and basal cell hyperplasia. Taken from “Oral and Maxillofacial Pathology” 2nd Ed. page 344
Figure 9 – Severe epithelial dysplasia: changes are found throughout the epithelium. There is total loss of differentiation between basal and spinous cells with many irregular pleomorphic, hyperchromatic nuclei. Taken from “Oral Diseases” 3rd Ed. page 14.23

Figure 10 – Severe full thickness dysplasia. Taken from “Lucas’s Pathology of Tumours of the Oral Tissues” 5th Ed. page 224
Variations to this system are abundant, with some pathologists preferring to grade dysplasia into the categories of “focal mild”, “mild”, “moderate”, and “severe”. The designation of “focal mild” was used in cases which displayed histologic features of dysplasia only in isolated microscopic foci. Other pathologists use just two categories — “mild” and “severe”.

The new WHO system:

The new WHO oral epithelial dysplasia grading system is being finalized for publication in late 2004/ early 2005. This aims to update the traditional system, including the addition of the category of “carcinoma in situ”, and also includes the Ljubljana classification for laryngeal lesions. These guidelines recommend an initial assessment of the architecture of the lesion followed by an assessment of the cytological disturbance. For a diagnosis of epithelial dysplasia, changes in both elements must be observed.
<table>
<thead>
<tr>
<th>Architectural Features</th>
<th>Cytological Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irregular stratification</td>
<td>Anisonucleosis</td>
</tr>
<tr>
<td>Loss of basal cell polarity</td>
<td>Abnormal variation in nuclear shape</td>
</tr>
<tr>
<td>Drop shaped rete ridges</td>
<td>Anisocytosis</td>
</tr>
<tr>
<td>Increased numbers of mitotic figures</td>
<td>Abnormal variation in cell shape</td>
</tr>
<tr>
<td>Abnormally superficial mitotic figures</td>
<td>Increased nuclear:cytoplasm ratio</td>
</tr>
<tr>
<td>Premature keratinisation in single cells</td>
<td>Increased nuclear size</td>
</tr>
<tr>
<td>Keratin pearls in rete ridges</td>
<td>Atypical mitotic figures</td>
</tr>
<tr>
<td></td>
<td>Increased number and size of nucleoli</td>
</tr>
<tr>
<td></td>
<td>Nuclear hyperchromatism</td>
</tr>
</tbody>
</table>

Table 1 – Criteria for the new WHO dysplasia grading system. Taken from Odell E. “Recognition and Grading of Oral Dysplasia”. Page S407

The new WHO guidelines describe the minimum criterion for a diagnosis of mild dysplasia as an architectural disturbance limited to the lower third of the epithelium.

Moderate dysplasia shows architectural disturbances extending into the middle third of the epithelium. The lesion may then be designated to the severe dysplasia category if there is marked cytological atypia. Conversely, it may be designated to the mild dysplasia category if only mild cytological atypia is observed. Severe dysplasia encompasses lesion where the architectural disturbances affect more than two-thirds of the thickness of the epithelium. The term “carcinoma in situ” implies that there is full thickness cytological atypia, and that malignant transformation has occurred but invasion is not present.

The new system does not detail how individual features should be scored, or combined to make the final grade, and so the issue of subjective grading still exists.
Other Grading Systems:

Many pathologists in the United States have adopted the grading system of oral intraepithelial neoplasia (OIN), which shares similarities to the cervical intraepithelial neoplasia (CIN) system. The basis of the CIN system is that one or more clones of transformed cells slowly replace normal keratinocytes, starting from basal and parabasal layers to progressively invade the whole epithelial height. This concept has been used to grade changes in various other tissues, and more recently, oral mucosa (OIN). As for CIN, there are three grades of OIN: OIN 1 – true mild dysplasia, OIN 2 – moderate dysplasia, OIN 3 – severe dysplasia and carcinoma in situ.

Certain groups have modified this OIN system to further correlate with grading of cervical lesions by following the Bethesda classification, which merges the different grades into two categories: low grade squamous intraepithelial lesions (LSIL) corresponding to CIN 1, and high grade squamous intraepithelial lesions (HSIL), corresponding to CIN 2 and CIN 3. Articles have reported an improvement in the consistency of grading in this two category system, and consequently, some groups have chosen to modify the OIN system in the same manner. Low grade oral intraepithelial neoplasia (LOIN) correlating with OIN 1 (mild dysplasia) and high grade oral intraepithelial neoplasia (HOIN) correlating with OIN 2 (moderate dysplasia) and OIN 3 (severe dysplasia and CIS). 14

This grading system has also come under close scrutiny, with very similar issues raised as for the traditional WHO grading method. Subjectivity of grading, lack of consistency between pathologists, as well as by the same pathologist on different occasions, and also
the lack of clear guidelines as to which features of epithelial change are more important in terms of prognosis.

The inclusion of OIN 2 (moderate dysplasia) in the HOIN category along with OIN 2 (severe dysplasia and CIS) also seems inappropriate as these lesions have been shown to have a markedly lower potential for malignant transformation.\textsuperscript{15,16}

Recently, the term “squamous intraepithelial neoplasia” (SIN) has been proposed to include all parts of the upper aero-digestive tract. The grading system proposed using this terminology is very similar to the OIN system, with SIN 1 designated to lesions showing mild dysplasia, SIN 2 for moderately dysplastic lesions, and SIN 3 encompassing both severely dysplastic lesions as well as CIS. This system represents a change in terminology rather than an alteration to grade.\textsuperscript{17}

The Ljubljana system was initially introduced to grade laryngeal hyperplastic epithelial lesions.\textsuperscript{18,19} Its recent application has been to the grading of oral hyperplastic lesions, in an attempt to correlate the degree and type of hyperplasia with the risk of malignant transformation, thus indicating prognosis as well as carrying implications for treatment. All lesions are categorised as follows:\textsuperscript{20}
<table>
<thead>
<tr>
<th>Category</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple hyperplasia</td>
<td>Acanthosis, no cellular atypia</td>
</tr>
<tr>
<td>Abnormal hyperplasia</td>
<td>Hyperplasia in up to half of the epithelial thickness with some cells showing enlarged nuclei but normal chromatin. normal stratification, typical basal layer mitoses. &lt;5% dyskeratotic cells</td>
</tr>
<tr>
<td>Atypical hyperplasia/“risky” epithelium</td>
<td>Cellular and nuclear pleomorphism, increased nuclear:cytoplasm ratio, nuclear hyperchromatism, increased mitoses (may be abnormal or suprabasal), dyskeratosis, stratification still generally preserved</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>Features of carcinoma without invasion</td>
</tr>
</tbody>
</table>

It should be noted that in this system, (in contrast to some other classifications), the presence of surface keratinisation does not influence the grading. It has been postulated that this feature has no prognostic significance. Dyskeratosis, on the other hand, most often displays abnormal premature keratinisation of the individual epithelial cells throughout the epithelium, and should be considered as a histological feature of premalignancy.\textsuperscript{20}

The clinical applicability and prognostic value of this grading system have been confirmed for laryngeal lesions, and it is believed that it could be successfully applied to oral epithelial hyperplastic lesions. The biological behaviour of epithelial hyperplasia may vary according to its location in the upper aero-digestive tract, however, there is little doubt that these lesions have more in common with laryngeal lesions than with those observed in the uterine cervix. Another advantage is that the histological criteria are clearly defined and well illustrated,\textsuperscript{18,19} which should decrease interobserver variation. This system has shown prognostic value in oral epithelial hyperplasia since the
progression to invasive carcinoma has only been observed in lesions showing atypical hyperplasia and CIS.\textsuperscript{20}

**Critical Evaluation of Grading Systems:**

The main problem that arises from many of these grading systems is one of inter- and intra-observer reliability. The issue of subjectivity in assessing oral epithelial dysplasia has often been raised, and is usually attributed to the lack of well-defined criteria and guidelines for reporting these lesions. No guidelines have been published recently as to which features of dysplasia indicate a poorer prognosis, or if all features should be weighted equally. Oral epithelial dysplasia and neoplasia comprise a continuum from mild dysplasia to carcinoma. The different grading categories represent arbitrary points along this continuum. In its very nature, this lends itself to subjectivity, with individual variations existing among observers. There have been also been disagreements as to which criteria contribute more to a lesion’s diagnosis. Most pathologists use the same list of features to grade dysplastic lesions, however, some characteristics are open to individual interpretation. This variability can be particularly problematic, as incorrect or inconsistent diagnoses may lead to over- or under-treatment and different prognoses for the same lesion.

A recent study involving four pathologists, each examining 100 histological slides reported and interobserver percentage agreement ranging form 49-69% between different examiner pairs.\textsuperscript{21}
Similar studies reported interobserver agreement percentages between 54-94%.

Intraobserver agreement was also found to be poor in these studies.⁹,²²

In 1985, Pindborg et al²³ reported displaying nine photomicrographs of oral lesions at a scientific meeting of oral pathologist. Each of the 72 participants submitted a diagnosis of the lesions ranging from no dysplasia to frank carcinoma. The range of agreement for different histological diagnosis varied from 1-78% (e.g. A case of CIS of the buccal mucosa had 1% agreement, and a CIS in the floor of mouth had 78% agreement).

Another study showed that interobserver reliability differed according to the site of the lesion, with buccal mucosa/vestibule and tongue showing good agreement, and lip and labial mucosa showing poor agreement. The presence of inflammation was also regarded as contributing to a poor agreement.²⁴

A study by Brothwell et al²⁵ showed a substantial improvement in inter- and intra-observer reliability, with the intra-observer agreement level surpassing that of inter-observer agreement. The authors concluded that observer bias can arise from the use of slightly different diagnostic thresholds resulting in a slightly different proportion of cases assigned to each diagnostic group. This type of bias was explained as difference in training, as well as different opinions on which histological features are most important.

The current grading systems are imperfect, however, the issue of reproducibility may not be as dismal as initially thought.

As pointed out by Professor Edward Odell, “The kappa statistic used to measure reproducibility in almost all research studies is a non-parametric test designed for
nominal categories (eg separating different diagnoses, sorting cats from dogs) whereas grading dysplasia is the arbitrary division of a continuum of severity of change (eg separating colours of a rainbow.) Reproducibility as judged by the kappa statistic should not necessarily be the aim. There are a number of features which might explain lack of agreement in grading; it is primarily subjective, the severity of individual features is not defined by grade, the prevalence of individual features differs between mild, moderate and severe categories, between sites in the mouth and with candidal infection.

However, when data are compared with other scoring systems for premalignancy, oral dysplasia grading appears relatively good. For instance, grading premalignancy in the breast has a kappa score of 0.17 rising to 0.25 for the most experienced pathologists and cervical dysplasia of only 0.41 indicating poor agreement. However, no one suggests that this invalidates clinical use, only that interpretation must take this into account.¹³

One other issue that can arise in grading dysplasia is that of specimen sampling. Pathologists traditionally base their histological diagnosis on the worst area of the specimen. It is possible that an area designated “severe epithelial dysplasia” may be adjacent to an area of invasive carcinoma adjacent to the biopsy margin. Most pathologists will keep this in mind, with recommendations of surgical excision of the areas involved often suggested.
Implication For Prognosis And Treatment:

The relationship between the various grades of epithelial dysplasia to the development of cancer is not clear cut. The histological diagnosis does not always correlate with clinical behaviour. Some severely dysplastic lesions remain the same for many years. Other lesions, mainly classified as mild-moderate dysplasia, have been known to regress, and on re-biopsy, no sign of dysplasia is found. On the other hand, mildly dysplastic lesions have occasionally been reported to rapidly become frankly malignant. However, in general, the degree of dysplasia is linked to the degree of probability of malignant transformation. Mildly dysplastic lesions do not indicate any major danger for the patient (although special reference should be made to high risk sites, such as the floor of the mouth and the ventral surface of the tongue, where even the slightest sign of dysplasia is significant). A more cautious approach should be taken for moderately dysplastic lesions, and severe dysplasia and CIS indicates that there is a very considerable risk of the development of cancer.¹

Difficulties arise when dysplastic lesions are inaccurately graded. This has implications for treatment, with some lesions potentially being over or under treated, with this significantly impacting on prognosis.

The Way Of The Future:

Currently, histological grading of oral epithelial dysplasia is most recognized predictor of malignant transformation. In recent times, numerous studies have explored the use of molecular markers as indicators of prognosis and risk of malignant change. Examples of
this include the over-expression of p53 and Ki-67 in precursor lesions being strongly linked with progression to cancer.\textsuperscript{26,27,28,29}

Studies by Sudbo et al\textsuperscript{30,31} have investigated the role of ploidy as an indicator of chromosomal stability, with aneuploid lesions showing an increased risk of malignant transformation compared with diploid cells. The predictive accuracy of these findings is far superior to that of histological grading.

All these techniques are currently being researched in the hope that a more accurate and reproducible method of predicting the risk and rate of malignant transformation will be developed. So far, the results are promising, but until these methods are fully trialed, reliance lies in the histological guidelines that are currently in use.
References:


6. Warnakulasuriya S. Histological grading or oral epithelial dysplasia: revisited. *J Pathol* 2001; 194 (3); 294-297


MDSc Oral Medicine/Oral Pathology

Year 1, Essay 2

By Sue-Ching Yeoh

November 2004

Outline the management of a patient presenting with xerostomia and unilateral salivary gland swelling.
**Introduction:**

Xerostomia is the abnormal reduction of saliva and can be a symptom of certain diseases or an adverse effect of certain medications.\(^1\) On average, a person produces 500ml of saliva in 24 hours. Daily flow rates vary depending on the demand or the current physiologic status of the patient. The unstimulated flow rate is 0.3 mL/min, whereas the flow rate during sleep is 0.1 mL/min. This can increase to 4.0 to 5.0mL/min during eating.

The submandibular glands produce the most saliva, accounting for about 70% of the total volume. 20% of the total volume of saliva is produced by the parotid glands, and only 1-2% from the sublingual glands. The remainder comes from the minor salivary glands.\(^2\)

Saliva contributes to all the functions of the mouth, and has a vital protective role in maintaining the health of oral tissues. It is a lubricant, protecting the mucosa from abrasions and dehydration, as well as aiding in swallowing and speech. Saliva has a mechanical cleansing action, contains lysozymes, histatins, lactoferrin, lactoperoxidase and immunoglobulins which contribute towards its antibacterial activity. Saliva plays an important role in protecting teeth against acid dissolution, by buffering acid with its high bicarbonate content. This buffering capacity is further increased by the supersaturation of calcium phosphate, which act to reduce demineralization and increase remineralisation of enamel. It can act as a solvent for food, contributing to the sensation of taste. Saliva contains amylase, which acts to digest carbohydrates, however this has a limited digestive function on the mouth and this may continue in the stomach within the food bolus. It
plays a role in the thirst signaling pathway, and has also been known to play a significant role in accelerating the clotting of blood.  

Salivary gland secretion is mainly under autonomic nervous control, but various hormones may also modulate salivary composition. Secretion is triggered by parasympathetic or sympathetic pathways, and when both are active, their effects are synergistic. Parasympathetic stimulation produces a copious flow of saliva containing little protein, whereas sympathetic stimulation produces little saliva but it contains a high protein concentration which may give a sensation of dryness. Excitation of either sympathetic or parasympathetic nerves to the salivary glands stimulates salivary secretion, but the effects of the parasympathetic nerves are stronger and longer lasting.

The aetiopathogenesis of xerostomia is complex and may be multifactorial.

**History and Examination:**

The management of a patient presenting with xerostomia and unilateral salivary gland swelling begins with taking an accurate history, and thorough clinical examination.

The history of the presenting complaint will indicate the cause and severity of the xerostomia, as well as the swelling, and may also indicate if the two are interlinked (ie. swelling has lead to xerostomia, or vice versa) or separate issues.
The clinician needs to ascertain when the salivary gland swelling commenced, and what alerted the patient to it. Note must be made of the presence of any triggering or relieving factors, and if the swelling is enlarging/reducing in size. Any associated co-morbidities (eg. pain, xerostomia, paraesthesia) need to be noted. If there is an element of pain/discomfort, further questions need to be asked as to the onset, nature, distribution, trigger/relieving factors. The patient should be asked if the onset of xerostomia preceded or followed the onset of swelling, and what was timing of this relationship. Any prior treatment for this complaint, and its effectiveness should also be noted.

A thorough and systematic medical history must also be taken. This helps in the diagnosis of oral manifestations of systemic diseases. Additionally, it also identifies the potential for any medical emergencies; assesses the patient’s fitness for treatment; indicates the type of pain control that may be needed; advises of possible modifications that need to be made to treatment (eg. antibiotic prophylaxis, timing of treatment); and alerts the clinician as to contraindications of the use of certain medications (eg. drug allergies, pregnancy). The patient’s medical history will also identify possible risks to staff, and carries medico-legal implications. The physical act of taking the medical history also helps the clinician to establish rapport with the patient.² (An example of the format for a systems review medical history is included in Appendix 1.)

This should be followed by a social history, as well as a dental history. The dental history will reveal the patient’s level of dental awareness, as well as indicate how well a patient may cope with any procedure (eg biopsy) that may be needed in the future.
Examination of the patient, should commence when they enter the room. Abnormalities of gait, mobility, or other general observations need to be recorded. Extra-oral examination should precede intra-oral examination.

Extra-orally, the presence or absence of facial swelling or asymmetry, regional lymphadenopathy and limitation in mouth opening, paraesthesia, as well as any other obvious pathology need to be noted. If swelling or lymphadenopathy is observed, the nature of this must be investigated. For instance, the location, size, consistency, mobility/fixation, overlying ulceration or redness, tenderness to palpation, displacement of other anatomical structures, need to be recorded.

Intra-orally, thorough examination of the soft tissues must be performed. Note any areas of abnormalities, including site, size, colour, texture (both surface and body), fixation/mobility, tenderness to palpation, displacement of other structures. Drying the mucosa and attempting to express saliva from all major glands is important, making note of the ease and amount of saliva expressed and whether this is clear or cloudy. Pooling of saliva in the floor of mouth should be noted. In a xerostomic patient, intra-oral examination typically demonstrates a reduction in saliva flow, with the residual saliva appearing either foamy or thick and ropey. The mucosa appears dry and “sticky”. The dorsal tongue is often fissured with atrophy of the filiform papillae. The patient may complain of difficulties with mastication and swallowing. The clinical findings, however, do not always correspond to the patient’s symptoms. Some patients who feel
that they have a dry mouth may appear to have an adequate saliva flow and oral
moistness. Other patients who clinically appear to have a dry mouth have no
complaints.\textsuperscript{6} Hard tissue examination should include complete dental charting as well as
periodontal assessment. Vitality testing of suspicious teeth may be carried out.

From the history and clinical examination, combined with the application of the
“diagnostic sieve”, a clinical diagnosis may be reached. (See Appendix 2)

<table>
<thead>
<tr>
<th>Inflammatory</th>
<th>Autoimmune – Sjögren’s syndrome (usually bilateral parotid swelling, although unilateral swelling may indicate suppurative parotitis) Non-specific – sialolithiasis and obstruction Sarcoidosis, Wegener’s granulomatosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infective</td>
<td>Ascending bacillary sialadenitis</td>
</tr>
<tr>
<td>Neoplastic</td>
<td>See Appendix 3</td>
</tr>
</tbody>
</table>

Table 1 – Diagnostic sieve – aetiology of xerostomia and unilateral salivary gland swelling.

With regards to a patient presenting with xerostomia and a unilateral salivary gland
swelling, there are many possible scenarios, each with a different history and clinical
features. The two features may be unrelated, and if this is the case, they both need to be
investigated separately. If they are related, then it must be decided which caused the
other.

For example, the patient may have developed a sialolith, which caused obstruction of the
salivary duct, possibly causing swelling in the gland, as well as slight xerostomia.
Alternatively, the sialolith may have caused obstruction and xerostomia, leaving the patient more prone to ascending infection, resulting in swelling.

In this clinical scenario, the main differential diagnoses would include: Neoplasm within the salivary gland, sialolithiasis, sialadenitis, Sjogren’s syndrome with associated glandular enlargement, radiation induced xerostomia with contralateral gland hypertrophy, sarcoidosis and Wegener’s granulomatosis.

The presence of a tumour in the salivary gland may manifest as a swelling. It may cause glandular dysfunction as well as ductal obstruction, leading to xerostomia. In general, tumours found in salivary glands can be classified as benign or malignant depending on their behaviour and histology. Clinically, there are certain features that would indicate either a benign or malignant tumour. These are shown in the table below:

<table>
<thead>
<tr>
<th>Benign salivary gland tumours</th>
<th>Malignant salivary gland tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow growing</td>
<td>Sometimes fast growing and painful</td>
</tr>
<tr>
<td>Soft of rubbery consistency</td>
<td>Sometimes hard consistency</td>
</tr>
<tr>
<td>Compromise 85% of parotid tumours</td>
<td>Comprise 45% of minor gland tumours</td>
</tr>
<tr>
<td>Do not ulcerate</td>
<td>May ulcerate and invade bone</td>
</tr>
<tr>
<td>Not associated with nerve signs</td>
<td>May cause cranial nerve palsies</td>
</tr>
</tbody>
</table>

Table 2 – Typical features of salivary gland tumours. Taken from “Cawson’s Essentials of Oral Pathology and Oral Medicine” 7th Ed. page 264

Tumours of the parotid glands make up 78% of all salivary gland tumours, and 75% of all pleomorphic adenomas. Overall, 15% of tumours found here are malignant. 12% of all salivary gland tumours occur in the submandibular glands, 30% of which are malignant.
Sublingual gland tumours constitute 0.3% of all salivary gland neoplasms, with 86% being malignant. The minor salivary gland tumours account for 10% of total tumours, with 45% malignant.7

Tumours occurring in the glands may be derived from the glandular tissue (ie salivary gland origin), from the overlying tissue, or metastatic deposits from tumours elsewhere in the body.

Sialolithiasis is a common disease of the salivary glands characterized by obstruction of salivary secretion by a calculus (sialolith), associated with swelling (especially at meal time or when the gland is stimulated), pain, and subsequent infection of the affected gland. This may lead to xerostomia, although it is rare that a small sialolith would cause a marked decrease in saliva flow.8 Sialolithiasis can occur at any age, but patients in their third to sixth decade represent most cases and it is considered rare in children.9,10 Most salivary calculi (80% to 95%) occur in the submandibular gland, whereas 5% to 20% are found in the parotid gland. The sublingual gland and the minor salivary glands are rarely (1% to 2%) affected.10 The size of the salivary calculi may vary from less than 1 mm to a few cm in largest diameter. Most of the calculi (88%) are less than 10 mm in size, whereas only 7.6% are larger than 15 mm. Parotid calculi are usually smaller than those in the submandibular gland.9 If the sialolith is located toward the terminal portion of the duct, a hard mass may be palpated beneath the mucosa.
Figures 1 and 2 taken from Barmash HD. Submandibular salivary stones; current management modalities. 11

Sialadenitis (inflammation of the salivary glands) can arise from various infections and noninfectious causes. Most bacterial infections arise as a result of ductal obstruction or decreased salivary flow, allowing retrograde spread of bacteria throughout the ductal system. Blockage of the duct can be caused by sialolithiasis, duct strictures, or compression by an adjacent tumour. Decreased flow may result from dehydration, graft versus host disease, as well as medications that inhibit secretions.

Most cases of acute bacterial sialadenitis are caused by Staphlococcus aureus, but they may arise from streptococci or other organisms. Non-infections causes of salivary
inflammation include Sjogren’s syndrome, sarcoidosis and radiotherapy. These patients are also at risk of developing acute bacterial sialadenitis as a result of the xerostomia. Acute bacterial sialadenitis is most common in the parotid gland, and is unilateral in 75-90% of cases. The affected gland is swollen and painful, and the overlying skin may be erythematous. An associated low grade fever may be present, as well as trismus. A purulent discharge is often observed from the duct orifice when the gland is stimulated. Xerostomia may also be noted.

Chronic sialadenitis may be due to recurrent or persistent ductal obstruction. Periodic swelling and pain occur within the affected gland, usually at meal times when the glands are stimulated.

Figure 3 – Left – clinical photograph of a patient with chronic recurrent parotitis of her right parotid gland showing a marked swelling of the affected gland. Right – clinical photograph of a young woman with chronic recurrent parotitis of her left parotid gland showing redness and induration of the overlying skin. Taken from Nahlieli O. et al. Management of chronic recurrent parotitis: current therapy.12
Sjogren’s syndrome is a chronic, systemic autoimmune disorder that principally involves the salivary and lacrimal glands, resulting in xerostomia and xerophthalmia. There are two forms of the disease: Primary – only salivary and lacrimal glands affected; Secondary – as for the primary form, with the addition of another autoimmune disease. (Criteria for the classification of Sjogren’s syndrome\textsuperscript{13} – Appendix 4) The prevalence of the disease is estimated at 0.3 – 3.0\%, with 80-90\% of individuals affected being female, predominantly of middle age. The principal oral feature is xerostomia, however, one third to one half of patients have diffuse, firm enlargements of the major salivary glands. This swelling is often bilateral, however, unilateral cases have been described, may be non tender and intermittent or persistent in nature. The likelihood of salivary gland enlargement increases with disease severity. In addition, the reduced saliva flow places these patients at increased risk of retrograde bacterial sialadenitis.

In patients with a long standing history of Sjogren’s syndrome, an increased risk of developing lymphoma has been reported. Mucosa-associated lymphoid tissue (MALT) lymphoma, as well as other types of lymphoma have been know to affect the salivary glands, manifesting as a swelling.\textsuperscript{14,15,16,17}

Sarcoidosis is a multisystemic granulomatous condition, thought to be caused by improper degradation of antigenic material. There is a variety of systemic features, however the most common oral finding is salivary gland enlargement and xerostomia. Other oral manifestations are rare, and include submucosal masses, isolated papules or areas of granularity on any oral mucosal site.\textsuperscript{6}
Wegener’s granulomatosis is an uncommon disease, postulated to occur because of either an abnormal immune reaction secondary to non specific infection, or an aberrant hypersensitivity response to an inhaled antigen. Its primary oral manifestations include “strawberry gingivitis” – a florid granular hyperplasia of the attached gingiva, as well as mucosal ulcerations. Enlargement of one or more major salivary glands from primary involvement of the granulomatous process has also been reported, with possible associated xerostomia.6

Salivary glands are very sensitive to radiation, and xerostomia is a common complication of radiotherapy for head and neck cancers. When a portion of the salivary glands is included in the fields of radiation, the remaining glands undergo compensatory hyperplasia in an attempt to maintain function.18,19 The changes begin within one week of initiation of radiation therapy, with a dramatic decrease in salivary flow noted during the first 6 weeks of treatment.

The amount of damage to the gland, and subsequent xerostomia depends on the dose of radiation given.20,21,22

Serous glands have a higher degree of radiosensitivity when compared with mucous glands. With significant irradiation, the parotid glands are affected dramatically and irreversibly. In contrast, mucous glands partially recover and over several months, may achieve a flow rate of 50% of pre-radiation levels.23
Further Investigations:

The need for further investigations is obvious, and the decision as to which investigations are most helpful will depend on what clinical diagnosis is reached. Investigations would include imaging; any combination of plain radiographs, sialography, computer tomographic (CT) scans, magnetic resonance imaging (MRI), ultrasonic scans, and scintigraphy. Fine needle aspiration (FNA) biopsy or open biopsy (incisional or excisional) may be performed, as well as blood tests.

If a tumour was suspected, routine plain radiographs should be taken, including an OPG. This may not be useful to visualize the mass, however, it is an essential tool for treatment planning, especially if the tumour is malignant, and the patient needs to undergo radiotherapy or chemotherapy. Other imaging tools may include head, neck and chest CT scans to ascertain the extent and exact location of the tumour, as well as to check for metastatic lymph nodes and lung metastasis. Commonly, FNA may be performed. The overall type-specific diagnostic accuracy of FNA biopsy for benign and malignant neoplasms of the salivary glands proceeding to surgery is high. (> 80%). The accuracy is higher for benign than malignant tumours, with the sensitivity for the diagnosis of malignancy approximately 80%, and the specificity over 98%. Incisional or excisional biopsy may still be needed for a definitive diagnosis.

If the presence of a sialolith is suspected, an OPG, and mandibular occlusal film (if suspected in the submandibular/sublingual gland or duct), or a lateral oblique view (if suspected in the parotid gland or duct) can be taken. Axial CT scans may be used as an
adjunct, and sialography may be helpful in confirming the presence of a radiolucent sialolith, or duct stricture.

Figure 4 – Sialography showing a sialolith (arrow) in an enlarged Wharton’s duct of the left submandibular gland. Taken from Nakayama E et al. Interventional sialendoscopy: a new procedure for noninvasive insertion of a minimally invasive sialolithectomy.25

Serology may be used to confirm a diagnosis of Sjogren’s syndrome. Screening for ANA, RF, anti-SSa, anti-SSb, are often useful. Sialography may show the classic “snow storm” pattern (sialectasis), due to the contrast medium leaking through the destroyed glandular tissue. Labial minor salivary gland biopsy may also be performed, although this is often omitted as serological findings are often enough to confirm the diagnosis.
Figure 5 – Different stages of sialectasia in Sjogren’s syndrome. Taken from Kalk WWI et al. Parotid sialography for diagnosing Sjogren’s syndrome.26

Tests for sarcoidosis include: blood test results of elevated serum angiotensin converting enzyme, eosinophilia, leucopenia, anaemia, thrombocytopenia, raised serum alkaline phosphatase level, ESR and serum calcium, as well as chest radiographs to check for hilar lymphadenopathy. Biopsy of affected salivary glands may be diagnostic.

A diagnosis of Wegener’s granulomatosis can be made by indirect immunofluorescence for serum antibodies directed against cytoplasmic components of neutrophils. There are two reaction patterns of these antineutrophil cytoplasm antibodies (ANCA) – perinuclear (p-ANCA), and cytoplasmic (c-ANCA). c-ANCA is the most useful and is present in 90-95% of cases.

Once all the necessary investigations have been completed, a working diagnosis is reached and the appropriate management in commenced.
Management:

Management may entail non-pharmacological or pharmacological means, either singly or in combination. Non-pharmacological management may be surgical or non-surgical.

If the investigations confirm the diagnosis of a tumour causing xerostomia and swelling, the management will depend on whether it is benign or malignant. If benign, the patient should be referred to the appropriate clinician for surgical removal. If malignant, treatment may involve surgical removal with or without adjuvant radiotherapy or chemotherapy. The treatment for malignant tumours will depend on the extent of the lesion as well as if there are nodal or distant metastasis. There will be resulting xerostomia from surgery and/or chemotherapy/radiotherapy which will also need to be treated.

Treatment of sialolithiasis depends on the location of the calculus, as well as its size. If the sialolith is small and located within the saliva duct, the patient may be advised to stimulate the gland by applying lemon juice or manually massaging the area in order to “flush” the calculus out. Endoscopic guided basket retrieval techniques\textsuperscript{25} as well as lithotripsy\textsuperscript{27} have been used in recent times with great success, although if the calculus is large, basket retrieval may cause trauma to the duct, resulting in more inflammation and eventual fibrosis. If the sialolith is located in the gland, surgery (conservative removal or total removal of gland) may need to be performed. If this occurs, the resulting xerostomia needs to be addressed.
Bacillary sialadenitis is usually treated with antimicrobials, especially flucloxicillin. Again, manual stimulation of the gland will aid in flushing bacteria from the duct.\textsuperscript{28,29}

If the sialadenitis was caused by stasis in the duct due to systemic medication, the patient’s medical practitioner should be contacted regarding the possibility of substituting a different medication that will decrease the xerostomia.

The mainstay for patients with Sjogren’s syndrome is symptomatic therapy. Drugs such as pilocarpine and cimicifugine have been used for their cholinergic action, however, patients must be warned about the potential adverse effects (e.g. diarrhea, sweating). Patients with Sjogren’s syndrome need to be monitored regularly as they have an increased risk of developing lymphoma.

Sarcoidosis has been known to resolve spontaneously in 60% of affected individuals. 20% respond well to systemic corticosteroid therapy, however, this is not without side effects. Specific treatment for the oral manifestations of xerostomia and salivary gland enlargement are mainly symptomatic.\textsuperscript{6}
The treatment for Wegener’s granulomatosis is systemic medication, usually cyclophosphamide or prednisone. Again, the oral manifestations of the disease are only treated symptomatically.

An important aspect of the treatment of any patient affected by xerostomia is patient education, so that they understand their condition and its implications for their general and oral health.

Simple measures such as increasing water intake, decreasing alcohol and caffeine intake, and chewing sugar free gum are very effective. The patients should be made aware of the various salivary substitutes available, such as the Biotene and Oral Balance products.

Xerostomic patients are at a higher risk of developing oral candidal infections, and topical antifungal medication can be used should this occur.

The patients should also be educated about the effects of xerostomia on their teeth and other oral tissue. The increased risk of developing dental caries, especially cervical caries can be reduced by regular oral hygiene measures as well as the use of concentrated fluoride gel in trays, or fluoride mouthrinses, as well as chlohexidine rinses. The patients need have regular dental follow-up, and the frequency of these will depend on what the cause of the xerostomia and swelling was, as well as its severity and treatment.
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2. Miles TM. Basic and applied physiology in dentistry- A text for the Primary Examination in Physiology. Royal Australasian College of Dental Surgeons, Sydney. 2000


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### Appendix 1:

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>Heart attack, angina, stroke, hypertension, rheumatic fever, heart murmur, heart operations/valve replacements, cholesterol</td>
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<tr>
<td>Respiratory</td>
<td>Asthma, shortness of breath, tuberculosis, smoking history</td>
</tr>
<tr>
<td>Haematological</td>
<td>Acquired or inherited coagulopathies, family history</td>
</tr>
<tr>
<td>Neural</td>
<td>Epilepsy, faints, depression/anxiety</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>Ulcers, reflux, Crohn’s disease, Coeliac disease, ulcerative colitis</td>
</tr>
<tr>
<td>Endocrine</td>
<td>Diabetes mellitus, thyroid, hormone replacement</td>
</tr>
<tr>
<td>Renal</td>
<td>Kidney, dialysis, bladder probles</td>
</tr>
<tr>
<td>Hepatic</td>
<td>Liver, jaundice, hepatitis, ETOH consumption</td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>Osteoarthritis, rheumatoid arthritis, osteoporosis</td>
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<tr>
<td>Immune</td>
<td>Systemic lupus erythematosus, Sjogren’s syndrome, lichen planus, HIV</td>
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<tr>
<td>Cancer</td>
<td>Past/present treatment</td>
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<td>Allergies</td>
<td>True versus adverse effects</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Due date</td>
</tr>
<tr>
<td>Other</td>
<td>Skin, genital, sensory (eg. ears, eyes, nose)</td>
</tr>
<tr>
<td>Hospitalisation</td>
<td>Reason, duration, treatment and outcome</td>
</tr>
<tr>
<td>Current medications</td>
<td>Prescribed, over the counter, herbal</td>
</tr>
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</table>
Appendix 2:

Diagnostic sieve:

<table>
<thead>
<tr>
<th>Category</th>
<th>Proforma</th>
<th>Xerostomia</th>
<th>Unilateral Salivary Gland Swelling</th>
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</thead>
<tbody>
<tr>
<td>Developmental/genetic</td>
<td>inherited</td>
<td>Salivary gland agensis</td>
<td>Polycystic parotid (usually bilateral)</td>
</tr>
<tr>
<td></td>
<td>congenital, acquired</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>de novo, late onset</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td>acute versus chronic</td>
<td>Autoimmune: Sjogren’s Syndrome, GVHD</td>
<td>Autoimmune: Sjogren’s syndrome (usually bilateral)</td>
</tr>
<tr>
<td></td>
<td>non specific</td>
<td>Non-specific: sialolithiasis and obstruction</td>
<td>Non-specific: sialolithiasis and obstruction</td>
</tr>
<tr>
<td></td>
<td>specific, autoimmune</td>
<td>Sarcoïdosis, Wegener’s granulomatosis</td>
<td>Sarcoïdosis, Wegener’s granulomatosis</td>
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<tr>
<td></td>
<td>(type I-IV hypersensitivity)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>T cell, B cell, mixed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infective</td>
<td>bacterial</td>
<td>Mumps, HIV, HCV</td>
<td>Mumps (usually bilateral)</td>
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<tr>
<td></td>
<td>viral</td>
<td></td>
<td>HIV salivary cyst</td>
</tr>
<tr>
<td></td>
<td>fungal</td>
<td></td>
<td>Ascending bacillary sialadenitis</td>
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<tr>
<td></td>
<td>parasitic</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>prion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutritional</td>
<td>deficiency or excess</td>
<td>alcoholism, bulimia dehydration</td>
<td>Alcohol or bulimia induced sialosis (usually bilateral)</td>
</tr>
<tr>
<td>Endocrine/Metabolic</td>
<td>deficiency or excess</td>
<td>Diabetes mellitus</td>
<td>Diabetes mellitus induced sialosis (usually bilateral)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Iron deposition (haemochromatosis, thalassemia)</td>
<td></td>
</tr>
<tr>
<td>Ischaemic/Hypoxic</td>
<td></td>
<td>haemorrhage</td>
<td></td>
</tr>
<tr>
<td>Neoplastic</td>
<td>benign or malignant</td>
<td>(Appendix 3)</td>
<td>(Appendix 3)</td>
</tr>
<tr>
<td>Trauma</td>
<td>mechanical</td>
<td>stricture and fibrosis of salivary duct</td>
<td>Ranula</td>
</tr>
<tr>
<td></td>
<td>chemical</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>physical (baro, radiation, thermal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iatrogenic</td>
<td></td>
<td>Medication induced (Appendix 5)</td>
<td>Pneumoparotid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouthbreathing, exercise induced</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Radiation or chemotherapy induced</td>
<td></td>
</tr>
<tr>
<td>Psychogenic</td>
<td></td>
<td>Anxiety</td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td></td>
<td>Old age</td>
<td>Kimura disease (usually bilateral)</td>
</tr>
</tbody>
</table>
Appendix 3:

World Health Organisation Histological Typing of Salivary Gland Tumours

1. Adenomas
   Pleomorphic adenoma
   Myoepithelialioma
   Basal cell adenoma
   Warthin tumour
   Oncotyroma
   Canalicular adenoma
   Sebaceous adenoma
   Ductal papilloma – inverted ductal papilloma, intraductal papillom, sialadenoma
   papilliferum
   Cystadenoma – papillary cystadenoma, mucinous cystadenoma

2. Carcinomas
   Acinic cell carcinoma
   Mucoepidermoid carcinoma
   Adenoid cystic carcinoma
   Polymorphous low grade adenocarcinoma
   Epithelial-myoepithelial carcinoma
   Basal cell adenocarcinoma
   Sebaceous carcinoma
   Papillary cystadenocarcinoma
Mucinous adenocarcinoma
Oncocytic carcinoma
Salivary duct carcinoma
Adenocarcinoma
Malignant myoepithelioma
Carcinoma in pleomorphic adenoma
Squamous cell carcinoma
Small cell carcinoma
Undifferentiated carcinoma
Other carcinomas

3. Non-epithelial tumours
4. Malignant lymphomas
5. Secondary tumours
6. Unclassified tumours
7. Tumour like lesions
Sialadenosis
Oncocytosis
Necrotising sialometaplasia
Benign lymphoepithelial lesion
Salivary gland cysts
Chronic sclerosing sialadenitis of submandibular gland (Kutter tumour)
Cystic lymphoid hyperplasia in AIDS
Appendix 4:

Classification of Sjogren’s syndrome

I. Ocular symptoms—A positive response to at least 1 of the following questions:
   1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?
   2. Do you have a recurrent sensation of sand or gravel in the eyes?
   3. Do you use tear substitutes more than 3 times a day?

II. Oral symptoms—A positive response to at least 1 of the following questions:
   1. Have you had a daily feeling of dry mouth for more than 3 months?
   2. Have you had recurrently or persistently swollen salivary glands as an adult?
   3. Do you frequently drink liquids to aid in swallowing dry food?

III. Ocular signs (ie, Objective evidence of ocular involvement defined as a positive result for at least 1 of the 2 following two tests):
   1. Schirmer’s test, performed without anesthesia (<5 mm in 5 min)
   2. Rose bengal score or other ocular dye score (>4 according to van Bijsterveld’s scoring system).

IV. Histopathology—In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score >1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm² of glandular tissue.

V. Salivary gland involvement—Objective evidence of salivary gland involvement defined by a positive result for at least 1 of the following diagnostic tests:
1. Unstimulated whole salivary flow (<1.5 mL in 15 min)

2. Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary
   or destructive pattern), without evidence of obstruction in the major ducts.

3. Salivary scintigraphy showing delayed uptake, reduced concentration, and/or delayed
   excretion of tracer.

VI. Autoantibodies—Presence of 1 or both of the following autoantibodies in the sera:
   1. Antibodies to Ro (SSA) antigens.
   2. Antibodies to La (SSB) antigens.

Revised rules for classification:

For primary SS:

In patients without any potentially associated disease, primary SS may be defined as
follows:

a. The presence of any 4 of the 6 abovementioned items is indicative of primary SS, as
   long as either item IV or VI yields positive results.

b. The presence of any 3 of the 4 following objective criteria: III, IV, V, or VI

c. The classification procedure represents a valid alternative method for classification,
   although it should be more properly used in a clinical-epidemiologic survey.

For secondary SS:

In patients with a potentially associated disease (for instance, another well-defined
connective tissue disease), the presence of item I or item II plus any 2 from among items
III, IV, and V may be considered indicative of secondary SS.
Exclusive criteria:

Past head and neck radiation treatment

Hepatitis C infection, Acquired immunodeficiency syndrome

Pre-existing lymphoma

Sarcoidosis

Graft-versus-host disease

Use of anticholinergic drugs (for a time shorter than the 4-fold life of the drug)
PUBLICATIONS
Chronic suppurative osteomyelitis of the mandible: Case report

SC Yeoh,* S MacMahon,† M Schifter‡

Abstract

Background: Osteomyelitis of the maxillofacial skeleton is rare in developed countries such as Australia. This case report describes the successful surgical treatment of chronic suppurative osteomyelitis (CSO) of the mandible in a 75 year old man. The precipitant factor was thought to be a retained tooth root in the (right) posterior body of the mandible.

Methods: Treatment included a pre-surgical course of antibiotics (clindamycin 300 mg, p.o. q.i.d. for two weeks) followed by removal of the retained root, surgical débridement of the affected bone, the intra-oral draining sinus, and resection of the cutaneous sinus tract. Specimens were taken for bacterial cultures and antibiotic sensitivity testing, and the resected tissue sent for histopathological review.

Results: On clinical and radiographic review at three months, the patient was well, completely symptom free and the osteomyelitis had fully resolved.

Conclusion: This case report demonstrates the typical features of CSO. The combination of antibiotic therapy and surgical débridement was effective in the treatment of chronic suppurative osteomyelitis of the mandible utilizing intravenous sedation, and so averting the need for a general anaesthetic.

Key words: Osteomyelitis, chronic, surgery, clindamycin, débridement.

Abbreviations and acronyms: CSO = chronic suppurative osteomyelitis; p.o. = per oral (by mouth); q.i.d.* = quarter in die (four times a day).

(Accepted for publication 5 November 2004.)

INTRODUCTION

Osteomyelitis of the maxillofacial skeleton, in particular, of the mandible is rare in developed countries such as Australia. Osteomyelitis is an inflammation of bone and bone marrow that develops in the jaws usually after a chronic infection.¹ It may be classified as acute, subacute or chronic, depending on the clinical presentation. This decline in prevalence can be attributed to the increased availability of antibiotics and the progressively higher standards of oral and dental health. Despite these advances, there remain select groups of patients who have an increased risk of developing osteomyelitis: specifically those who have undergone radiotherapy affecting the mandible (which may result in a specific form of osteomyelitis termed osteoradionecrosis),¹¹ the immunocompromised,¹¹ including uncontrolled diabetics, and patients on immunosuppressive therapy, such as high dose corticosteroids, needed for transplant recipients and the treatment of auto-immune disorders.

As the general population ages and retain their teeth for longer, combined with the declining availability of oral health professionals, particularly in the public sector, as well as in remote rural and regional centres, it is thought that the incidence of osteomyelitis may increase. Therefore, dentists will need to be aware of clinical features and management of this uncommon disease.

The primary cause of chronic osteomyelitis of the jaws is infection by odontogenic microorganisms.¹ It may also arise as a complication of dental extractions and surgery, maxillofacial trauma and the subsequent inadequate treatment of a fracture, and/or irradiation to the mandible.¹⁵

The typical age of presentation is in the fifties to the sixties, with males more likely to be affected. The commonest site is the posterior body of the mandible. The incidence, outside of those who have received head and neck radiotherapy and the immunocompromised, is increased in patients who have poor oral hygiene and are abusers of alcohol or tobacco.¹

CSO can develop without an intervening acute phase. Some authors have suggested that osteomyelitis must be present for at least one month before it is
termed 'chronic', as this suggests that the disease is refractory to the host defences, or to initial therapy – usually oral antibiotics (as in this case).6,7

Several reports have concluded that CSO can only be treated successfully by a combination of antimicrobial therapy with surgery – either sequestrectomy or decortication of the affected bone.4,5 The aim of surgery is to eliminate all of the infected and necrotic bony tissue, and if incomplete may lead to persistence of the osteomyelitis.

Case report

A 73 year old man was referred to the Department with a five month history of an enlarging swelling that was discharging pus from a cutaneous sinus present on the right inferior border of the mandible (Fig 1). On examination, the patient was asymptomatic, afebrile, with normal pulse and blood pressure, and there was no regional lymphadenopathy. There was no limitation of mouth opening, and on specific testing there was no paraesthesia of the right lower lip and mental area.

His medical history was essentially non-contributory. He had mild cardiovascular disease, namely well controlled angina and hypertension managed with a Nitroflingual Pumpspray (glyceryl trinitrate) and Atacand (candesartan cilexetil) respectively. Significantly, this did not represent a contraindication to the use of intravenous sedation for the subsequent oral surgery that he needed. He had no known allergies, and denied any tobacco or alcohol use.

On intra-oral examination, the patient had a partial, reasonably well maintained dentition. A draining sinus was noted on the crest of the right edentulous alveolar ridge in the area that one would expect to find the 46
This area was slightly tender to palpation. These clinical features were typical of CSO as described by Koobusch et al. and Hudson.

**Investigations**

OPG, periapical and mandibular occlusal radiographs demonstrated, in the area of the right posterior body of the mandible, a localized mottled area of mixed radiolucency/radio-opacity which was ovoid in shape, and measured 20mm at its greatest diameter. It extended from the crest of the alveolar ridge to the inferior alveolar canal (Fig 3). This was consistent with the radiologic features of osteomyelitis described in the literature. The radiographs suggested that there may have been a retained tooth root in the centre of the affected area. This was confirmed on subsequent surgical débridement.

A clinical diagnosis of CSO of the mandible was made. Management entailed a two week course of oral clindamycin (300mg p.o. q.i.d., followed by surgical débridement of the affected area (Fig 4), removal of the tooth root and resection of the cutaneous sinus tract (Fig 5) utilizing intravenous sedation, at which time histological samples and microbial cultures were also taken. Clindamycin was chosen because of its broad antibacterial coverage, including activity against anaerobic organisms, commonly present in chronic ‘mixed’ odontogenic infections, and its established potential to penetrate well, and achieve high therapeutic concentrations, in bone.

**RESULTS**

The results of the microbiological cultures showed normal oral flora and some aerobic Gram-negative bacilli, which were sensitive to clindamycin. This was consistent with the microbiological findings reported by Gentry. The histopathology demonstrated chronic inflammation and fibrosis. These findings, in combination with the clinical picture, were consistent with chronic supplicative osteomyelitis.

Three months after the original surgery repeat radiographs were taken. There was no clinical or radiological evidence of residual infection (Fig 6 and 7).

**DISCUSSION**

This case report demonstrates the typical features of CSO, a rare but well-described potential complication of chronic odontogenic infections, that dentists may more frequently encounter. Management entailed a course of antibiotics in combination with surgical débridement. This is consistent with the published protocols of van Merksteijn et al., Kim and Jang, and Koobusch et al.

It has been suggested that the minimum duration of antibiotic therapy to treat CSO is two weeks. However, it has been suggested by Bamberger that a minimum of four weeks is indicated. Some reports have also advocated the use of hyperbaric oxygen in the treatment of this condition, especially in the irradiated mandible. In the present case, the patient was prescribed a four week course of oral clindamycin, which, in combination with surgical débridement was successful.
REFERENCES


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AN OVERVIEW OF TUMOUR MARKERS IN HEAD AND NECK CANCER AND THEIR USE IN CLINICAL PRACTICE

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ABSTRACT

The prevalence of oral squamous cell carcinoma is increasing in Australia. Consequently, the development of new methods to identify and predict the behaviour of cancer has become increasingly important. Identification of tumour markers which are produced in the body in response to the presence of cancer has many implications for clinical practice. Potential uses include screening patients at risk of developing cancer, identifying tumour types and origins, monitoring the effectiveness of therapy, determining recurrence, as well as indicating prognosis. This understanding of cancer at the molecular level has also led to research into the use of gene therapy to improve prognosis. This involves introducing genetic material into a person’s cells to fight or prevent disease. Currently, this is only available in a clinical trial. The use of tumour markers and gene therapy holds promise for the treatment of oral cancer; however there is still the need for further research.

The prevalence of tumours of the head and neck region is increasing and, in particular, squamous cell carcinoma represents the most frequent malignant tumour of the oral cavity. Oral squamous cell carcinoma represents 3 - 4% of all human cancers and 40% of all head and neck cancers. The prevalence in Australia has increased by 29% in men, and 3% in women between 1975 and 1995. The total number of NSW citizens dying of oral cancer has increased by 43% in the same time period.

According to the model of cancer by Hanahan and Weinberg, a cell must acquire a minimum of six alterations in order to become malignant. Broadly these are: independence of external proliferative growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis and the ability to invade.

The clinical behaviour of head and neck squamous cell carcinoma is difficult to predict based on classical histopathological parameters and TMN staging alone. Consequently, the identification of molecular markers that can accurately define cancer with a poor prognosis is of great importance.

A tumour marker is a substance in the body that may indicate the presence of cancer. They are usually found in the blood, urine or other tissue samples, and may correlate of tumour stage and grade. They may be valuable in monitoring response to treatment and predicting recurrence. They are produced either by the tumour itself, or by the body in response to the presence of cancer (Table 1).

Tumour markers can be used in a variety of situations to aid in the management of patients.

Screening: This is the systematic application of a clinical or laboratory-based test to identify individuals at sufficient risk of a specific disorder, and who have not sought prior medical attention for that disorder, to enable them to benefit from further investigation or direct preventive action. This is different from diagnosis in that it attempts to identify a disease or condition at an early stage prior to the appearance of clinical symptoms.

Disease monitoring: This is used to determine the effectiveness of therapy during the course of management. Tumour markers can provide a significant lead time in the detection of recurrent disease prior to the routine clinical diagnosis. They can also potentially detect the persistence of malignancy after primary chemotherapy.

Disease recurrence: Tumour markers can be used serially to determine recurrences. A baseline level can be obtained and then followed up. A changing level may be indicative of recurrence and the alterations in level often precedes clinical or radiographic determination. A therapeutic decision can therefore be made prior to extensive recurrences.

Differential diagnosis: The diagnostic gold standard is still histopathology; however, this is not always conclusive, and often requires additional testing for a definitive result. Tumour markers can be used to help distinguish many tumour types and origin as well as distinguish primary from metastatic tumours.

Prognosis: Traditionally, this is based on the TMN system. Tumour markers can be used in addition to other methods to forecast a patient’s response to therapy, thereby enabling the physician to appropriately adjust or determine the level of treatment needed to manage the disease.

One marker which has been studied extensively is p53. This is a protein product of a tumour suppressor gene. It is located on the short arm of chromosome 17, and appears to have a pivotal role in many cancers, including those of the head and neck. p53 monitors the integrity of DNA prior to replication.
Table 1

<table>
<thead>
<tr>
<th>Tumour marker category</th>
<th>Examples</th>
</tr>
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<tbody>
<tr>
<td>1. Oncogenes</td>
<td>Ras, c-myc, Cyclin D1, Bel-1</td>
</tr>
<tr>
<td>2. Tumour suppressor genes</td>
<td>p53, pRb, p16, p21</td>
</tr>
<tr>
<td>3. Cell proliferation markers</td>
<td>Ki-67, Cyclin dependant kinase</td>
</tr>
<tr>
<td>4. Cell differentiation markers</td>
<td>p27 protein, Mini chromosome maintenance proteins</td>
</tr>
<tr>
<td>5. DNA content</td>
<td>Ploidy/Aneuploidy</td>
</tr>
<tr>
<td>6. Cell adhesion molecules</td>
<td>CD44, E-cadherin, Integins, Laminin</td>
</tr>
<tr>
<td>7. Metabolic/anti-metabolic factors</td>
<td>Glutathione-S-transferase, Thymidylate synthase</td>
</tr>
<tr>
<td>8. Immunosurveillance markers</td>
<td>T cell receptors, Lymphocyte signalling molecules</td>
</tr>
<tr>
<td>9. Growth factors and their receptors</td>
<td>Epidermal growth factor receptor, Vascular endothelial growth factor</td>
</tr>
<tr>
<td>10. Circulating markers</td>
<td>Human leukocyte antigen, Squamous cell carcinoma antigen, p53 antibody</td>
</tr>
<tr>
<td>11. Salivary markers</td>
<td>p53 antibody</td>
</tr>
<tr>
<td>12. Telomerase activity</td>
<td></td>
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<tr>
<td>13. Carcinogen metastatic markers</td>
<td>Nm 23</td>
</tr>
<tr>
<td>14. Antioxidant molecules</td>
<td>Malondialdehyde (end product of lipid peroxidation)</td>
</tr>
<tr>
<td>15. Apoptotic/anti-apoptotic factors</td>
<td>p63, p73, Bak, Bel-2, Pin-1</td>
</tr>
</tbody>
</table>

( Arresting the cycle in the event of abnormality) and also coordinates the cascade of intracellular events culminating in repair or in apoptosis if the damage is irreparable. Overall, approximately half of all the cancers of the mucosa of the head and neck are believed to contain mutations in a specific region of the p53 gene. Cells that contain the mutant p53 protein can be detected by simple immunohistochemical staining. Disruption to p53 function appears to be an early event in head and neck oncogenesis and has been linked to exposure to mutagens, such as benzopyrenes in tobacco. It has been found that p53 expression can predict tumour recurrence, with the absence of expression associated with lower rates of recurrence.

In head and neck cancer, the outcome improves when the completeness of resection is confirmed by molecular studies of the surgical specimen. This is also important in managing oral leukoplaikia, in which margins are often very narrow, non-existent, or not assessed. Wide surgical margins are difficult or impossible to achieve in patients with diffuse, multifocal oral leukoplaikia. The finding of molecular evidence that margins are positive may explain the substantial rates of recurrence of leukoplaikia and the development of cancer at the site of resection, despite histologic evidence of negative margins. In a recent study, the presence of p53 gene mutations in histologically negative tumour margins, identical to those in the primary tumour, was reliable in predicting recurrence, and overexpression of p53 in precursor lesions has been strongly linked with progression to cancer.

Ki-67 is a nuclear proliferation antigen. It is expressed in the nuclei of proliferating cells in all phases of the cell cycle except G0. It is encoded on chromosome 10 and the protein helps to regulate cell proliferation. This marker can be detected in frozen tissue as well as paraffin-embedded tissue. Recent investigations have used this marker to determine the proliferative rate of head and neck cancers. p53 and Ki-67 have both been used to help predict the response to treatment, organ preservation, and the survival in patients with advanced squamous cell carcinoma of the head and neck treated with chemoradiotherapy.

CD44 is another tumour marker that is significantly related to prognosis. It is a cell membrane molecule, found in cells of haematopoietic, epithelial, and mesothelial origin. Its multiple normal functions include cell-cell adhesion, lymphocyte activation, and cell-substrate interaction. CD44 binds hyaluronan. Therefore, the tumour advantage may depend on low expression or on a lack of function of CD44. In both cases there is a low CD44 expression and high metastatic potential of some tumours and positive association of this marker with increased survival time.

Cyclin D1 is a cell cycle regulator located on chromosome 11. It has been found to be overexpressed in head and neck squamous cell carcinoma. This overexpression has been correlated to a poor prognosis. Deletion of the p16 gene on chromosome 9 has also been observed in a significant number of head and neck squamous cell carcinomas. This gene regulates cyclin D1 activity, ultimately downregulating cellular proliferation. Both the amplification of cyclin D1 and the deletion of p16 can be detected by using fluorescence in situ hybridization. It has been found that these two genetic aberrations together indicate a poorer outcome than either of the abnormalities alone. Some of the cyclins appear to offer the best prospects as prognostic indicators for those with cancer of the head and neck, and as predictors of radiotherapy and drug resistance.

Measurements of tumour marker levels alone are not sufficient indications of cancer for several reasons. Tumour marker levels can be elevated in people with benign conditions, and are not elevated in every person with cancer. Also, many current tumour markers are not specific to a particular type of cancer; the level of a tumour marker can be raised by more than one type of cancer.
There has been much research into the use of tumour markers in head and neck squamous cell carcinoma. So far, the results are promising, with several markers already in use. However, further study needs to be completed in order for these markers to become a routine part of the management of these patients.

The advances in understanding genetic aberrations in carcinogenesis have set the stage for scientists to alter patients' genetic material to fight or prevent disease. Gene therapy is an experimental treatment that involves introducing genetic material (DNA or RNA) into a person's cells to fight disease. Gene therapy is being studied in clinical trials for many different types of cancer and for other diseases. It is not currently available outside a clinical trial. Researchers are studying several ways to treat cancer using gene therapy. Some approaches target healthy cells to enhance their ability to fight cancer. Other approaches target cancer cells, to destroy them or prevent their growth.

Current research is aimed at:

- Replacement of missing or altered genes with healthy genes. Because some missing or altered genes (e.g., p53) may lead to cancer, substituting working copies of these genes may keep cancer from developing.

- The improvement of the patient's immune response to cancer. In this approach, gene therapy is used to stimulate the body's natural ability to attack cancer cells.

- In some studies, researchers inject cancer cells with genes that make them more sensitive to chemotherapy, radiotherapy or other treatment. In other studies, genes have been placed into healthy haematopoietic stem cells to make them more resistant to the side effects or high doses of anticancer drugs.

- Another approach is to inject cancer cells with genes that can be used to destroy the cells. In this technique, apoptotic genes are introduced into cancer cells. Later, a pro-drug (an inactive form of a toxic drug) is given to the patient. This is activated in cancer cells containing the apoptotic genes leading to the destruction of those cancer cells.

- Other research is focusing on the use of gene therapy to prevent cancer cells from developing new blood vessels.

Knowledge and understanding of cancer at the molecular level has not only opened the way for the use of tumour markers in the assessment of cancer patients, but has also led to research into the potential use of gene therapy to improve prognosis.

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Title: A Proposed Clinical Scoring System for Monitoring Oral Lichen Planus (OLP): a preliminary study

Authors: Yeoh S-C, Schifter M, Shepherd M, Coleman H, Zoellner H

Purpose: To develop a reliable and reproducible clinical scoring system for oral lichen planus (OLP).

Methods: A scoring system for OLP was developed, based on a previously published system (Piboonniyom S, et al. 2005). Our modified clinical scoring system aimed to record the site of involvement, the clinical features (reticular/hyperkeratotic, erythematous and ulcerative), and clinical severity relative to the size of the lesions. Weighted scores were given to erythematous and ulcerative lesions, with these areas scaled by 1.5x and 2.0x respectively. A 10-point visual analogue scale (VAS) were used to record the patients’ own assessment of their oral discomfort as related to the OLP. A second 10-point VAS was used to record the clinician’s assessment of the severity of the patient’s oral discomfort. Scores for lesions from each site were totalled to give an overall weighted score for each patient. The oral cavity was divided into 16 sites. Lesions were graded as reticular (R) scoring 0 or 1 (0 = no lesion, 1 = presence of a lesion); erythematous (E) scoring from 0 to 3 (0 = no lesion, 1 = less than 1cm², 2 = 1-2 cm², 3 = 2-3cm²); and ulcerative (U) areas scoring from 0 to 3 (0 = no lesion, 1 = less than 1cm², 2 = 1-2 cm², 3 = 2-3cm²). Total scores were calculated using $\Sigma R + \Sigma(E \times 1.5) + \Sigma(U \times 2.0)$. To date, 20 patients with OLP were examined by four independent observers. Each observer had a different level of clinical experience, ranging from that of a final year dental student to an Oral Medicine specialist.

Results: This scoring system has proven to be a useful clinical tool. Further modifications are needed. Concerns were raised regarding both intra- and inter-observer reliability.

Conclusions: This scoring system allows for monitoring the progress of OLP and response to therapy, and appears to by reproducible, reliable and user-friendly. It could potentially be adapted to record oral lesions in chronic graft versus host disease as well as other ulcerative and vesiculobullous conditions.
Gene expression profiling of two oral squamous cell carcinoma (OSCC) cell lines compared with normal oral mucosal epithelium using cDNA microarray.

Yeoh S-C, Chan S, Schifte M, Zoellner H, Coleman H.

Oral squamous cell carcinoma (OSCC) represents the most frequent malignancy of the oral cavity. The overall 5 year survival rate of oral cancer patients has not improved significantly over the past two decades, despite the significant advances with multimodality treatment. The identification of genetic alterations associated with OSCC has been the basis of understanding of how normal cells become malignant. The aim of this study was to identify genes associated with oral carcinogenesis by comparing the relative gene expression of OSCC with normal oral epithelium using cDNA microarray.

Two OSCC cell lines were grown in culture, and a normal oral keratinocyte cell line was established from a primary explant of normal human gingiva. Total RNA was isolated, and converted to cDNA by reverse transcription. Samples were labelled with fluorescent dyes, and hybridised onto an 8K human genome array (AGRF). Arrays were scanned and the data analysed.

Several genes were identified to be relatively over expressed in the OSCC cell lines compared to the normal oral keratinocytes. These genes were mainly associated with cell growth and proliferation, as well as angiogenesis.

cDNA microarray analysis is a useful technique that allows the comparison of a large number of previously identified genes simultaneously, showing the relative over- or under-expression of genes in malignant cells compared with normal cells. Gene profiling by microarray shows promise in being able to better predict the prognosis of patients with OSCC, and to identify genes that could serve as biomarkers and as potential targets for biological therapy.

Oral Sub-Mucous Fibrosis (OSMF) in Australia?

Cox S, Zoellner H, Schifer M, Coleman HG, Yeoh S-C.

OSMF is an established pre-malignant condition. OSMF is related to the habitual chewing, for protracted periods of Betel “quid” or “paan”, which contains a mixture of areca nut and/or betel leaves, various flavourings, including spices, to which tobacco is often added. OSMF contributes to the high incidence of oral squamous cell carcinoma (OSCC), throughout the Indian Sub-Continent, Southeast Asia, and locations in the western Pacific. Migrants from these countries to Australia continue to practice this habit. Use of quid and/or paan has increased with its export from India, as a commercially prepared food
product, for sale worldwide, including in Australia. The consequences are an expected increase in the incidence of OSCC.

**Method:** Case series, with literature review.

**Results:** Three cases of OSMF are reviewed, in particular addressing the concern about access to betel quid/paan products in Australia.

**Conclusions:** Betel quid/paan chewing is an identified risk factor for the development of OSCC. OSMF is a known pre-malignant condition, with recognisable oral mucosal features. Action is required to prevent this potential worsening of the incidence of OSCC. Education of health care providers is required to question their patients about Betel nut use, and identify OSMF. This seems particularly important for those practitioners who cater to immigrants from countries where this habit is entrenched. A preventative program to target this population and limit their acceptance and use of Betel nut products is also needed.

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**Title:** Dental Extractions/Oral Surgery Safely Undertaken in Patients on Bisphosphonate Therapy: a prospective trial.

**Authors:** Schifter M, Yeh S-C, Zoellner H, Coleman HG, Cox S

**Purpose:** A review of a prospective trial of patients who have undergone oral surgical procedures – dental extraction - whilst being on bisphosphonate therapy. Bisphosphonates have a high affinity for mineralised bone, leading to the inhibition of osteoclastic activity, therefore they are now widely used in the prevention and management of metastatic disease to the bone and in the treatment of all forms of osteoporosis. Worldwide, there have been reports of osteonecrosis of the jaw (“bis-phossy jaw”) occurring in patients receiving bisphosphonates, particularly those patients in which bisphosphonates have been part of their cancer therapy. Most have been associated with dental extractions and associated local infection. This has led to recommendations that dental extractions/oral surgery should not be undertaken in those patients taking bisphosphonates. At Westmead we have developed a protocol, involving the pre- and post surgery administration of antibiotics, gentle extraction techniques, with primary closure for those patients who have been on long term bisphosphonate therapy.

**Results:** To date 10 patients, median age of 64 (range 60-69) taking various bisphosphonates, have had teeth extracted, and on follow-up of up to 3 months, have not developed osteonecrosis of the jaw(s).

**Conclusions:** There are an increasing number of patients who are likely to need and benefit from the use of bisphosphonate agents, particularly the more potent later generation agents. Prospective surveillance of such patients, and removal of teeth with a poor prognosis prior to the commencement of bisphosphonate therapy is considered ideal. Nonetheless, protocols need to be developed for the safe oral surgery on occasions when such treatment is unavoidable. The protocol developed by us and outlined above may satisfy this need.
The Clinical Utility of the Carbon-Dioxide (CO₂) Laser in Oral Medicine Practice

Authors: Georgiou A, Schiffter M, Yeoh S-C, Palme C, Coleman H, Zoellner H, Cox S, Walsh L.

Purpose: A prospective clinical review of the utility of the carbon-dioxide (CO₂) laser in oral medicine practice.

Results: 22 patients, of which, 14 had leukoplakia with varying grades of dysplasia (mild to severe) underwent CO₂ laser ablation of their oral mucosal lesions. Results were favourable in terms of acute morbidity, with improved healing, and less scarring, in comparison to scalpel biopsy. The complication rate was low.

Conclusion: The CO₂ laser shows excellent promise as another useful tool for the management of oral mucosal lesions. Further, ongoing, long-term studies are required to determine its utility in the definitive management of oral mucosal dysplasia and leukoplakia.

Title: Kepivance® (recombinant keratinocyte growth factor (rHuKGF)) in the management of oral mucositis in patients undergoing conditioning therapy for haematopoietic stem cell transplantation: a case report

Authors: Schiffter M, Yeoh S-C, Coleman H, Zoellner H, Cox S.

Purpose: To review the use of Kepivance in the prevention of oral mucositis. Kepivance has shown excellent early results to decrease the incidence, severity and duration of severe oral mucositis (OM) in patients with hematologic malignancies receiving myelotoxic chemotherapy and hematopoietic stem cell support. It appears to be well tolerated. The use of Kepivance in such patients could potentially decrease the use of opioid analgesics and expensive total parenteral nutritional support, avoid complications such as catastrophic airway embarrassment, and possibly limit life-threatening septicemia.

Method: Case report with literature review.

Conclusions: This case together with laboratory-based experiments and early clinical trials, to date, with admittedly small numbers of patients, does suggest that Kepivance may limit mucositis in patients receiving intensive chemotherapy or even radiotherapy to the head and neck region. The aetio-pathogenesis of oral mucositis and the mechanisms by which Kepivance is thought to act to limit the development of mucositis will be reviewed.