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The Changing Aetiology of Oral Cancer and the Role of Novel Biomarkers to Aid in Early Diagnosis

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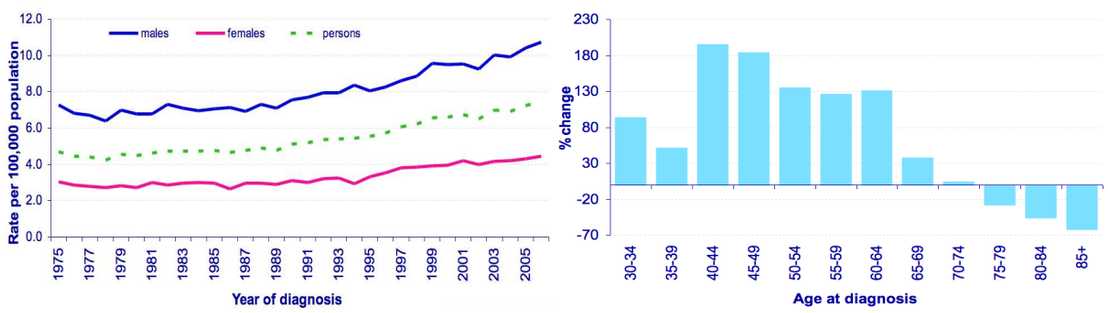
1. Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most prevalent neoplasm in the world, with approximately 900,000 cases diagnosed worldwide (Chin, Boyle et al. 2006). The chronic use of tobacco and alcohol consumption has long been recognized as prominent risk factors in the development of oral cancer (Hashibe, Brennan et al. 2009). Oral Cancer is the 8th and 13th most common malignancy in the world for males and females respectively. Up to 80 % of these cancers occur in Asia (Cheong, Chandramouli et al. 2009). Precancerous and cancerous oral lesions may mimic any number of benign oral lesions, and as such may be left without investigation and treatment until well advanced.

The five year survival following the diagnosis of oral malignancy can be as low as 15-50% as most cancer are advanced and associated with lymphatic spread at the time of discovery (McCullough and Farah 2008). Most patients with oral cancer or a potentially malignant oral mucosal lesion are often asymptomatic at the time of diagnosis (Baranovsky and Myers 1986). Some patients do not seek care until pain, persistent ulceration, unexplained bleeding or an oral or neck mass is discovered at which time the disease is very advanced.



Most cancer deaths occur in patients 55 years or older (Silverman and Gorsky 1990). Thus, oral cancer is predominantly a disease of the elderly and for those with known epidemiologic risk factors, sufficient time exists to examine patients, detect precursor lesions and treat prior to the development of malignancy.

2. Aetiology

There are several known risk factors in the development of oral cancer with the most studied and well-established being the use of tobacco (Marder 1998; Hashibe, Brennan et al. 2009). In the developing world, tobacco and areca nut use, either alone or in combination, account for the majority of leukoplakias, whereas the majority of oral leukoplakias in the developed world are associated with just the use of tobacco (Napier and Speight 2008). Heavy smokers have been shown to be seven times more likely than non-smokers to have leukoplakias. Further, the importance of tobacco is reinforced by the regression and / or disappearance of many lesions following cessation with a recent study showing that 56% regressed at 3 months and 78% regressed a year after smoking cessation (Napier and Speight 2008).

2.1 Alcohol

There is increasing evidence of the role of alcohol consumption in the development of oral cancer (Hindle, Downer et al. 2000; Hashibe, Brennan et al. 2009). In a recent large pooled study undertaken by the International Head and Neck Cancer Epidemiology (INHANCE) consortium, analyzed 11,221 patients with head and neck cancer and 16,168 controls and showed a greater than multiplicative joint effect of both tobacco and alcohol (Hashibe, Brennan et al. 2009). Further, this study estimated the population attributable risks for smoking and drinking combined to be 64% (95% CI: 45-75%) showing that the joint effect of tobacco and alcohol is responsible for a large proportion of head and neck cancers (Hashibe, Brennan et al. 2009). Interestingly though, this study also concluded that a proportion of head and neck cancers cannot be attributed to either tobacco or alcohol, particularly for oral cavity cancer, among women and below age 45 (Hashibe, Brennan et al. 2009).

The ability of alcohol to cause protein denaturation and lipid dissolution, as well as its anti-microbial activity against most bacteria, fungi and viruses has resulted in alcohol being used in mouthwashes as a solvent, preservative and antiseptic agent. Studies have shown that high concentrations of alcohol in mouthwashes may have detrimental oral effects such as epithelial detachment, keratosis, mucosal ulceration, gingivitis, petechiae, and oral pain (Hsu TC, Furlong C et al. 1991; Warnakulasuriya S, Parkkila S et al. 2008) Furthermore, there is increasing evidence that there may be a direct relationship between the alcohol content of mouthwashes and the development of oral cancer, specifically, an increased risk of acquiring cancer (oral cavity, pharynx, larynx) by over 9 times for current smokers, over 5 times for those who also drink alcohol, and almost 5 times for those who neither smoke nor drink alcohol (Guha, Boffetta et al. 2007). A recent review of the literature suggested that it would be inadvisable for oral health care professionals to recommend the long-term use of alcohol-containing mouthwashes (McCullough and Farah 2008).

While alcohol was initially described as only a risk enhancer in smokers, there is now sufficient epidemiological evidence to suggest that chronic alcohol consumption is an independent risk factor (Rothman and Keller 1972; Herity, Moriarty et al. 1981; Maserejian, Joshipura et al. 2006). The exact mechanism of alcohol on the development of oral cancer remains unclear, as alcohol in itself is not clastogenic, mutagenic or carcinogenic. There is growing evidence that the local oxidation of alcohol to its toxic metabolite, acetaldehyde (AA), may be the ultimate mechanism for mediating the carcinogenic effect of alcohol in the mouth (Seitz, Matsuzaki et al. 2001).

AA is the first metabolite of alcohol and is a well-known mutagenic and carcinogenic agent (Feron, Kruysse et al. 1982). The 1999 International Agency for Research on Cancer Monographs evaluated the carcinogenic risk of AA and concluded that there was inadequate evidence in humans, but sufficient evidence in experimental animals of the carcinogenic nature of AA. Although the bulk of alcohol metabolism is carried out in the liver, extra-hepatic metabolism of alcohol to AA has been shown to occur elsewhere in the body including the oral cavity. An early study demonstrated considerable AA production in saliva (up to 143μM) after moderate consumption of alcohol (0.5g/kg body weight) (Homann, Karkkainen et al. 1997). The level of AA formed was well above endogenous AA level of 1μM (Lachenmeier, Kanteres et al. 2009) and was within the range that is capable of inducing mutagenic changes 50-150μM (Salaspuro 2007). Furthermore, studies have also found that ingested alcohol may in fact be metabolized to AA by commensal organisms in the oral cavity via microbial alcohol dehydrogenase. Microorganisms that have been documented to be significantly associated with higher AA production include *Streptococcus* spp., particularly *S. Salivarius*, *Neisseria* spp and *Candida albicans* (Homann, Tillonen et al. 2000; Muto, Hitomi et al. 2000; Kurkivuori, Salaspuro et al. 2007). The known toxic effect of AA as well as its local production in the mouth has led to increasing interest in the level of salivary AA formed after alcohol-containing mouthwash use and its association with oral cancer development.

Epidemiological studies have not led to a definitive consensus on the association of alcohol-containing mouthwashes and oral cancer. Lachenmeier et al (2009) tested the salivary AA content of four healthy individuals after rinsing with alcohol-containing mouthwash (Lachenmeier, Gumbel-Mako et al. 2009). Using headspace gas chromatography, this study found salivary AA concentrations significantly above endogenous levels.

To further our understanding of the level of salivary acetaldehyde after rinsing with alcohol containing liquids, we have recently completed an study with 30 healthy dentate dental students from the University of Melbourne participated in this study. They were selected based on the following criteria: 1) >18years of age; 2) good over-all health; 3) non-smoker; 4) non-intraoral prosthesis; 5) healthy dentition with no oral problems. The four test liquid samples selected were red wine (14% vol. alcohol), scotch whiskey (43% vol. alcohol), low alcohol mouthwash with chlorhexidene (11.5% vol. alcohol) and high alcohol mouthwash (26.9% vol. alcohol).

Saliva samples for each test liquid were collected on different days. Participants were randomly allocated a test liquid depending on the day of sampling. Participants were excluded if they had consumed food or drink, or performed oral hygiene in the previous 2 hours or consumed alcohol in the previous 24 hours.

Baseline salivary samples were collected before participants rinsed with any test liquid. Participants were then instructed to rinse 20ml of the selected test liquid vigorously for 30 seconds and expectorate immediately. Salivary samples were collected after 1 and 5 minutes after the expectoration of test liquid. Patients were required to chew on cotton rolls for 1 minute before sampling. The cotton rolls were centrifuged for 2 minutes at 3000 g in Salivette saliva collection tubes (Sarstedt, Australia Pty Ltd). A 450 µL sample of the clear saliva supernatant was then transferred to a 10 mL headspace vial containing 50 µL of perchloric acid (20% w/w) (Eriksson et al, 1982). The effect of sample storage conditions were tested by comparing AA concentrations in a set of spiked samples frozen for 2 days against a batch of freshly spiked samples.

Salivary AA concentrations were measured using headspace gas chromatography similar to previously reported methods (Lachenmeier, Gumbel-Mako et al. 2009). A gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionization detector (FID) and headspace autosampler (AOC-5000, CTC analytics) was used for analysis. Data acquisition and analysis were performed using GC Solutions software. Sample vials were first incubated for 5 min at 50^{o}C in the autosampler's oven to achieve a favourable vapour-liquid equilibrium of the AA in the headspace vials. Thereafter, 1 mL of sample headspace was automatically drawn into the injection syringe by means of the electropneumatic dosing system and injected into the GC using a split ratio of 10:1. The GC conditions used to separate the volatile components in the saliva were as follows: column: 30 m x 0.25 mm I.D. x 0.25 µm film thickness (DB-23, J&W Scientific Inc.); temperature program: 40^{o}C hold for 2 min, 80^{o}C/min up to 200^{o}C and hold for 3 min; injection port temperature: 200^{o}C; FID temperature: 250^{o}C; carrier gas (He) flow rate: 30 cm/sec.

The suitability of the internal standards method, the calibration curve method and the standard addition method for calculating the unknown AA concentration in the saliva samples was studied. The results shown in Table 1 indicate that the analyte concentration detected from the saliva standards is in good correspondence to that with standards made up in deionized water. The calibration curve prepared from aqueous AA standards (1, 2, 5, 10 mg/L) was linear over the whole range and the limit of detection was 0.5 mg/L (11.4µM). Preliminary experiments indicated that the method developed had good reproducibility (%RSD = 1.05%, n = 6).

All mouthwashes caused a statistically significant ($p < 0.05$) increase in production of AA at both the 2 minutes and 5-minute samples when compared to the pre-mouthwash sample. Statistically significantly more AA was present at the 2-minute interval than at the 5-minute sample (Table 1). However, even 5 minutes after rinsing there was statistically significantly more AA present for all four mouthwashes when compared to the pre-mouthwash sample. There was no significant difference ($p = 0.218$) between the levels of AA produced 5 minutes after rinsing with any of the 4 solutions.

There are of course limitations to this preliminary study in particular the small sample size with only nine subjects for each test liquid. Nevertheless, these results indicate that rinsing with alcohol produced an increase in oral AA above endogenous levels and in the range capable of inducing mutagenic change (50-150µM) and that alcohol containing mouthwash has a similar effect on acetaldyde levels within the oral cavity as recreational alcohol containing liquids. These results indicate that further investigation is required into the levels of AA produced by a wide range of alcohol containing liquids, particularly those in common use.

	Alcohol (%)	Average AA concentration (μM)		
		Baseline	1 minute	5 minutes
Scotch	43	8.1 ± 8.1	97.0 ± 33.2	41.9 ± 21.2
Wine	14	11.2 ± 6.4	59.4 ± 31.0	33.8 ± 20.2
Listerine	26.9	9.1 ± 5.9	53.5 ± 19.3	25.1 ± 11.6
Savacol	11.5	18.5 ± 11.2	43.8 ± 13.8	31.4 ± 7.5

Table 1. Average AA concentration after rinsing with test liquids.

We have further assessed whether alcohol-containing mouthwashes (ACM) can induce cellular toxicity and/or genotoxicity in human cells in vitro with the single-cell gel (comet) assay and morphological assessment of apoptosis and mitosis, in order to clarify suggestive but inconclusive epidemiological data concerning a potential oral cancer risk associated with regular use of alcoholic mouthwash. Normal (OKF6/TERT-2 cells) (Dickson MA 2000) and dysplastic (DOK cells) (Chang Se 1992) human oral epithelial cells were treated for 30 seconds with a 1:5 alcohol-containing mouthwash (26% ethanol) dilution and equivalent concentration of ethanol (5.4%) for comparison. The negative control group was treated with phosphate buffered saline solution and the positive control group was treated with 650μM H2O2. Recovery time points of 5, 10 and 20 minutes were allowed before trypsinisation and layering onto slides with low-melting point agarose for electrophoresis. DNA damage was scored using the visual method established by Speit and Hartmann (Speit G 2006). Parameters from the comet assays and cytotoxicity testing were analysed by one-way ANOVA and Tukey's test.

ACM and ethanol treatment groups consistently demonstrated significantly greater DNA damage ($P < 0.001$) in the comet assay when compared to negative controls. No significant difference was noted between recovery time points. No significant differences in apoptosis and mitosis were noted between treatments and controls in the cytotoxicity assay, however OKF-6 cells (normal) were shown to have increased apoptosis and decreased mitosis when compared to DOK cells (dysplastic). These results indicate that ACMs do have a genotoxic effect in oral epithelial cells, however they do not induce cellular toxicity in these short-term laboratory conditions.

Although separate assays exist to detect damage through each specific mechanism discussed above, the comet assay is a general test for detection of DNA breakage- the end result of all damage pathways. It is sensitive to DNA single- and double-strand breaks, interstrand cross-links, and apurinic/apyrimidinic (AP) sites which relax the supercoiled structure of genomic DNA under alkaline conditions (Speit G 2006). Multiple studies have established that visual scoring is both efficient and accurate, bearing a linear correlation to computer scoring (Garcia O 2004) (Panayiotidis MI 2004). Results indicate that ACMs cause significant fragmentation of DNA, which over many cycles can lead to mutation. Furthermore, it was found that no DNA repair occurred within the 20 minutes allowed for recovery following treatments, allowing carcinogenic effects to compound more rapidly.

Selecting two cell lines (DOK and OKF-6) for experiments permitted testing of ACM effects on normal oral epithelial cells, as well as allowing comparisons to be made with dysplastic cells. Results from the comet assay indicate that DNA damage is significantly greater in

DOK cells compared to OKF-6 cells, supporting the idea that ACM use may be a greater risk in smokers or patients with pre-existing dysplasia. Previous research shows that risk associated with alcohol consumption is not necessarily constant over the multistage pathway to oral cancer (Franceschi S 2000) (Franceschi et al., 2000). Traditionally, alcohol has been thought to play a role in the later stages of oral cancer progression (Day & Brown, 1980), a view supported by our results. However newer studies are finding that drinking may exert earlier effects, as cessation does not lower oral cancer risk for up to 9 years (Franceschi S 2000) Temporal mechanisms of alcohol carcinogenicity may be more complex and multifactorial than previously thought. Although experiments used were not sensitive to a concurrent synergism between ACM and tobacco carcinogens, it was possible to ascertain the possibility of a sequential synergistic effect with tobacco because DOK cells were developed from the tongue mucosa of a smoker.

Thus, it would appear that the effect of alcohol consumption on oral epithelial cells is likely to be local, topical and temporally important in oral carcinogenesis.

2.2 Human Papilloma Virus

For many years, HPV has been accepted as an important cofactor in the development of cervical cancer, originating from a mucous membrane with similarities to the oral mucosa. It has long been postulated that oncogenic HPV subtypes (specifically, HPV 16 and 18) can have a tumorigenic effect on oral epithelia. Evidence to support a role for HPV was found in analysis of the presence of HPV in biopsy specimens, the majority concluding that it was likely that HPV may be a cofactor in the development of oral cancer (Nielsen, Norrild et al. 1996; D'Costa, Saranath et al. 1998; Al-Bakkal, Ficarra et al. 1999). The INHANCE consortium mentioned above reported on the analysis of a large cohort assessing links between cancer and specific sexual behaviours, including practice of oral sex, number of lifetime sexual partners and oral sex partners, age at sexual debut, a history of same-sex contact and a history of oral-anal contact. This study concluded that these sexual behaviours are associated with increased risk of cancer, particularly of the tongue, tonsil and oro-pharynx and reinforce the association with infection by HPV sub-types (Heck, Berthiller et al. 2009). Furthermore, it has been shown that there is an overall increase in the incidence of base of tongue cancer over the past 30 years and further that there is an increase in the prevalence of HPV in these tongue cancers (Attner, Du et al. 2009). Thus, it would appear that there is an increased understanding of the causative role for HPV in oral and oro-pharyngeal cancer with an urgent need for further research in the role of that this virus may play in the propagation of potentially malignant mucosal lesions.

A recent extensive collaborative study recently reported the prevalence of HPV types in the oropharynx in men who have sex with men (MSM) and compared sampling methods as well as identifying risk factors. This cross-sectional study enrolled 250 HIV negative and 250 HIV positive MSM who were sampled by either a self-collected flocked throat/mouth swab agitated in RNAlater or collected gargled saline. Further, a questionnaire about sexual behaviour, oral hygiene and smoking was collected.

HPV PCR was undertaken after DNA extraction using MagNA Pure LC (Roche Molecular Systems) the HPV DNA amplification was undertaken using L1 consensus primers & beta-globin control via PCR. An ELISA using biotin-labeled probe was used to identify PCR

products and subsequently a line-blot hybridization (Linear array - Roche) used to genotype the PCR product.

The results of this study showed a prevalence of oral HPV with 74 positive in one or both samples. The self-collected flocked throat and mouth swab was positive in 33 of the 74 (44.6% sensitive) while the oral rinse was positive in 65 of the 74 positive samples (87.8% sensitive). High-risk genotypes (16 and 59) were found in 21 (4.2%) samples. The HPV prevalence in this MSM population depended on the HIV status with those HIV negative showed 8% (0.8% HPV 16) positive samples while those MSM who were HIV positive had 22% (4.8% HPV 16) positive samples. Oral rinse method of sample collection was nearly twice as sensitive as self-collected swab. Significant risk factors included smoking, age greater than 40 and lifetime oral sex partners greater that 100.

Thus, it would appear that high-risk HPV sub-types are present in the oral cavity, and this presence is associated with risk behaviours, in particular smoking and the number of lifetime oral sex partners. It could be postulated that there is synergism of these risk factors, particularly if alcohol consumption was also included. There is therefore a need for the development and clinical validation of a simple salivary method for the assessment of risk of developing oral cancer that is not associated with smoking as this is likely to enhance the effectiveness of oral cancer screening services and improving oral cancer outcomes.

3. Novel biomarkers

Currently the gold standard in diagnosis of malignant and potentially malignant oral mucosal lesions is incisional biopsy and histo-pathological assessment. However, histopathological examination has concerns related to sampling errors and errors in interpretation, and lacks sensitivity to determine lesion progression.(Holmstrup, Vedtofte et al. 2006) That is, the level of dysplasia of an oral lesion may not necessarily correlate with the lesion's potential for malignant transformation. Hence, there is a need for a more accurate system to predict the progression to cancer and currently there is significant work being undertaken in identifying markers in patients with oral cancer and pre-cancer that may serve as a valuable resource in finding markers for the early diagnosis of these conditions.

3.1 DNA ploidy

Several studies have already shown that DNA ploidy is of prognostic importance in some human malignancies such as carcinomas of the ovary (Brescia and et al. 1990), prostate (Badalament and et al. 1991), urinary bladder (Norming and et al. 1992) and malignant melanomas (Sorensen and et al. 1991). Importantly, DNA ploidy has been shown to be an early marker of malignant transformation in oral dysplasia (Sudbo and et al. 2001). DNA ploidy can be classified into three clinically relevant categories, diploid, tetraploid and aneuploid. The positive correlation that has been reported to exist between DNA ploidy and prognosis of oral dysplasia (Sudbo and et al. 2001), suggests that DNA ploidy may well be a key factor in the diagnosis, prognosis and treatment of oral malignancies in the future and thus requires full investigation.

The use of DNA ploidy for oral mucosal conditions has attracted considerable controversy (Curfman GD 2006; Reed KD 2007). There was considerable promise in the early part of this decade that the use of DNA ploidy could better predict outcome for potentially malignant oral mucosal disease. Irrespective of the controversies associated with a large portion of this work multiple independent and meticulous studies have been undertaken to attempt to put the investigations of the utility of oral mucosal DNA ploidy on a sound scientific basis. Interestingly, the results of these studies have been varied with some reporting guarded success at predicting outcome of malignant transformation (Torres-Rendon and et al. 2009 June), while others have shown little benefit from this technique in assessing the malignant potential of mucosal lesions (Neppelberg and Johannessen A.C. 2007).

The majority of previous studies assessing DNA ploidy in oral mucosal lesions have used nuclei isolated from formalin fixed, paraffin embedded, archival material 19-22. These nuclei were extracted, processed to form a monolayer on glass slides and, after Feulgen staining, individual nuclei selected for integrated optical density analysis. The number of nuclei analyzed was usually in the range of 200-300 per sample 22. In each of these retrospective studies, internal control nuclei within each monolayer, such as inflammatory cells or "unsuspicious" epithelial cells, were used to assess for significant variations from DNA diploidy (aneuploidy) of nuclei from each specimen. However, as a prospective clinical tool, such methodology is limited.

Over recent years there has been increased use of liquid-based cytology (LBC) for cervical smears and this method has been analyzed for utility with oral cytology (Kujan and et al. 2006). The initial study evaluating LBC for oral cytology assessed 150 specimens from 50 healthy volunteers and found that this technique distributed cells evenly, optimized fixation, improved and unbiased sampling, enhanced nuclear detail and eliminated air-drying artifacts. These researchers also reported that the specimens showed cells from two populations, superficial and intermediate cells with only six (4%) of specimens containing parabasal or basal cells (Kujan and et al. 2006). This latter finding has significant impact as these superficial cells represent keratinocytes that are terminally differentiating and thus nuclei have become non-functional with condensed and fragmented chromatin.

In a recent study (McCullough MJ 2009) assessing the variability of ploidy present in oral cytological material using nuclear analyses of integrated density via Feulgen staining, liquid based processing of oral cytological samples and the establishment of a large database of normality. This study found that there was more variabilitiy observed in patients with normal mucosa than in oral mucosal dysplasia and neoplasia. It may well be that these nuclear alterations present in the superficial cell layers resulted in increased optical integrated density after Feulgen staining observed and the observed lack of differentiation between normal and abnormal samples was likely to be due to sampling, with inadequate numbers of basal cells whose DNA could not be assessed separately from the large number of superficial nuclei (McCullough MJ 2009).

An attempt to circumvent this tissue-sampling problem has been reported by obtained representative tissue specimens by scraping with a dermatological curette (Navone R 2008), thus producing "micro-biopsies" rather than cytological samples from brushing mucosa. Such a technique, which included liquid cytology for tissue handling, has been reported to

be a non-invasive, rapid method that has little patient discomfort and is able to sample a broader area than a single biopsy (Navone R 2008). Ideally, the analyses of the cells collected would include multiple markers, not only assessing the presence of basal cells in the sample, but also the extent of a number of genetic changes known to be linked to the development of oral neoplasia. This is unlikely to be feasible using ploidy assessment via the Feulgen reaction as this reaction requires acid hydrolysis of DNA significantly altering both cytological and nuclear attributes. Such markers may well include the recently reported centrosomal abnormalities reportedly to occur universally in oral dysplasia.

Thus, there still remains the need for a robust method to assess molecular changes known to be associated with the early changes in neogenesis to aid in the early detection of oral mucosal lesions with increased malignant potential.

3.2 mRNAs

The identification of tissue markers that aid assessment of malignant potential have been investigated, and reviewed by Scully (Scully 1993). Such markers include cell surface and cytoplasmic components.

Loss of Heterozygosity (LOH) is considered loss of genetic material at microsatellite loci, and has been shown to be an important event in tumorigenesis (Ng IO, Xiao L et al. 2000). Investigations of LOH in genes and chromosomal regions, has shown that defined regions are affected in oral carcinogenesis/HNSCC and that these may be used as a possible prognostic marker.

A model for HNSCC progression has been proposed suggesting deletions at 3q, 9q and 17q are associated with a morphological transition of cells from normal to dysplastic cells, and that carcinoma development was promoted by further deletions at 4q, 6p, 11q, 13q and 14q (Califano J, van der Riet P et al. 1996).

Single nucleotide polymorphisms (SNPs) are single base alteration or point mutations in DNA and are considered the most common form of genetic variation, occurring approximately every 1200 base pairs in human chromosomes (Sherry 2001). Hence, many studies have investigated the incidence of SNPs in particular genes to determine their role in HNSCC carcinogenesis.

TP53 is an extensively characterised TSG, which encodes the protein p53. TP53 is located on the short arm of chromosome 17 and has 11 exons, one of which is non functional (Liao PH, Chang YC et al. 2000). p53 is a sequence specific transcription factor that is important for DNA maintenance.

A study of 94 oral squamous cell carcinomas (OSSC) found that 43% had TP53 mutations (Ostwald, 2000), similarly another study observed 48.66% of OSSC had p53 mutations (Hsieh, Wang et al. 2001). An analysis of 18 oral tumours found 72% had TP53 mutation, however this high incidence may have been due to the small sample size (Partridge, 2000). Generally, TP53 mutations are considered a common genetic change, being evident in 40-50% of HNSCC (Nylander, 2000).

Other studies have documented associations between TP53 polymorphisms and HNSCC. One investigation observed the highest number of mutations occurred in exon 7 and that the

type of SNP was characteristic of lesion location. Lip lesions exhibited G:C to A:T transitions while intra-oral lesions exhibited an equal frequency of transitions and transversions (G:C to T:A) (Ostwald C, Gogacz P et al. 2000). It has been proposed that these transversions are preferentially induced by breakdown products of benz[a]pyrenes, suggesting a strong correlation with tobacco usage (Ostwald C, Gogacz P et al. 2000). A study of sequential epithelial dysplasias and squamous cell carcinomas suggested TP53 mutations correlated with an invasive tumour phenotype (Shahnavaz SA, Regezi JA et al. 2000). A further study reported greater than 90% of HNSCC samples had TP53 mutations, with the type of mutation varying among tumours (van Oijen MG, Vd et al. 2000 Feb). They also demonstrated primary tumours and metastases showed the same TP53 mutation, suggesting that TP53 mutations are relatively stable within a given tumour.

SNPs in other genes have been investigated including the CYP1A1 polymorphism in the gene encoding cytochrome p450. This polymorphism results in an amino acid substitution from Isoleucine to Valine. It has been proposed that the Valine form of the enzyme has a greater catalytic and mutagenic activity towards benz[a]pyrenes and therefore may be associated with high risk of OSSC development (Sato M, Sato T et al. 2000; Sreelekha TT, Ramadas K et al. 2001). However, other studies have not observed an association between this polymorphism and HNSCC (McWilliams JE, Evans AJ et al. 2000).

3.3 MicroRNAs

An area of interest for markers of potentially malignant mucosal lesions is MicroRNAs (miRNAs). MiRNAs are small non coding RNAs that mediate gene expression at the post-transcriptional level by degrading or repressing target messenger RNAs (mRNAs). They act by binding to partially complementary sites in the 3` region of the mRNA target. MiRNAs are approximately 18 - 22 nucleotides in length and are predicted to regulate at least 30% of the mRNA transcripts (Gomes and Gomez 2008).

MiRNAs are transcribed by RNA polymerase II or III as an independent gene unit or as part of an intron on a larger mRNA molecule. This mRNA can be up to 1000 nucleotides in length and has a stem-loop structure. This initial transcript is cleaved into a shorter stem-loop structure of less than 100 nucleotides by a type III RNAse (Drosha). This pre – miRNA is exported out into the cytoplasm by exportin-5 where another RNAse III (Dicer) cleaves it, resulting in a 18 – 24 nucleotide long mature miRNA. The mature miRNA is bound to a protein complex called an RNA induced silencing complex (RISC – RNA) formed by four argonaute family proteins (Ago 1-4). This active miRNA – protein complex binds to specific sites present in a number of mRNA's resulting in their inactivation. To date, 1048 different human miRNAs have been identified in the miRBase database It has been shown that each mirNA regulates a number of mRNAs(Gomes and Gomez 2008).

MicroRNAs bind to the 3' untranslated region (30 UTR) of target mRNA. Depending on the degree of sequence complementary, the mRNA is either inhibited or degraded. Studies conducted up to date suggest that miRNAs function either as oncogenes or tumor suppressors in various cancer types, as well as OSCC.

MiRNAs have been associated with almost all types of human malignancies including haematological and solid cancers. When the classification accuracy of types of cancer were

compared, variation from the normal profile of miRNAs were shown to more pronounced in poorly differentiated tumors (Hui, Shi et al. 2009).

A reduction in miRNAs that suppress tumours is thought to result in an increase of oncogenic proteins and hence accelerates oncogenic transformation. On the other hand, an increase in miRNAs during oncogenises may be associated with inactivation of tumour suppressor genes thus accelerating oncogenic transformation as outlined in Fig.1. (Gomes and Gomez 2008).

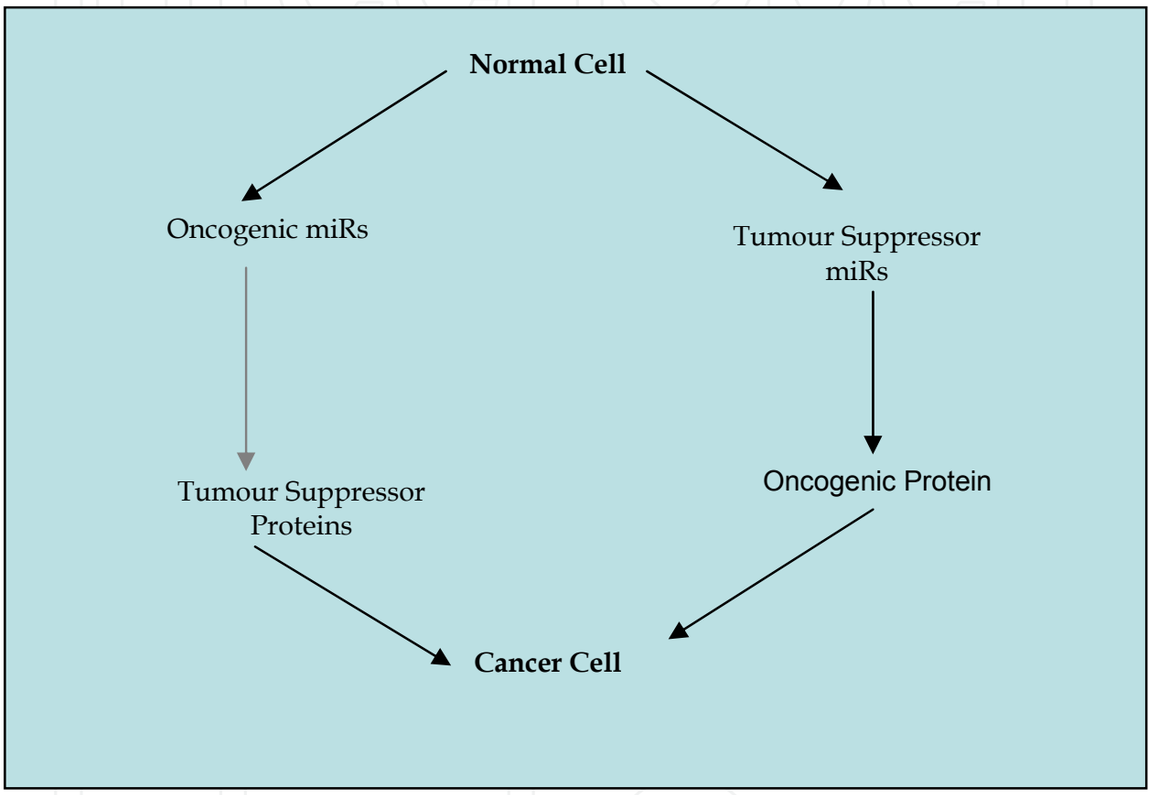


Fig. 1.

Abnormal regulation of these miRNAs in OSCC induces cell proliferation and anti-apoptosis, promotes cancer metastasis and potentiates resistance to chemotherapy. miRNA-regulated pathways in OSCC (Bo-hai Wua, Xue-peng Xiong et al. 2011).

In the past, a great deal of focus has been placed on messenger RNAs (mRNAs) and their ability to act as biological markers in cancer. Recent studies have shown that miRNAs more accurately cluster different types of solid tumors than mRNA, suggesting that miRNAs may be an alternative early marker of malignancy (J, G et al. 2005) Furthermore, the extent of change in mRNA between cancer cells and normal cells is relatively small, whereas, many miRNAs exhibit large changes between normal and cancer cells (in the order of ten to hundred fold changes) hence potentially enhancing detection of differences.(Jinmai Jiang, Eun Joo Lee et al. 2005)

It has been reported that miRNAs may be less prone to degradation and modification than mRNAs due to their small size, hence allowing the use of formalin fixed paraffin embedded (FFPE) samples (Martina, Magotra Amber A. et al. 2008). There is thus a potential rich source of retrospective information available for comparative genomics and investigation of potential biomarkers that is likely to provide biological insights far more expeditiously than the prospective collection of frozen samples.

Abnormal miRNA expression has been found in both premalignant and malignant cells (Clague j, Scott M. Lippman et al. 2010). Hence, there is a need for the investigation of miRNA expression in potentially malignant oral mucosal lesions as well as OSCC as deregulated miRNAs may be an early reliable marker for malignancy as well as a potential target for cancer prevention. Although there are no specific patterns of miRNA expression till date, certain core miRNAs should be considered in tumorigenesis of the head and neck region. This core set includes, mir -21, mir -205 and mir – 155 which have shown to be constantly upregulated (Tran, O'Brien et al. 2010).

It is evident further investigations to elucidate a marker or markers of malignant potential are required to aid detection of malignant and pre-malignant lesions, as well as to better predict the prognosis of individuals with HNSCC. With the advent of accessible gene sequence information and high throughput genetic methods, genetic alterations may act as such a marker.

3.4 Cytokines

Cytokines are a group of small, mainly secreted proteins that affect the behaviour of cells in a diverse number of ways. The binding of cytokines to specific cytokine receptors can induce a number of activities in the cell, such as growth, differentiation, or death (Janeway CA 1996). Although most cytokines have pleotrophic effects, some are generally considered pro-inflammatory, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) (Dinarello CA 1997), whereas others are associated with anti-inflammatory effects, such as transforming growth factor-beta-1 (TGF-β1) (Ling EM 2002).

Over the last 5 years or so, a considerable effort has been undertaken analysing the salivary proteome. There has been a very large number of non-redundant proteins recognised in saliva with one study (Scarano E 2010) reporting over 1400, while a further (Xiao H 2011) almost 2,000, reflecting the potency of salivary biomarker profiles in the identification and management of a range of diseases (Bandhakavi S 2009). Salivary biomarkers have the potential to serve as non-invasive, widely available screening tools that do not rely on the localization of a lesion for diagnosis. This advantage over other detection methods gives salivary biomarker screening the potential to identify patients with premalignant lesions.

Of particular interest has been the use of salivary cytokine levels as markers of cell proliferation and oral cancer (Schapher M 2011) with the most studied cytokines including epidermal growth factor (EGF), interleukin 6 and 8, vascular endothelial growth factor (VEGF), interleukin 4 and 10, tumour necrosis factor (TNF) and endothelin.

A large body of work has assess interleukin 6 (IL-6), a multifunctional cytokine that participates in the inflammatory and immune responses shown to directly promote the growth of certain types of cancer as well as being associated with an increased rate of

metastasis (St John MA 2004) (Brailo V, 2006 #143). Interestingly, IL-6 would appear to have different effects on different cell populations, stimulatory for some cell types, while inhibitory for others (Wang YF 2002) as well as demonstrable direct effect on cancer cells due to inactivation of the p53 tumour suppressor gene (Hodge DR 2005). Irrespective of the role of IL-6, there is an increasing body of evidence to support that there is higher level of IL-6 in saliva of patient with oral cancer as well as oral potentially malignant lesions, than in normal controls. In a recently trial of 29 consecutive patients being treated for oral cancer it was shown that these patients had much higher salivary concentration of IL-6 than in controls and that this concentration increased during the treatment period returning to baseline levels at discharge (Sato J 2010).

Other studies however have assessed a panel of pro-inflammatory cytokines as makers of malignancy. In a recent study of levels of IL-1a, IL-6, IL-8, VEGF-a and TNF-a in saliva were measured using quantitative ELISA in a grop of 18 patinets with tongue SCC. These biomarkers were increased in patients with oral cancer, significantly increased in a sub-group of patients with endophytic tongue cancer and IL-8 levels particularly shown to correlate with poor prognosis as well as controls who smoke and consumed alcohol daily (Korostoff A 2011).

These findings indicate that salivary cytokine levels is very likely to provide useful information of epithelial behaviour and carcinogenesis and the potential of a panel of these cytokines being able to be used as a screening tool for oral cancer is currently underway, the results of which are eagerly awaited as this is likely to have a profound impact on the early detection of oral cancer and thus morbidity and mortality.

3.5 Chemokines

Chemokines are a superfamily of structurally related cytokines, which share an ability to chemotactically attract their target cells along a concentration gradient. It is through this ability that these molecules play an integral role in the migration of immune cells to areas of pathogen challenge. Chemokines also mediate the movements of cells to allow interactions between immune cells that are essential for mounting immune responses (Zlotnik A 2000).

All chemokines are small proteins, ranging in weight from 6-14KDa. There are now over 40 identified chemokines which can be classified into 4 main structural families, dependent upon the position of cysteine residues near the N-terminus. These families are the CC, CXC, C and CX3C, with the X denoting the number of amino acids between the cysteine residues (Olsen TS 2002). Nearly all chemokines are secreted from the site of production and they often bind with glycosaminoglycans (Hoogewerf AJ, Kuschert GS et al. 1997 Nov 4). It is thought that this is the method in which the chemokines form the concentration gradient that target cells migrate towards, as a higher concentration is formed on the connective tissue nearest the area of chemokine production.

The function of chemokines can be subdivided into two main families; those that are induced after inflammatory stimuli, the inflammatory chemokines, and those produced constitutively in tissues, the homing chemokines (Kunkel EJ 2002). There appears to be some overlap between these chemokines as some of the inflammatory chemokines appear to be produced constitutively in some areas of the body (Izadpanah A 2001) and some of the

chemokines designated as homing chemokines can be upregulated by inflammatory stimuli (Morales J 1999) .

Although the detection of chemokine levels by enzyme-linked immunosorbent assay (ELISA) has become a sensitive and specific method to determine the chemokine profile in patient fluids, this does not fully represent the actual inflammatory conditions in vivo. Indeed, many chemokines are post-translationally modified by proteolytic cleavage, which can render an agonist more active, inactive or even convert the active chemokine into a receptor antagonist of the intact molecule (Struyf S 2003).

A recent study analysed, using ELISA, the saliva of patients with oral cancer for the presence of both inflammatory chemokines (CXCL8, CXCL10 and CCL2) as well as homeostatic chemokines (CXCL4, CCL14 and CCL18) (Michiels K 2009). Patients with and without periodontitis were used as controls and it was found that H&N carcinomas give rise to a change in the chemokine composition of the oral fluid with a significant increase in CXCL8, CXCL10, and CCL14 before, but not after, therapy. However, the levels detectable by ELISA were very low and it is likely that more refined methods should indicate not only intact chemokines, but also those modified post-translationally. These authors conclude that it can be expected that specific truncated chemokines and the proteases involved are linked to a particular disease state and postulate that further proteomic analysis of biological fluids will help us to learn more about the pathogenesis of specific diseases and can provide solutions for new diagnostic and treatment options.

4. Conclusion

Oral cancer is a significant problem with low rates of mortality and an enormous impact on quality of life and morbidity. In the past this has been predominantly a disease of the elderly with known epidemiologic risk factors, however this appears to be changing. The principle recognized risk factor is smoking; however, there is growing evidence that the local effect of alcohol, most likely via its toxic metabolite acetaldehyde, may be increasing in importance. There is now evidence that alcohol containing mouthwash show similar oral acetaldehyde levels as recreational alcohol containing liquids. Further, these mouthwashes have been shown to have genotoxic effect in oral epithelial cells. Therefore be inadvisable for oral health care professionals to recommend the long-term use of alcohol-containing mouthwashes.

Particular high-risk HPV sub-types are now known to be implicated in a large proportion of oro-pharyngeal cancers and these same sub-types are present in the oral cavity. It has been postulated that there is synergism of risk factors, such as smoking and alcohol consumption. As these previously under-recognized risk factors may be becoming of increasing important aetiological agents, there is therefore a need for the development and clinical validation of a simple salivary method for the assessment of risk of developing oral cancer that is not associated with smoking as this is likely to enhance the effectiveness of oral cancer screening services and improving oral cancer outcomes. Identifying a combination of salivary biomarkers that predict at- risk and cancerous states with sufficient sensitivity and specificity for widespread use will have a profound impact on improving the morbidity and mortality of the disease.

A number of cellular and molecular markers are currently under ongoing investigation to aid in the detection of early changes to aid in the detection of oral mucosal lesions with increased malignant potential. With the advent of accessible gene sequence information and high throughput genetic methods, genetic alterations may act as such a marker. Furthermore, there is evidence that salivary cytokine levels can provide useful information of oral epithelial behaviour and carcinogenesis and the potential of a panel of these cytokines being able to be used as a screening tool for oral cancer is currently underway, the results of which are eagerly awaited as this is likely to have a profound impact on the early detection of oral cancer and thus morbidity and mortality. Finally, further genomic and proteomic analysis of oral tissue, whether oral biological fluids or cells, will help us to learn more about the pathogenesis of oral cancer and it is anticipated that this will provide solutions for new diagnostic and treatment options.

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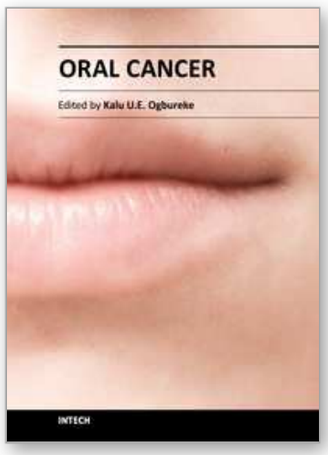

Oral Cancer

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Oral cancer is a significant public health challenge globally. Although the oral cavity is easily accessible, early diagnosis remains slow compared to the enhanced detection of cancers of the breast, colon, prostate, and melanoma. As a result, the mortality rate from oral cancer for the past four decades has remained high at over 50% in spite of advances in treatment modalities. This contrasts with considerable decrease in mortality rates for cancers of the breast, colon, prostate, and melanoma during the same period. This book attempts to provide a reference-friendly update on the etiologic/risk factors, current clinical diagnostic tools, management philosophies, molecular biomarkers, and progression indicators of oral cancer.

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