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## Blood Groups and Oral Lesions Diagnostics

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### 1. Introduction

Cancer incidence in humans has gradually increased over the last century. Surgical, radio, chemotherapeutic and biological treatments have experienced important advances, with concomitant reduction in the morbidity associated with the radical surgical practices of the past. The term “oral cancer” includes a diverse group of tumors arising from the oral cavity (Khalili, 2008). Usually included are cancers of the lip, tongue, pharynx, and oral cavity. The World Health Organization (WHO) reported oral cancer as having one of the highest mortality ratios amongst all malignancies (Parkin et al., 2000). Although oral cancer is rare and attracts little attention, it is more common than Hodgkin’s disease tumours of brain, liver, bone, thyroid gland, stomach, ovaries, or cancer of the cervix. It ranks 12th among all cancers (Jemal et al., 2002). The vast majority of malignant neoplasms in the mouth are squamous cell carcinomas. Oral cancer incidence and mortality rates vary widely across the world. Mortality rate is an important tool that provides implicit information about incidence, diagnosis stage, solving capacity of health services, available technology and health programs to be applied. Although globally oral cancer represents an incidence of 3% (males) and 2% (females) of all malignant neoplasm, it has one of the lowest survival rates – 50 percent, within a five-year period (Greenlee et al., 2001).

It is important to diagnose oral cancer in its early stages, since the management of small and localized tumors involves less morbidity and mortality than more advanced-stage disease, where treatment must be more aggressive. Indeed, the stage in which the disease is diagnosed is directly correlated to long-term survival. (Onizawa et al., 2003). It is generally accepted that when diagnosed in its early stages, a favorable prognosis is expected, with a survival rate exceeding 90% at the 5-year follow-up. However, reviewing the literature exposes a less optimistic picture, because lymph node metastases seem to occur in 5% to 20% of cases (Regev et al., 1992, Khalili, 2008). However, in practice many malignancies are diagnosed and treated in advanced stages and/or once the patients have already experienced symptoms causing them to seek medical help. This explains the great interest in improving multidisciplinary therapies, and particularly in establishing more reliable techniques for diagnosing and prognosis of the illness (Miller & Kearney N, 2001).

The membrane, which defines the extent of the cell, is not only a physical boundary but also has many specific functions, among which is the capacity to react with other cells and the intracellular matrix (Ebnet & Vestweber, 1999, Hascall, 2000). Carbohydrates are structures found on the cell surface bound to either lipid or protein embedded in the membrane.

Changes in the carbohydrate structure of these cell-surface glycolipids and glycoproteins have been demonstrated during development, during cell maturation in adult tissue, and in relationship to malignant development.

Biochemical and molecular genetic studies have contributed to our molecular knowledge of blood group-associated molecules in the past few years (Dabelsteen, 1996, Fenderson et al., Fukuda, 2002, 1986, Hakomori, 1999, 2002, 2003, Le Pendu *et al.*, 2001). Among the 30 blood group systems presently identified, almost all have a molecular basis and present investigations are oriented towards the analysis of genetic polymorphisms, tissue-specific expression and structure-function relationships. Antigens defined by carbohydrate structures, among which ABO, Hh, Lewis and Secretor are the main representative species, are indirect gene products (Hakomori *et al.*, 1967). They are synthesized by Golgi-resident glycosyltransferases, which are the direct products of the blood group genes. Cell-surface carbohydrates are built up in a stepwise fashion when monosaccharides are transferred from their sugar nucleotide derivatives to appropriate acceptors. Each particular type of transfer is catalyzed by a unique specific glycosyltransferase. In tumors, changes in glycosylation are found in both glycolipids and glycoproteins (Hakomori, 1999; Le Pendu *et al.*, 2001). Most studies have dealt with alteration of carbohydrates at the cell surface. However, several recent studies have shown that altered glycosylation plays a major role in most aspects of the malignant phenotype, including signal transduction and apoptosis. These studies have recently been reviewed (Hakomori, 2002; Hakomori & Handa, 2002, Dabelsteen & Gao, 2004). Historical studies associating the Lewis system antigens and/or ABH system secretory antigens with disease are varied and generally inconclusive. Critical analysis of these studies reveals that in many instances the serology is inadequate, mainly as a result of unappreciated difficulties in accurately phenotyping diseased individuals (Svensson, 2000).

Before a detailed account of the immunochemistry and genetics is presented, a brief summary will be given in order to orient the general reader. The A and B antigens were originally detected on erythrocytes by means of isoagglutinins in the serum of persons lacking these determinants. These antigens are synthesized from a common intermediate, H substance, by addition of a single sugar to the non reducing end of H oligosaccharide chains, and the immunologic reactivity of the H antigen is markedly decreased by the additional sugar. Group O erythrocytes and the saliva of group O secretors contain the H antigen. Even though the O antigen does not exist, the designation group O erythrocytes have been retained for historical reasons. The blood group H antigen is an oligosaccharide molecule whose expression is normally restricted to the surfaces of human erythrocytes and a variety of epithelial cells, including those that line the gastrointestinal, urinary, and respiratory tracts (Larsen et al. 1990). The H antigen is a fucosylated structure of the form Fucal-2Gal $\beta$ 3-, whose expression is determined by GDP-L-fucose:P-D-galactoside 2- $\alpha$ -L-fucosyltransferases [ $\alpha$ (1,2)Frs; EC 2.4.1.69]. These enzymes catalyze a transglycosylation reaction between their sugar nucleotide substrate GDP-L-fucose and oligosaccharide acceptor substrates with terminal type I (Gal $\beta$ 1-3GlcNAc-) or type II (Gal,  $\beta$ 1-4GlcNAc-) moieties. The secretor status is defined by the presence of H type 1 antigen in body secretions such as milk and saliva. H type 1 antigen belongs to both the Lewis and the ABO(H) histo-blood-group systems and is expressed in erythrocyte membranes and in several epithelial tissues, namely the gastric mucosa, the upper respiratory tract and the lower genito-urinary tract. Approximately 75 per cent of white persons secrete glycoproteins containing the same A, B or H antigens present on their erythrocytes (Moreno et al., 2009).

The Lewis antigens, Le<sup>a</sup> and Le<sup>b</sup>, are also found on erythrocytes and glycoproteins. These antigens appear on the same glycoproteins as the ABH determinants, but their synthesis is regulated by the independent gene *Le*. The operation of these independent genes on a common substrate results in a complex phenotypic interaction (Henry et al., 1995).

It is well established that the large array of functions that a tumour cell has to fulfill to settle as a metastasis in a distant organ requires cooperative activities between the tumour and the surrounding tissue and that several classes of molecules are involved, such as cell-cell and cell-matrix adhesion molecules and matrix degrading enzymes, to name only a few. Cell adhesion molecules are found on the surfaces of all cells, where they bind to extracellular matrix molecules or to receptors on other cells. Cell adhesion is critical in the dynamic processes necessary for tissue morphogenesis in development and the maintenance of complex differentiated tissues in adult organisms. Adhesion molecules have originally been thought to be essential for the formation of multicellular organisms and to tether cells to the extracellular matrix or to neighbouring cells (Marhaba & Zöller, 2004). CD44 is the major human cell surface receptor for hyaluronate and functions in a diverse range of physiological processes. CD44 may play a role in stimulating *in vivo* aggressiveness of tumors through hyaluronate-rich stroma (Hudson et al., 1996). Expression of CD44 has been described to correlate with metastasis formation in various tumors, although evidence in oral cavity cancers is inconclusive.

## 2. ABO antigens

Although the ABO blood group antigens were initially identified as erythrocyte substances with a significance mainly ascribed to serology, it soon became clear that these antigens were found on most epithelial cells and in secretions (Landsteiner, 1900). These ABH antigens are carbohydrate antigens which in epithelia are expressed in a highly regulated way that correlates with the pattern of epithelial differentiation and with cell maturation (Ravn & Dabelsteen, 2000).

Profound changes in expression have been documented during epithelial cell migration in wound healing and in pathological processes such as malignant development, including oral carcinoma (Dabelsteen, 1996, Dabelsteen et al., 1998, Hakomori, 1996, Le Pendu et al., 2001). Tumor progression is often associated with altered glycosylation of the cell-surface proteins and lipids (Hakomori, 1996). The peripheral parts of these cell-surface glycoconjugates often carries many of the target molecules that reside in blood are also present in oral fluids, albeit at lower concentrations. Oral fluids are, however, relatively easy and safe to collect without the need for specialized equipment and training. Thus, oral fluids provide convenient samples for medical diagnostics, carbohydrate structures related to the ABO and Lewis blood-group antigens. The expression of histo-blood-group antigens in normal human tissues is dependent on the type of differentiation of the epithelium. In most human carcinomas, including oral carcinoma, a significant event is the decreased expression of histo-blood-group antigens A and B (Hakomori, 1999). The mechanisms of aberrant expression of blood-group antigens are not clear in all cases (Hamokori & Handa 2002, Le Pendu et al., 2001, Gao 2004a, 2004b). A relative down-regulation of the glycosyltransferase that is involved in the biosynthesis of A and B antigens is seen in oral carcinomas in association with tumor development (Hakomori, 1999, Le Pendu et al., 2001). However, several recent studies have shown that altered glycosylation plays a major role in most

aspects of the malignant phenotype, including signal transduction and apoptosis. Studies of associations between various cancers and the ABO blood groups have shown elevated relative risks for some categories of disease (Campi et al., 2007, Khalil, 2008).

To investigate the association of expression of ABH antigens and oral cancer, we conducted a study of premalignant lesions and diagnosed malignant tumors. The patients analyzed in this study presented to the Stomatology Department of The Odontology Faculty of the National University of Rosario, Argentina during two years. In total 132 subjects were examined, half of whom suffered from oral pre-cancerous and cancerous lesions, while the other half were the control group (benign lesions: mucosceles, papiloma, etc). All of them were subjected to clinical oral examinations. In the group of patients with oral pre-cancerous and cancerous lesions (experimental group), a pathohistological examination of the oral mucosa was performed (Biondi et al., 2008).

All biopsies were fixed in 4% buffered formaldehyde, paraffin embedded, sectioned at 4µm, and stained with hematoxylin and eosin. Sections (4 µm) from the tumor biopsies were placed on gelatine-coated slides. Sections were deparaffinized in xylene and brought to water through graded ethanol (100%).

### 2.1 Specific red cell adherence test

Specific red cell adherence test was performed on paraffin embedded sections to detect the intensity of isoantigens A, B and H (O) on the epithelial cell surface by a three layer sandwich technique, as described in (Vengelen-Tyler, V. 2002, Strauchen et al., 1980). Commercially available Anti A, Anti B, and Anti AB antisera from Span Diagnostic Limited and *Ulex europaeus* lectin (Anti H) were used. Slides of 4-5 micron section were deparaffinized and brought to water, immersed in Tris buffered saline 0.05 M (pH 7.4) for 30 minutes, covered with isologous antisera according to patients' blood group, and incubated for one hour with Anti- A, -B and -O antisera in a moist chamber at room temperature. The slides were then dipped in Tris buffered saline three times with occasional stirring to remove the unreacted antisera. A few drops of 2-5% isologous indicator RBC's suspension were added to the sections and incubated for 20 minutes in group A or B and one hour for group O. The slides were inverted over a support in a petridish containing Tris buffered saline such that the undersurface of the slide just touched the solution, and kept for five minutes to settle unreacted RBCs down. The slides were observed under low power magnification and photographed immediately.

Normal tissues containing blood group antigens, endothelium of blood vessels and RBCs acted as inbuilt positive controls, and adipose tissues acted as inbuilt negative controls.

In the present study the isoantigenicity of the epithelium was graded according to degree of adherence of indicator RBCs as strongly positive adherence (++++) to negative adherence (-). Intermediate levels were graded as + for 25% of adherence, ++ for 50% of adherence, and +++ for 75% of adherence.

The immunoadherence reaction to tissue sections using antibodies and red blood cells showed a significant loss of A, B or H antigens related to the stage of tumor development and the histological grade of malignancy (Table 1).

In the tissue sections studied, the endothelium of blood vessels was reactive with the erythrocytes (positive control), and adipose tissues did not react with the red blood cells

(negative controls). Loss of A, B, and H antigens from the surface of red blood cells was observed in patients with oral malignancy (89,4%), while the other 10,6% conserved the ABH expression. 39.4 % of the benign lesions which were diagnosed anatomopathologically lost the antigenic reactivity.

	PRECANCEROUS CANCEROUS	BENIGN LESIONS
Partial or total deletion	59	26
Antigenic conservation	7	40

Table 1. Expression of the ABH antigens in fixed tissue sections of oral lesions

Blood-group antigens can be present on key receptors controlling cell proliferation, adhesion, and motility, such as epidermal growth factor receptor, integrins, cadherins, and CD44 (Gao et al., 2004). The expression patterns of these various receptors differ according to the type of normal epithelium and the type of cancer, and therefore the role of ABH antigens in the biology of human cancers may also vary. The function of the expression of ABO antigens in normal stratified oral epithelium is unclear.

In routine diagnostic histopathology, classification of tumor type is based on the histological appearance of the most differentiated parts of the tumor. The prognosis of the tumor, on the other hand, is based partly on properties within the less differentiated parts. In most cases, the degree of differentiation is determined by cellular and tissue morphology and by the ability of the cells to synthesize certain specific products such as keratin and mucins. It has previously been demonstrated that the expression of cell surface carbohydrates in oral stratified epithelium is related to cell differentiation (Ravn & Dabelsteen, 1999). Most studies have dealt with alteration of carbohydrates at the cell surface.

The results we obtained have demonstrated that the patients examined showing benign lesions expressed the ABH antigens in the tissues analyzed but there were significant differences in the experimental group (Fig.1, 2). We also found a higher intensity of oral disease in the group with total ABH deletion, and the occurrence of epithelial dysplasia was most frequently found in this group. Within the most invasive tumors sites, a deletion of ABH reactivity correlated significantly with the stage of tumor development and histological malignancy grade.

In the sections studied, the endothelium of blood vessels was reactive with the erythrocytes (positive control) and adipose tissues did not react with the red blood cells (negative controls).

We used the loss of the expression of ABH antigens as a marker of differentiation.

As the expression of these antigens can be detected by monoclonal antibodies, they are a better objective marker of differentiation than the more commonly used subjective histological assessment. The presence or absence of blood group antigens has been used to predict the clinical course of patients with superficial transitional cell carcinoma of the bladder (Foresto et al., 2000). The red-cell adherence test has been the most widely accepted method of antigen determination, but this technique has inherent weaknesses. Recently, the immunoperoxidase assay has been used to detect antigens on tumor cells. We compared patients using the red-cell adherence and immunoperoxidase methods on adjacent micro cut

sections. The red-cell adherence and immunoperoxidase methods performed similarly (89%) when assessing the presence or absence of antigen (Boileau et al., 1985).

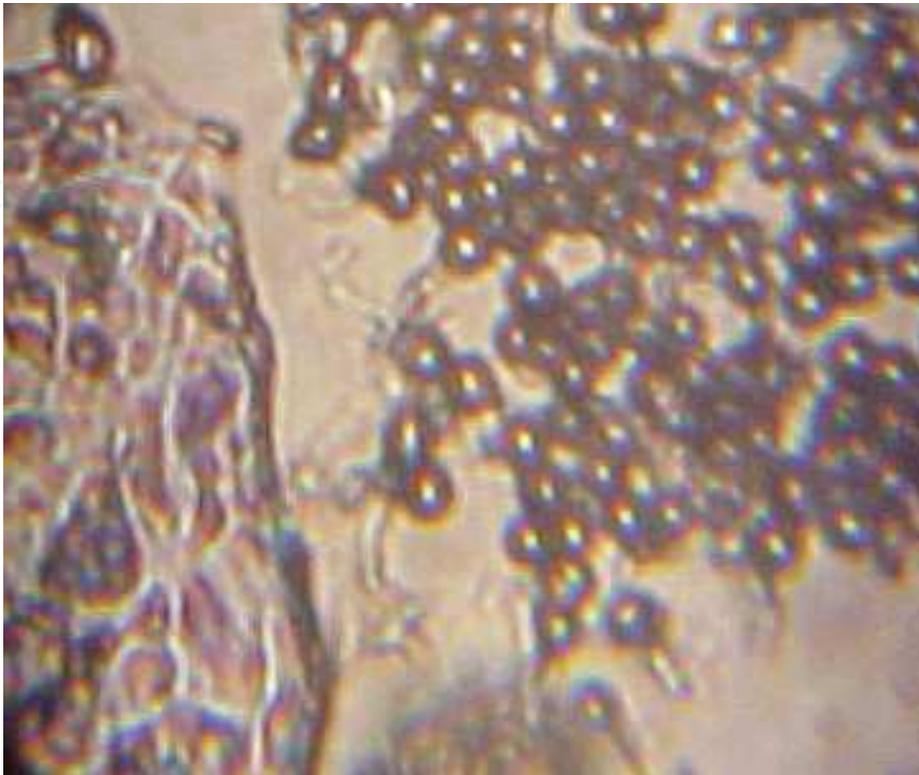


Fig. 1. Cancerous lesion: non immuno-adherence of red blood cells

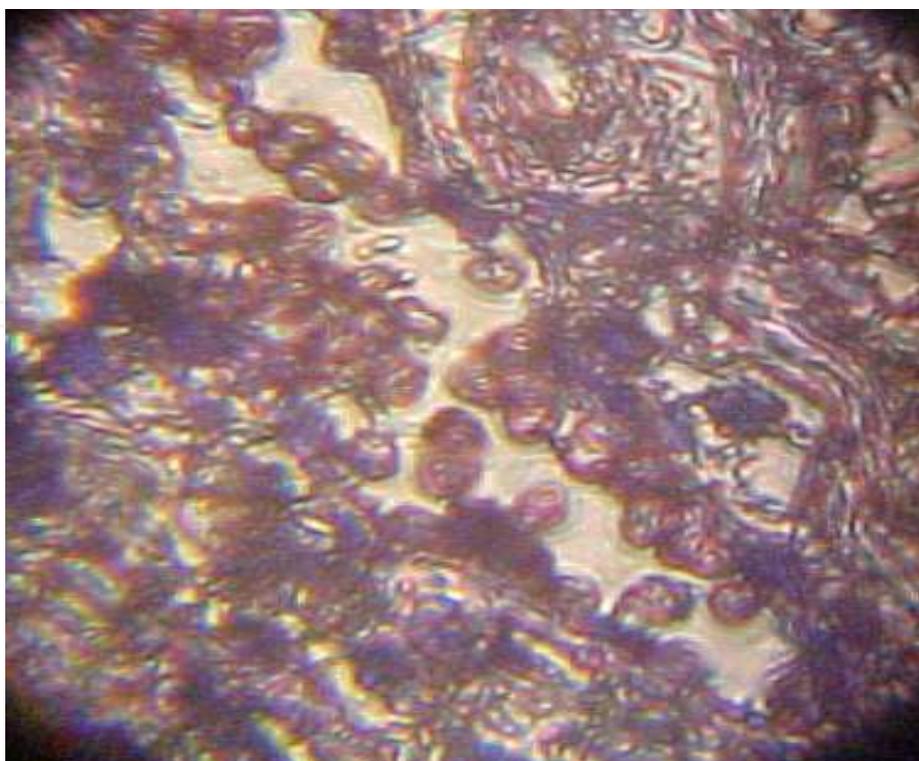


Fig. 2. Oral benign lesion: immuno-adherence of red blood cells to the tissue

Oral cancer often develops clinically as a two stage process, the first step being the appearance of a potentially malignant lesion and the second step the development of carcinoma. Leukoplakia and erythroplakia are clinical changes in the oral mucosa regarded as potentially malignant lesions (Gao et al., 2004). It is generally accepted that tumors are composed of heterogeneous cell populations with different biological behaviors. To obtain optimal prognostic information about the tumor, therefore, the entire tumor cell population should be studied. Despite the somewhat non representative nature of the biopsy material, it was possible to show that loss of ABH antigens was associated with the spread of tumor (stage). This could be of diagnostic and prognostic value.

Immunohistochemical studies of oral squamous cell carcinomas have shown loss of expression of A or B antigens in more than 80% of cases, all of which showed concomitant loss of A/B transferase (Gao et al., 2004a, 2004b). Studies of potentially malignant lesions have shown loss of A/B antigen in most lesions with epithelial dysplasia and in half of the lesions clinically. In the normal oral cavity, keratinized epithelium in the palate or gingiva shows little or no expression of A or B blood group antigen. Since a change from a non-keratinized to a keratinized differentiation pattern is a characteristic of many oral carcinomas and potentially malignant lesions, the lack of expression of blood-group antigens in such lesions could be due to a change in the differentiation pattern of the epithelium (Ravn & Dabelsteen, 2000, Dabelsteen et al., 1975). Other study has showed that the sequential expression of antigen is lost in carcinomas but retained in lesions with epithelial dysplasia and in lesions which clinically and histologically are regarded as benign. It also showed that although the sequential expression of carbohydrate antigens are retained in lesions with epithelial dysplasia, these lesions differ from normal and benign lesions due to an extended distribution of one of the carbohydrate structures (Dabelsteen et al, 1988). Some findings have also demonstrated that malignant development in stratified oral epithelium is associated with aberrant glycosylation of cellular glycoconjugates and that there are differences between premalignant lesions and carcinomas which may prove to be of diagnostic significance (Dabelsteen et al, 1988).

### 3. Secretor status and Lewis histo-blood group antigens

Although the ABO blood group antigens were initially identified, by Landsteiner, as erythrocyte substances with a significance mainly ascribed to serology, it soon became clear that these antigens were found on most epithelial cells and in secretions. Today the molecular and genetic basis of the ABH and Lewis systems and the associated secretory phenotypes has been resolved (Kelly 1995). The secretor gene (*FUT2*) codes for an  $\alpha(1,2)$ fucosyltransferase that determines the ABH secretor status and influences the Lewis phenotype of an individual. The secretor status is defined by the presence of H type 1 antigen in body secretions such as milk and saliva H type 1 antigen belongs to both the Lewis and the ABO(H) histo-blood-group systems and is expressed in erythrocyte membranes and in several epithelial tissues, namely the gastric mucosa, the upper respiratory tract and the lower genito-urinary tract (Torrado, et al., 2000). Although the synthesis of H type 1 antigen is dependent on the sequential action of several glycosyltransferases, the secretor enzyme (*FUT2*), an  $\alpha$ -1,2-fucosyltransferase, is responsible for the transfer of fucose in an  $\alpha$ -1,2 linkage to form the terminal H type 1 structure (Oriol et al., 1986).

The Lewis histo-blood group antigens Lewis a ( $Le^a$ ) and Lewis b ( $Le^b$ ) are carbohydrate structures that form epitopes on glycolipids and glycoproteins (Nishihara et al., 1994). Two

independent genes determine the Lewis phenotype; the Lewis gene (*Le* and *le*), and the secretor gene (*Se* and *se*). Conventional Lewis blood grouping is difficult (e.g., in cancer patients and pregnant women) because of the presence of nongenuine Lewis negative individuals (Ørntoft, et al., 1991). The secretor status in Lewis-negative individuals is currently determined by a labor-intensive hemagglutination inhibition technique that uses heat-inactivated saliva. In Lewis positive individuals, the secretor status is deduced from the Lewis phenotype: i.e.: Le(a-b+) individuals are secretors, and Le(a+b-) individuals are nonsecretors (Nishihara et al., 1994). The ABO blood group antigens are among the well-known fucosylated glycans. The expression of them is regulated by several glycosyltransferases that add monosaccharides to a precursor molecule in a sequential fashion (Mandel et al., 1992). The  $\alpha(1,2)$  fucosyltransferase that forms the H antigen, an essential precursor of the A and B antigens, plays a regulatory role in the tissue expression of the ABO antigens.

The expression and secretion of ABO antigens in epithelial cells are controlled by secretor-type  $\alpha(1,2)$ fucosyltransferase activity, known as the Secretor (Se) transferase (*FUT2* gene product) (Narimatsu et al., 1996). Several different polymorphisms are known in the *FUT2* gene, some called as silent mutations, while others as to non-functional enzymes. Homozygous individuals with non-functional enzymes are termed non-secretors (*se*\_/\_*se*\_). About 20% of individuals are non secretors who fail to express the ABO antigen in saliva. On the other hand, heterozygous individuals carrying one functional allele, have secretion similar to the wild-type. These are termed secretors (Se) (Narimatsu et al., 1996, Koda, et al., 1997).

Tumor progression is often associated with altered glycosylation of the cell-surface proteins and lipids. The peripheral part of these cell-surface glycoconjugates often carries carbohydrate structures related to the ABO and Lewis blood-group antigens. We analyzed the *FUT2* gene and Se status in patients with oral lesions (benign, pre-cancerous and cancerous lesions) in order to determine whether these factors could be a marker risk of oral cancer. In total 178 subjects were examined, half of whom suffered from oral lesions (benign, pre-cancerous and cancerous), while the other half were the healthy control group. All of them were subjected to clinical oral examinations and standard evaluation tests in order to establish the secretor status of their saliva (agglutination inhibition technique (Vengelen-Tyler, 2002). In the group of patients with oral benign, pre-cancerous and cancerous lesions (experimental group), a pathohistological examination of the oral mucosa was performed.

Patients with benign oral lesions showed hyperplasia caused by diverse agents such as infectious, inflammatory, traumatic, hormonal, and drugs. The premalignant lesions included leukoplakia and lichen planus. The malignant lesions studied were squamous cell carcinoma.

Appropriate informed consent was obtained from all subjects and all procedures were performed according to the ethical standards established by the University of Rosario.

Saline erythrocyte suspensions were used for serological studies. The Lewis phenotypes of fresh blood samples were determined by a hemagglutination method (Vengelen-Tyler, 2002), using anti-Le<sup>a</sup> and anti-Le<sup>b</sup> monoclonal antibodies. In order to establish the secretor status we analyzed their saliva by the agglutination inhibition technique.

### 3.1 Inhibition test for secretor status

Two or 3 ml of saliva were collected into wide mouthed tubes. In order to eliminate the mucine protein they were treated with thermal shocks. They were then centrifuged and the supernatants were transferred to clean test tubes and placed in a boiling water bath for 10 minutes to inactivate salivary enzymes. To 1 drop of appropriately diluted blood grouping reagent (anti-A, anti-B, or *Ulex europaeus* lectin) we added 1 drop of the patient's saliva. After incubation for 10 minutes at room temperature, we added 2 drops of 2% to 5% saline suspension of washed indicator red cells. Then, the tube was incubated for further 30 minutes and centrifuged in order to macroscopically inspect for agglutination. Agglutination of indicator cells by antibody in tubes containing saliva indicates that the saliva does not contain the corresponding antigen (non-secretor status). Failure of known antibody to agglutinate indicator cells after incubation with saliva indicates that the saliva contains the corresponding antigen (secretor status).

### 3.2 Molecular studies

#### 3.2.1 DNA isolation

Genomic DNA was isolated from saliva samples. We designed a protocol for DNA extraction from these samples. They were subjected to thermal shock by successive freezing and thawing and centrifuged to work with the cell button. We used the technique CTAB-DTAB (dodecyltrimethylammoniumbromide/ cetyltrimethylammoniumbromide) adding CTAB directly without the addition of TE buffer (Yamamoto et al., 1990, Henry et al., 1995). The DNA concentration was measured spectrophotometrically at 260 nm and diluted in sterile water to a concentration of 100 ng per  $\mu\text{L}$ .

#### 3.2.2 G428A polymorphism

The DNA samples were analyzed by ASO-PCR (allele specific oligonucleotid - polymerase chain reaction) with specific primers (Operon Lab) for G428 allele and the wild type allele of *FUT2* gene (Table 2). A fragment of 132 bp was amplified as described by Henry et al. (Henry et al., 1995), except for the annealing temperature modifications. According to gradient of PCR the  $T_m$  of the primers chosen was 66°C. The PCR products (132 bp) were analyzed in 2 % agarose gel containing ethidium bromide. The categorical data were examined with a  $\chi^2$  test, and the ORs were calculated as measure of association.

Primers	$T_m$	Sequence	Specificity
FUT2-Se-428-s	68,8 °C	5'-CCGGTACCCCTGCTCGTG-3'	Se (direct)
FUT2-se-428-f	66,6 °C	5'-ACCGGTACCCCTGCTCGTA-3'	se (direct)
FUT2-all-523-as	66,7 °C	5'-CCGGCTCCCGTTCACCTG-3'	Non specific (reverse)

Table 2. Sequence of primers for the analysis of the G428A mutation

In our population the nonsense mutation (428 G-A) in the *FUT2* gene is the most frequent polymorphism. We studied the possible association between the 428 G-A in the *FUT2* gene and oral disease progression. The genotyping revealed that 18 (20.5%) of the 89 blood donors were found to be non-secretors (se/\_ ) and 79.5 % of the healthy individuals studied

presented the *Se* gene (*FUT 2*) that governs the secretion of water-soluble ABH antigens into saliva (control group). These secreted antigens can be demonstrated in saliva by agglutination inhibition tests with ABH antisera and molecular biology through analysis of the *FUT 2* gene. In contrast, twenty-eight patients (58%) with oral pre-cancerous and cancerous lesions were non secretors, OR = 2.43; CI 95% (1.03; 5.71) ( $p= 0.0407$ ) (Table 3). We found a higher intensity of oral disease in the non-secretor group, and epithelial dysplasia was found exclusively in this group.

	Benign Lesions (mucocelas, papilloma, etc)	Pre-cancerous + Cancerous
<i>FUT2 -Se</i> (Secretor Status)	26	20
<i>FUT2 -se</i> (Non Secretor Status)	15	28

Table 3. Secretor status in patients with oral lesions

The molecular analysis showed that 48.31% of patients was homozygous for the G428A mutation (the mutation present in the 2 alleles), while the other patients were homozygous for the secretor status (none of them presented the allele G428A), or heterozygous secretor (1 allele presented with the mutation G428A) (Fig. 3).

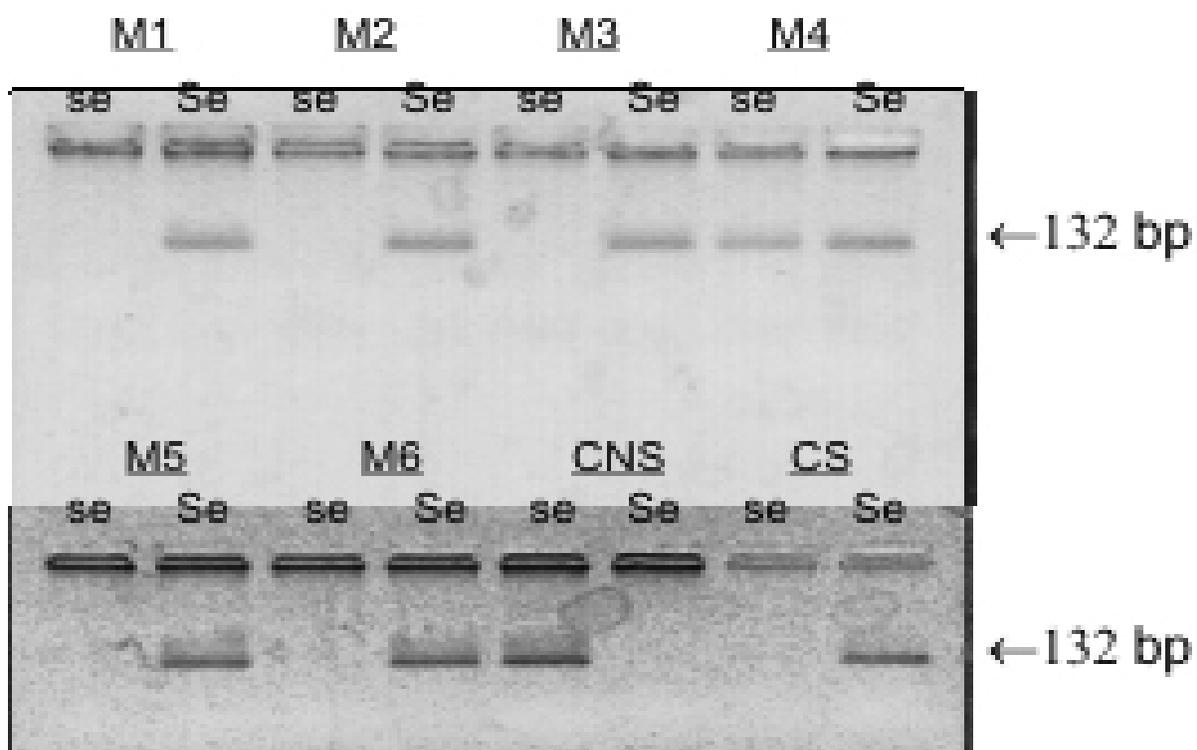


Fig. 3. The agarose gel shows the PCR products of 132 bp for the M1-M6 samples. Each sample was analysed for the wild type allele (*Se*) and for G428A allele (*se*) and was run together with secretor control (CS) and non-secretor control (CNS).

The secretor status is defined by the presence of H type 1 antigen in body secretions such as milk and saliva. H type 1 antigen belongs to both the Lewis and the ABO (H) histo-blood-group systems and it is expressed in erythrocyte membranes and in several epithelial tissues. The secretor enzyme (FUT2), an  $\alpha$ -1,2-fucosyltransferase, is responsible for the fucose transfer in an  $\alpha$ -1,2 linkage to form the terminal H type 1 structure. The cell-surface fucosylated oligosaccharides participate in several biological processes, such as embryogenesis, tissue differentiation, tumour metastasis, inflammation and bacterial adhesion (Dabelsteen, 2004). About 20% of the Caucasian population is non-secretor. Several disease correlations have been linked to non-secretor status. In general, being non-secretor results in several disadvantages regarding metabolism and immune function (Campi et al., 2007, Le Pendu et al., 2001, Daniels, 2007).

Our results have demonstrated that most of the individuals examined in the healthy group were secretor (have the *FUT2* gene) (79.5 %) and there were significant difference between secretors and non-secretors in the experimental group. We have also found a higher intensity of oral disease in the non-secretor group, and the occurrence of epithelial dysplasia was mostly found in the non-secretor group. This study evaluated the association between oral lesions and polymorphisms of the *Se* genes. We found that oral pre-cancerous and cancerous lesions were increased among individuals with non secretor status and nonsense mutation 428G→A (Trp143→stop) (58.33%). We found 20 patients diagnosed histopathologically as malignant lesions despite the secretory status. We also observed that the red cell Lewis antigen reactivity does appear to be associated with the secretor status in the saliva, a conclusion supported by the observation that some individuals with Le(a-b+) red cells show reactivity of ABH antigens in their secretions and they have the *FUT2* gene.

The studies of patients with premalignant and malignant oral lesions, in which non-secretor status predominates, appear to be an associated risk marker for the development of oral cancer. Leukoplakia and erythroplakia are clinical changes in the oral mucosa regarded as potentially malignant lesions (Clausen et al., 1994, Hakomori, 1999). Certain histopathological changes may indicate a malignant potential in a lesion. However, the presence of such changes is not a reliable predictor of malignant transformation, and their absence does not mean that the patient is out of risk of developing a tumour (Gao et al., 2004b).

Although the relationship between epithelial dysplasia in a leukoplakia and malignant transformation of the lesion is debatable, many workers consider that the finding of epithelial dysplasia indicates a higher likelihood to develop malignancy. It is, however, more probable that the antigen changes found in the dysplastic lesions are associated with other factors, such as cell movement and growth rate, rather than malignancy per se (Dabelsteen, et al., 1975).

Our study evaluated the association between oral lesions and polymorphisms of the *Se* genes and secretor status. We found that oral pre-cancerous and cancerous were increased among individuals with non-secretor status and nonsense mutation 428G→A (Trp143→stop). We also demonstrated that the Le (a+b-) antigen expression was present in the population showing greater risk. The studies of patients with pre malignant and malignant oral lesions, in which non-secretor status predominates, show that this status appears to be an associated risk marker for the development for oral cancer.

#### 4. CD44

CD44 is a transmembrane glycoprotein that binds hyaluronan, extracellular matrix proteins and growth factors. Alternative splicing of a single gene generates a family of splice variants (CD44v1-10) in addition to the standard isoform. Cell adhesion molecules are essential for maintaining the stable structure of stratified squamous epithelium. In normal epithelium, keratinocytes are attached to each other and to the underlying basement membrane. Cell adhesion, however, must be dynamic to facilitate the mobility and turnover of cells. In dynamic situations, keratinocytes alter their cell-cell and cell-ECM interactions by virtue of altered expression and function of cell adhesion molecules. The expression of cell adhesion molecules is normally tightly regulated-forming, persisting, or declining in an ordered fashion. This allows for controlled cell proliferation, mobility, differentiation, and survival. Many of these processes are misregulated in malignant tumours, and it has been shown that many of the characteristics of tumour cells are attributable to the aberrant expression or function of cell adhesion molecules. However, multiple CD44 isoforms are expressed by normal stratified squamous epithelia, such as the epidermis and the lining of the oral cavity (Hudson et al., 1996).

The neoplastic transformation of normal epithelial cells to metastatic tumour cells is a complex process involving a number of alterations in the expression of genes implicated in cell proliferation, cell adhesion and cell migration. Tumour progression is the process by which tumour cells acquire malignant properties, such as progressive growth, invasion and metastasis (Nowell, 1986). One of the genes involved in these processes is *CD44* which appears to be one of the most promising candidates as a cancer diagnosis marker (Otavia, et al., 2001). Several studies have provided evidence that the expression of CD44 is specifically altered in many types of tumours. They show aberrant expression and processing of CD44 transcripts and cell surface expression of CD44 appears to change profoundly during tumour metastasis, particularly during the progression of various carcinomas (Assimakopoulos et al., 2002). Numerous studies based on immunohistochemical analyses of paraffin-embedded or frozen tissue sections using different monoclonal antibodies to CD44 isoforms and molecular biological techniques have provided evidence that in many types of tumours there is overexpression of CD44 isoforms.

We investigate by confocal microscopy, the expression of CD44 protein in epithelial cells obtained from saliva samples from patients with oral lesions. We studied 28 patients with various oral lesions (benign, pre-cancerous and cancerous), and a control group (n = 32) who had no alterations. We worked with saliva samples subjected to thermal shock and washed with phosphate buffered saline. They were concentrated by centrifugation. Then  $10^6$  cells were incubated with anti-CD44 antibody suitable dilution for 30 min at room temperature. After washing with phosphate buffered saline, it was incubated with secondary antibody labeled with allophycocyanin (APC). Parallel internal controls were processed for each sample. The different cell suspensions were washed with phosphate buffered saline and observed by confocal microscopy (Nikon C1) using 639 nm red laser. The results obtained showed fluorescence corresponding to the presence of CD44 protein in samples from patients diagnosed with cancer and precancer. A higher intensity was observed in individuals with a pathological diagnosis of squamous cell carcinoma (Fig 4). In contrast, samples from patients with benign lesions showed no fluorescence images as samples of the control group (Fig. 5). These findings indicate that overexpression of CD44 molecule analyzed could be considered as a marker of risk in individuals with oral lesions.



Fig. 4. Image of squamous carcinoma cells obtained by confocal microscopy. The over expression of CD44 protein is noted by the red fluorescence observed on cell membranes and in cytoplasm.



Fig. 5. Image of benign lesions cells obtained by confocal microscopy. No red fluorescence is observed indicating the absence of CD44 protein expression.

Studies on early premalignant lesions and on early stage malignancies of several types of common tumours, such as breast, bladder and colon, have reported increased CD44 isoform expression and aberrant CD44 transcript processing, but also a marked heterogeneity in the pattern of expression within the tumour. These specific alterations in CD44 expression become clear and distinct with tumour progression, with higher expression levels achieved in invasive and metastatic tumour cells. Several mechanisms, based on the properties of CD44 as the major hyaluronan CD44 in squamous cell carcinomas receptor and as a signal transmitter and growth presenting molecule, have been proposed to explain the role of elevated CD44 expression during tumour development and progression (Knudson, 1998).

## 5. Conclusion

Clinical examination and histopathological studies of biopsied material are the classical and the most accepted diagnostic methods used for precancerous and cancerous oral lesions. While conventional oral examination may be useful in the discovery of some oral lesions, it does not identify all potentially premalignant and/or malignant lesions.

It has been shown that leukoplakias from patients who subsequently developed malignancy all demonstrated loss of expression of histo-blood group antigen in the lesions that preceded the carcinomas. This may indicate that the change in expression of A/B antigen is an early event in the malignant development process.

We propose that areas of SRCA-test negative epithelium are closely related to invasive carcinomas and may be their precursor lesions. However, as it is generally accepted that cancer cells must undergo a whole series of changes to become metastatic, it is remarkable the degree of expression of a single carbohydrate structure was significantly correlated with aggressive clinical behaviour of the tumour. It is therefore possible that further prognostic information can be obtained by detecting a group of other related carbohydrate structures at the cancer cell membranes.

Our findings also demonstrate that the *Se* genotypes affect the risk of developing malignant oral disease defined by the secretor status. The study also evaluated the association between oral lesions and polymorphisms of the *Se* genes. We found that oral pre-cancerous and cancerous lesions were increased among individuals with non secretor status and nonsense mutation 428G→A (Trp143→stop).

Thus, we think that CD44 might be a good candidate as a predictor of prognosis in this group of cancers. However, a larger series with clinical follow-up and study of other biological markers of tumor progression is needed to determine whether it is an independent prognostic factor or not.

In summary, our results indicate that at the same time as the morphological changes that occur during the process of oral carcinogenesis, another series of events occurs. Further follow-up studies are required to clarify the role of predictive markers of risk in precursor lesions of oral cancer

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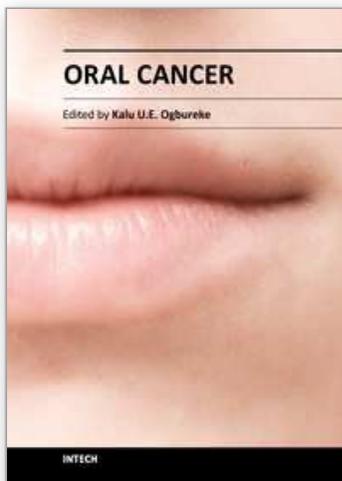
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## **Oral Cancer**

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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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