Oral Diseases (2005) 11, 95–99

http://www.blackwellmunksgaard.com

Oral Oncology

Statherin levels in saliva of patients with precancerous and cancerous lesions of the oral cavity: a preliminary report

AM Contucci[1], R Inzitari[2], S Agostino[1], A Vitali[2], A Fiorita[1], T Cabras[3], E Scarano[1], I Messana[3]

[1]*Department of Otolaryngology, Catholic University, Rome, Italy;* [2]*Department of Biochemistry and Clinical Biochemistry, Catholic University, Rome, Italy;* [3]*Department of Science Applied to Biosystems, Cagliari University, Cagliari, Italy*

OBJECTIVE: The aim of this study was to measure concentration of human salivary statherin in patients with oral cavity pathologies and salivary gland diseases.
SUBJECTS AND METHODS: Levels of statherin were analysed with High Performance Liquid Chromatography (HPLC) in following groups of subjects: group A: 24 patients with neoplastic diseases of salivary glands, group B: 13 patients with inflammatory lesions of salivary glands, group C: 13 patients with precancerous and cancerous lesions of the oral cavity excluding salivary gland tumors, group D: 20 healthy volunteers (control group).
RESULTS: Our preliminary data indicated a sensible reduction of the statherin level in the saliva of patients with precancerous and cancerous lesions of the oral cavity (group C) compared with the healthy subjects (group D). The statherin levels are not significantly reduced either in the inflammatory (group B) or in the salivary glands tumours (group A), compared with the healthy subjects (group D).
CONCLUSION: Statherin could play a protective effect in oral cavity in association with its other functions.
Oral Diseases (2005) **11**, 95–99

Keywords: statherin; saliva; oral diseases

Introduction

Saliva is a clear, slightly acidic mucoserous exocrine secretion, composed of a variety of electrolytes, immunoglobulins, proteins, enzymes and it plays an important role in the maintenance of oral health (Humphrey and Williamson, 2001). In fact, during evolution, several defence mechanisms have developed in the saliva against bacteria, viruses, fungi chemical or mechanical agents (Johansson *et al*, 2000; Nagler *et al*, 2002). They seem to be adaptative, i.e. immunological, primarily based on secretory IgA, enzymatic, based on lysozyme and constitutive, taking profit from the antiseptic properties of other salivary proteins such as histatins, cystatins, proline-rich proteins (PRPs), mucins, statherin, lactoferrin, etc. (Lamkin and Oppenheim, 1993; Kaplan *et al*, 2001; Nagler *et al*, 2002; Raj *et al*, 1992). The soft tissue integrity is maintained by the epidermal growth factor (EGF) (Rao *et al*, 1997). Currently, the defence system based on the antioxidant properties of various molecules and enzymes such as uric acid and peroxidase enzyme, is receiving growing interest (Mooe *et al*, 1994; Kondakova *et al*, 1999).

The four major salivary protein families are PRPs, statherin, cystatins and histatins that differ significantly from other host defence salivary proteins, such as IgA, lactoferrin, lysozyme and salivary peroxidase because the former group has specific functions in the oral environment while the second exhibits broad tissue distribution as well as broad activities (Lamkin and Oppenheim, 1993).

PRPs account for approximately 60% of the total secretory proteins and are subdivided into acidic, basic and glycosylated PRPs (Lamkin and Oppenheim, 1993). These proteins bind to hydroxyapatite and calcium ions, they inhibit crystal growth of calcium phosphate in supersaturated solutions and interact with several oral bacteria on adsorption to hydroxyapatite. Statherin, cystatins, and histatins also exhibit affinities for mineral surfaces, inhibit calcium phosphate precipitation and play a role in maintaining the integrity of teeth.

In particular, statherin is a multifunctional peptide that possesses a high affinity for calcium phosphate minerals, maintains the appropriate mineral solution dynamics of enamel, promotes selective initial bacterial colonization of enamel, and functions as a boundary lubricant on the enamel surface. In addition, statherin may function in the transport of calcium and phosphate during secretion of salivary glands. It was demonstrated that statherin concentration, differently from other

Correspondence: Dr Alessia Maria Contucci, Istituto di Clinica Otorinolaringoiatrica, Università Cattolica del Sacro Cuore, Largo A. Gemelli, 8, 00168 Rome, Italy. Tel.: 06/30154439, Fax: 06/3051194, E-mail: lucr2000@yahoo.com
Received 14 January 2004; revised 1 April 2004; accepted 15 June 2004

salivary peptides, is not subjected to circadian rhythms (Castagnola *et al*, 2002). However, the role and the function of this peptide are still not well-known.

The aim of this study was to measure the concentration of human salivary statherin by High-Performance Liquid Chromatography (HPLC), comparing a control group of healthy subjects and a group of patients with salivary gland diseases and oral cavity pathologies. Significant concentration differences between the groups could contribute to clarify the role of this peptide in maintaining the health of oral cavity.

Patients and methods

Reagents and instruments

All reagents were analytical grade purchased either from Merck (Darmastad, Germany), Carlo Erba (Milan, Italy) or Sigma-Aldrich (St Louis, MO, USA). Chromatographic eluents were from Carlo Erba (Milan, Italy). The HPLC used to analyze human saliva, was a Beckman (Palo Alto, CA, USA) Gold 125S solvent module equipped with a diode array 168 detector and Gold Noveau software. The chromatographic column Hypersil BDS-C18 (Hewlett-Pacard, Palo Alto, CA, USA) with 3 μm particle diameter (column dimensions 100×4 mm) protected by a guard column of ODS Hypersil resin (5 μm; 20×2.1 mm).

Patients and specimens collection

Between June 2001 and December 2002, 50 saliva specimens of 20 male and 30 female patients were collected and analyzed. The age range was 10–80 years (mean age: 51 years).

The patients were divided in the following groups (Table 1): group A: 24 patients with neoplastic (benign and malignant) diseases of the salivary gland, 15 smokers and nine non-smokers, group B: 13 patients with inflammatory lesions of the salivary glands, three smokers and 10 non-smokers; group C: 13 patients with precancerous and cancerous lesions of the oral cavity lacked salivary gland tumors, six smokers and seven non-smokers.

A control group of 20 subjects (group D) was selected. They were in healthy clinical conditions, non-smokers, non-drinkers, without either pathologies of the salivary glands or of the oral mucosa. They were 11 male and nine female patients. The age range was 27–68 years (mean age: 45 years).

The informed donors were advised regarding a common standardized specimen collection procedure. Donors avoided drug assumption for a week before specimen collection, coffee and tea were omitted from the diet at least 24 h before and then they were invited to maintain a normal life-style, with three meals a day and to brush their teeth 5 min before saliva collection. Collection time was established between 2 and 3 pm. Saliva specimens were collected from the anterior floor of the mouth with a small plastic aspirator. Saliva was immediately transferred in a plastic tube and 80 mmol l^{-1} phosphate buffer pH 2.2 was added to the sample (1:1, v/v). The sample was centrifuged at 8000 g, the precipitate was discharged and the upper solution was stored at −80°C until analyzed. Samples were thawed at room temperature and analyzed by HPLC method.

Table 1 Sample breakdown by group

Group A (24 patients) (15 smokers, nine non-smokers)	
Pleomorphic adenoma	7
Warthin tumor	12
Adenoid cystic Ca	1
Lymphoma	1
Lipoma	1
Mature Teratoma	1
Carcinosarcoma	1
Group B (13 patients) (three smokers, 10 non-smokers)	
Calcolosis	6
Chronic sialoadenitis	7
Group C (13 patients) (six smokers, seven non-smokers)	
Precancerous lesions:	
Leukoplakia	4
Malignant lesions:	
Squamous cell Ca (Trigone 2; oral cavity 7)	9
Group D (20 patients, 20 non-smokers)	
Healthy control group	

HPLC analysis

The following solutions were utilized: (eluent A) H_2O and trifluoroacetic acid (TFA) 0.2%, pH 2.2; (eluent B) acetonitrile/eluent A (80:20, v/v). Gradient development was as follows: 0% B for 1 min, linear gradient from 0 to 75% B in 45 min, linear gradient from 75 to 100% B in 1 min, 100% B for 8 min, linear gradient from 100 to 0% B in 1 min The flow-rate during the whole gradient development was 1.0 ml min^{-1} the DAD window was established between 190 and 300 nm. The volume injected corresponded to 50 μl. Quantification of statherin was carried out using the peak area at 276 nm in comparison with standards of tyrosine and on the knowledge of the tyrosin content of salivary peptides, as previously described (Castagnola *et al*, 2002).

In order to check possible loss of statherin because of precipitation, two salivary samples from normal subjects were acidified immediately after collection with 0.2% TFA and the soluble fraction of each sample was subdivided in two fractions. One fraction of each sample was submitted to freezing at −80°C and analyzed after thawing at room temperature after 1 day, the other immediately analyzed by HPLC and statherin concentrations were compared.

Statistical analysis

Statistical analysis was performed using STATA 6.0 (Stata Corporation, College Station, TX, USA) by variance analysis and *t*-test to compare statherin levels in the groups of the study. The threshold for significance was 0.01.

Results

In Figure 1 the HPLC separation of salivary peptides/proteins is shown. The HPLC conditions applied

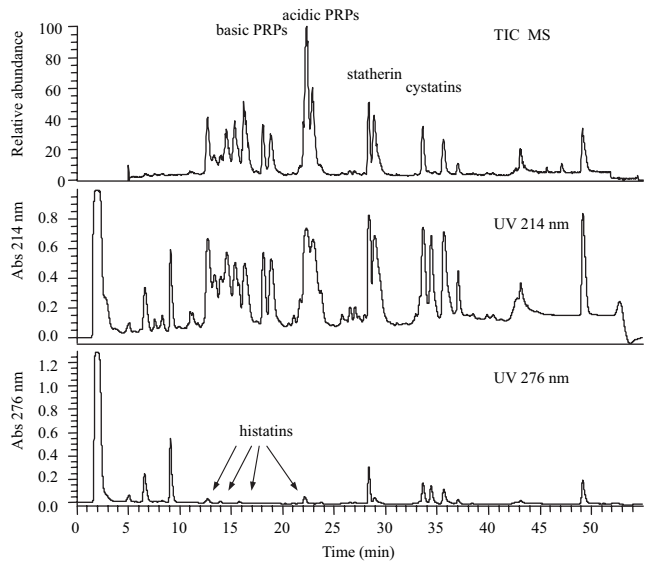

Figure 1 This figure shows the HPLC separation of salivary peptides/proteins with the identification of the principals peaks

(column and gradient) permitted to elute salivary statherin in a chromatographic zone free from relevant contaminants, as judged by ion-trap mass spectrometry. On this basis, quantification of statherin by its tyrosine content was possible (Castagnola *et al*, 2002). Possible loss of statherin by precipitation after freezing was checked by determining statherin concentration in two salivary samples before and after freezing alt −80°C. No significant differences were found between the two determinations for both samples.

HPLC analysis statherin levels ranged between 0 and 15.06 μM (mean value: 3.30 $\pm$ 3.70 μM) and group A patients, between 0 and 26.3 μM (mean value: 7.64 $\pm$ 9.366 μM) in group B patients and between 0 and 6.45 μM (mean value: 2.28 $\pm$ 2.86 μM) in group C.

In controls (group D) statherin levels ranged between 4.3 and 5.59 μM (mean value: 4.93 $\pm$ 0.61 μM).

Statistical analysis showed a significant reduction of statherin level in group C when compared with group D ($P \leq 0.01$) while there were no significant differences between groups A and B and the control group ($P > 0.05$) and between group A and group B ($P > 0.05$).

In group C, only two patients with cancer of the retromolar zone showed statherin levels higher than controls (6.45 and 8.61 μM).

In each group no significant difference ($P = 0.19$) was found in statherin levels between smokers and non-smokers.

Discussion

Whole saliva is a mixture of fluids produced by major and minor salivary glands and gingival crevicular fluid. It contains electrolytes, immunoglobulins, proteins, enzymes, mucins and nitrogenous products (Edgar, 1992; Humphrey and Williamson, 2001). About 65% of unstimulated saliva originates from the submandibular gland, 25% from the parotid, 4% from the sublingual and 8% from other salivary glands (Edgar, 1990).

Moreover, saliva plays an important role in maintaining oral health and creating an appropriate ecologic balance (Amerongen and Veerman, 2002). Its most important functions are: (1) lubrication; (2) buffering action and clearance; (3) maintaining tooth integrity; (4) antibacterial and antiviral activity and (5) taste and digestion (Humphrey and Williamson, 2001). Statherin specific IgG block adhesion of *Candida albicans* to saliva-coated hydroxyapatite and buccal epitelial cells (Johansson *et al*, 2000).

At present, the relationship among the various salivary components and the protective function against cancerogenic agents in the oral cavity is unclear. Various experimental models of oral carcinogenesis demonstrated the protective effect of saliva (Lekholm and Wallenius, 1976; Hawkins *et al*, 1994; Dayan *et al*, 1997; Kaplan *et al*, 2002). Several (Lekholm *et al*, 1975; Lekholm and Wallenius, 1976; Kaplan *et al*, 2002, Dayan *et al*, 1997) investigated the protective role of saliva in oral carcinogenesis in a desalivated rat model. They induced oral cancer with a topical application of carcinogen 4-nitroquinoline-1-oxide (4NQO) or dimetilbenzantracene (DMB) or administered with drinking water.

Maier *et al* (1986) studied patients with carcinomas of the oral cavity and oropharynx. If compared with healthy controls patients with tumors showed a significantly limited function of the large salivary glands reflecting a reduction of the protective mechanisms on oral cavity and enabling an increased penetration of environmental carcinogens through the mucosal surface.

Mizukawa *et al* (1998, 2000, 2002) analyzed saliva of patients with oral squamous cell carcinoma and with various oral diseases (oral lichen planus, leukoplakia, oral inflammation, oral candidosis and glossitis) and found that concentration of human α-defensin 1 (HNP-1) and β-defensin (HBD-2) are increased in patients with oral diseases (Mizukawa *et al*, 1999, 2002, Sawaki *et al*, 2002).

These studies suggested that some salivary components have a protective effect, which can both delay and decrease the level of proliferation induced by any carcinogen. These components and the mechanism by which this protective effect is obtained have not yet been discovered.

The functional role exerted by salivary proteins, in particular by statherin, in regulating calcium concentration in the oral cavity, might be at the basis of the observed protective effects of saliva. In literature, many studies demonstrated that intracellular Ca^{2+} plays an important role in the regulation of cancer cell proliferation mediated by the K^+ channels (Pardo *et al*, 1999; Ruff *et al*, 2001; Ashutosh *et al*, 2003, Jager *et al* 2003, Ouadid-Ahidouch *et al*, 2004).

We do not already know the relations existing between the intracellular and extracellular (salivary) Ca^{2+} and staherin and if this protein can influence the ion concentration. Further studies are necessary to highlight these relations.

Our preliminary data indicated a significant reduction of staherin levels in the saliva of patients with precancerous and cancerous lesions of the oral cavity (group C) compared with healthy subjects (group D), suggesting that this peptide is connected with this type of pathology. The finding that statherin levels are not significantly reduced either in inflammatory diseases (group B) or in salivary glands tumors (group A), compared with healthy subjects (group D) indicates that statherin level reduction could be in relation only with neoplastic diseases of the oral cavity. In fact statherin levels of the whole saliva do not show a significant change in several other pathologies of the oral cavity, like litiasis, chronic sialoadenitis or tumors of the salivary glands where the anatomy and the secretory function of the gland is often modified.

During maximal stimulation, achieved during mastication, the contribution of parotid gland saliva rises to 50% of the total salivary volume. We decided to collect resting saliva at the same approximate period of collection, because the measurements of salivary proteins provides less variations. However, in a previous study we demonstrated that salivary statherin concentration does not show circadian variations and it is practically constant, since any increase of flow rate is paralleled by an increase of statherin secretion (Castagnola *et al*, 2002).

The significant reduction of statherin in patients with precancerous and cancerous lesions of the oral cavity induces to argue whether statherin reduction is the cause or the effect of cancerogenesis of the oral cavity.

Today, it is unknown if the reduction of the peptide predisposes to cancerogenesis or if the tumor can, in some way, reduce statherin concentration (Abiko *et al*, 1999). This protein could exert a protective effect in oral cavity in association with its other functions: maintaining the integrity of teeth, promoting selective initial bacterial colonization of enamel and influencing the transport of calcium and phosphate ions during secretion in salivary glands (Raj *et al*, 2002).

Acknowledgements

This work was supported by Regione Autonoma Sardegna (Progetti Educazione Sanitaria, 2001–2002) funds.

References

Abiko Y, Mitamura J, Nishimura M *et al* (1999). Pattern of expression of beta-defensins in oral squamous cell carcinoma. *Cancer Lett* **143:**37–43.

Amerongen NAV, Veerman ECI (2002). Saliva-the defender of oral cavity. *Oral Dis* **8:**12–22.

Ashutosh SP, Michael JC, Murali G *et al* (2003). Effects of intermediate-conductance Ca^{2+}-activated K^+ channel modulators on human prostate cancer cell proliferation. *Eur J Pharmacol* **471:**157–164.

Castagnola M, Cabras T, Denotti G *et al* (2002). Circadian rhythms of Histatin 1, Histatin 3, Histatin 5, Statherin and Uric acid in whole human saliva secretion. *Biol Rhythm Res* **33:**213–222.

Dayan D, Hirshberg A, Kaplan I *et al* (1997). Experimental tongue cancer in desalivated rats. *Oral Oncol* **33:**105–109.

Edgar WM (1990). Saliva and dental health. Clinical implications of saliva: report and consensus meeting. *Br Dent J* **169:**96–98.

Edgar WM (1992). Saliva: its secretion, composition and functions. *Br Dent J* **172:**305–312.

Hawkins BL, Heniford BW, Ackermann DM *et al* (1994). 4NQO: a mouse model of oral cavity squamous cell carcinoma. *Head Neck* **16:**424–432.

Humphrey SP and Williamson RT (2001). A review of saliva: normal composition, flow and function. *J Prost Dent* **85:**162–168.

Jager H, Dreker T, Buck A *et al* (2004). Blockage of intermediate-conductance Ca^{2+}-activated K^+ channels inhibit human pancreatic cancer cell growth in vitro. *Mol Pharmacol* **65:**630–8.

Johansson I, Bratt P, Hay DI *et al* (2000). Adhesion of Candida Albicans, built not Candida krusei, to salivary statherin and mimicking host molecules. *Oral Microbiol Immunol* **15:**112–118.

Kaplan I, Engelberg J, Dayan D (2001). The effect of desalivation on 4-nitroquinoline-1-oxide-induced tongue carcinogenesis: a morphometric study of nucleolar organizer regions. *J Oral Pathol Med* **30:**48–52.

Kaplan I, Hochstadt T, Dayan D (2002). PCNA in palate and tongue mucosal dysplastic lesions induced by topically applied 4NQO in desalivated rats. *Med Oral* **7:**336–343.

Kondakova I, Lissi EA, Pizarro M (1999). Total reactive antioxidant potential in human saliva of smokers and nonsmokers. *Biochem Mol Biol Int* **47:**911–920.

Lamkin MS and Oppenheim FG (1993). Structural features of salivary function. *Critical Rev Oral Biol Med* **4:**251–259.

Lekholm U, Wallenius K (1976). Experimental oral cancer in rats with xerostomia. *Odontol Rev* **27:**11–18.

Lekholm V, Wallenius K, Heyden G (1975). Alterations in the lipidic content of rat palatal epitelium during carcinogenesis. *Histochemistry* **44:**147–154.

Maier H, Born IA, Adler D (1986). Limited function of the large salivary glands of the head. A new aspect for the pathogenesis of cancers of the oral cavity, oropharynx and hypopharynx. *Laryngol Rhinol Otol* **65:**195–200.

Mizukawa N, Sugiyama K, Fukunaga J *et al* (1998). Defensin-1, a peptide detected in the saliva of oral squamous cell carcinoma patients. *Anticancer Res* **18:**4645–4650.

Mizukawa N, Sugiyama K, Ueno T *et al* (1999). Defensin-1, an antimicrobical peptide present in saliva of patients with oral diseases. *Oral Dis* **5:**139–142.

Mizukawa N, Sugiyama K, Kamio M *et al* (2000). Immunohistochemical staining of human alpha-defensin-1 (HNP-1), in the submandibular glands of patients with oral carcinomas. *Anticancer Res* **20:**1125–1127.

Mizukawa N, Sugiyama K, Ueno T *et al* (2002). Levels of human defensin-1, an antimicrobial peptide, in saliva of patients with oral inflammation. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **87:**539–543.

Mooe S, Calder KAC, Miller NJ *et al* (1994). Antioxidant activity of saliva and periodontal disease. *Free Radic Res* **21:**417–425.

Nagler RM, Klein I, Karzhevesky N *et al* (2002). Caharacterization of the differentiated antioxidant profile of human saliva. *Free Radic Biol Med* **32:**268–277.

Ouadid-Ahidouch H, Roudbaraki M, Delcourt P *et al* (2004). Functional and molecular identification of intermediate conductance Ca^{2+}-activated K^+ channels in breast cancer

cells: association with cell cycle progression. *Am J Physiol Cell Physiol* **287:**125–34.

Pardo LA, del Camino D, Sanchez A *et al* (1999) Oncogenic potential of EAG K^+ channels. *EMBO (Eur Mol Biol Organ) J* **18:**5540–5547

Raj PA, Jhonsson M, Levine MJ *et al* (1992). Dependence on sequence, charge, hydrogen bonding potency, and helical conformation for adsorption to hydroxyapatiteand inhibition of mineralization. *J Biol Chem* **267:**5968–5976.

Rao RK, Thomas DW, Pepperl S *et al* (1997). Salivary epidermal growth factor plays a role in the protection of ileal mucosal integrity. *Dig Dis Sci* **42:**2175–2181.

Ruff K, Giehl K, Grissmer S (2001). The role of Ca^{2+}-activated K^+ channels in the proliferation of pancreatic carcinoma cells. *Biophys J* **80:**101a.

Sawaki K, Mizukawa N, Yamaai T *et al* (2002). High concentration of beta-defensin-2 in oral squamous cell carcinoma. *Anticancer Res* **22:**2103–2107.