Available online at www.sciencedirect.com

SciVerse ScienceDirect

British Journal of Oral and Maxillofacial Surgery 51 (2013) 394–398

BRITISH Journal of Oral and Maxillofacial Surgery

www.bjoms.com

Saliva collection methods for DNA biomarker analysis in oral cancer patients

April M. Matthews [a], Harpal Kaur [b], Michael Dodd [b], Jacob D'Souza [c], Triantafillos Liloglou [d], Richard J. Shaw [c,d], Janet M. Risk [d,*]

[a] *School of Dentistry, University of Liverpool and Regional Maxillofacial, Unit, Aintree University Hospitals NHS Foundation Trust, Liverpool, UK*
[b] *School of Dentistry, University of Liverpool, UK*
[c] *Regional Maxillofacial Unit, Aintree University Hospitals NHS Foundation Trust, Liverpool, UK*
[d] *Department of Molecular & Clinical Cancer Medicine & School of Dentistry, University of Liverpool, UK*

Accepted 24 September 2012
Available online 12 October 2012

Abstract

Patients with head and neck cancers are predisposed to local recurrence and second primaries because of the phenomenon of field cancerisation, and clinical detection of recurrence remains challenging. DNA biomarkers in saliva may prove to be an adjunct to current diagnostic methods, but irradiation of the primary site often leads to xerostomia. We assessed 3 methods of collecting saliva for their ability to generate DNA of sufficient quantity and quality to use in biomarker assays. Paired saliva samples were collected from 2 groups of patients with oral squamous cell carcinoma (SCC). In the first group saliva was collected in Oragene® vials and as saline mouthwash from non-irradiated patients ($n = 21$) (4 had had radiotherapy before collection); in the second group it was collected using Oragene® sponge kits and as mouthwash from irradiated patients ($n = 24$). Quantitative polymerase chain reaction (qPCR) showed that Oragene® vials contained DNA in significantly greater amounts (median 122 μg, range 4–379) than mouthwash (median 17 μg, range 2–194) ($p = 0.0001$) in the non-irradiated patients, while Oragene® sponge kits (median 4 μg, range 0.1–61) and mouthwash (median 5.5 μg, range 0.1–75) generated comparable concentrations of DNA from the irradiated group. All 90 samples contained DNA of sufficient quantity and quality for p16 promoter quantitative methylation-specific PCR (qMSP). While Oragene® vials contained the most DNA, all 3 methods yielded enough to detect DNA biomarkers using qMSP. The method of collection should depend on the compliance of the patient and oral competency.

Keywords: Saliva; Collection method; Mouthwash; Biomarker; Oral cancer; DNA; PCR

Introduction

Oral squamous cell carcinoma (SCC) is a molecularly heterogeneous disease[1] that often develops in areas of field change where there is a propensity for recurrence and second primary tumours.[2] Molecular biomarkers associated with oral SCC have been detected in saliva[3–5] despite evidence that more than 70% of the DNA contained in samples of saliva from normal people is from bacteria,[6] and that the human genomic DNA component is derived as much from immune as from epithelial cells.[7] Genomic DNA is a stable macromolecule which has been extracted by a variety of methods in normal people,[8–10] and has been successfully obtained from saliva returned by postal services and stored at room temperature without preservatives.[11,12] These robust features fit it for translation into the clinical setting as a source of biomarkers.

It is accepted that early diagnosis of oral SCC can significantly improve clinical outcome.[13] The main clinical value of saliva biomarkers in this disease would be in early detection and surveillance of disease when multiple,

* Corresponding author at: Department of Molecular & Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Daulby Street, L69 3GN, UK. Tel.: +44 0151 706 5265; fax: +44 0151 706 5809.
E-mail addresses: j.m.risk@liverpool.ac.uk, j.m.risk@liv.ac.uk (J.M. Risk).

0266-4356/$ – see front matter © 2012 The British Association of Oral and Maxillofacial Surgeons. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bjoms.2012.09.017

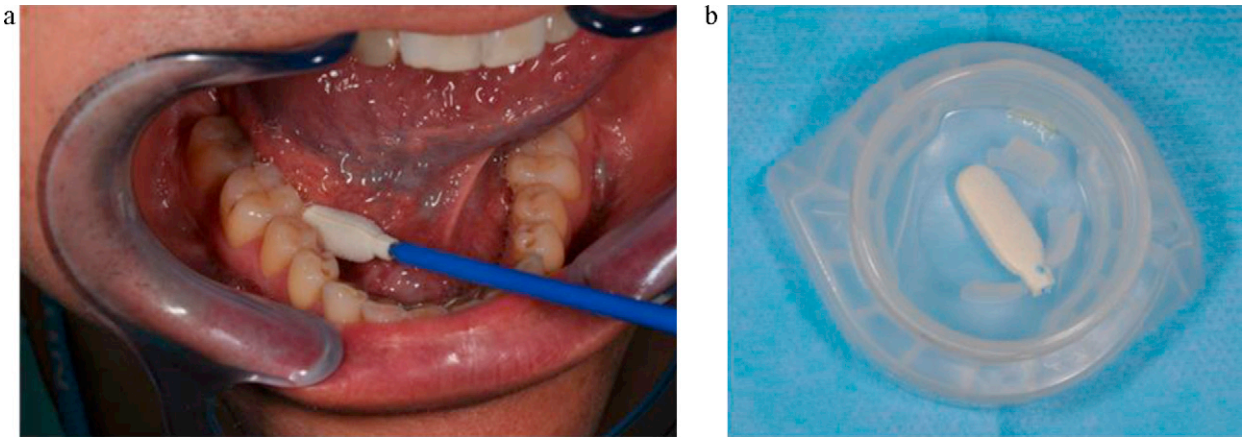

Fig. 1. (a) Collection of saliva using Oragene® sponge kit; (b) sponge tip loaded with saliva in the Oragene® collection pot.

chronological samples are required, and repeated biopsy examination is impractical. Collecting saliva is not invasive and is known to be more acceptable to patients than venipuncture.[14] Saliva bathes the whole oral cavity and is therefore more likely to be representative of the entire exposed field than invasive biopsy examination of local tissue, but concerns about sensitivity in relation to contamination by bacteria and immune cells remain unanswered.

Postoperative radiotherapy in the treatment of oral SCC identifies patients at risk of recurrence, and who need close follow-up,[15] but the clinical detection of early recurrence or second primaries in the surgically altered, irradiated field can be challenging. Xerostomia, a known sequela of radiotherapy, could make the collection of saliva for adjunctive molecular methods of detection difficult.

We aimed to establish the quality and quantity of DNA that could be extracted from the saliva of patients with oral cancer, including a group who had had radiotherapy, using 3 different methods of collection. One new technique uses a sponge that was developed for patients who find conventional methods difficult.

Methods

Two separate groups of 21 and 24 patients (45 in total) were recruited from consecutive patients attending Aintree University Hospital Maxillofacial Unit for treatment of oral SCC. The study was given ethical approval (EC 47.01) and all patients agreed to participate.

The first group comprised 10 preoperative and 11 postoperative patients, 4 of whom had had radiotherapy before the saliva was collected. Two samples were collected from each patient as follows: 0.9% normal saline 25 ml was swilled in the mouth for 30 s and deposited into a tube.[4] Ten minutes later, whole saliva was deposited directly into an Oragene® vial (DNA Genotek Inc, Ontario, Canada) until the 2 ml fill line was reached. Samples were immediately stored at 4 °C for a maximum of 3 h before being placed in a centrifuge at $1200 \times g$ for 5 min and the cell pellet stored at −80 °C. Oragene® vials do not require processing before storage.

The second group comprised 24 patients who had had operation and radiotherapy for oral SCC. Paired saliva samples were collected from each patient as follows: 5 individual sponges (DNA Genotek Inc, Ontario, Canada) were placed into pools of whole saliva in the patient's mouth (Fig. 1a), then removed and placed in a standard Oragene® vial (Fig. 1b). Mouthwash was collected and the samples stored as before.

DNA was extracted from the mouthwash in the cell pellets according to a spin column protocol (DNeasy Blood & Tissue Kit: Qiagen, Crawley, UK). Phosphate buffered saline was added to each pellet to make a final volume of 500 μl, then proteinase K (20 mg/ml) 25 μl was added to an aliquot (180 μl) of this, and separation continued using spin columns with elution in AE buffer (10 mM Tris–Cl, 0.5 mM EDTA; pH 9.0) 200 μl.

DNA was extracted from the Oragene® vials using the manufacturer's protocol for a sample of 0.5 ml. Vials were incubated at 50 °C for 1 h to lyse the cells and digest nuclear proteins, then Oragene® purifier 20 μl was added to 0.5 ml of sample before precipitation in ethanol and final resuspension in Tris–EDTA buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) 200 μl.

DNA was extracted from the Oragene® sponges using the manufacturer's protocol. The vials were incubated in a 50 °C water bath for 1 h as above, and the liquid was recovered from the sponges by centrifugation in a 5 ml plastic syringe placed in a 15 ml centrifuge tube. The manufacturer's Oragene®/saliva protocol for 4 ml samples was followed for the entire volume. DNA was resuspended in Tris–EDTA buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0) 200 μl.

To quantify accurately the total concentration of human DNA, a quantitative polymerase chain reaction (qPCR) using the human ribonuclease P (RNAse P) gene[16] was done using the Applied Biosystems 7500 Fast Real-Time PCR system on the standard curve program according to the manufacturer's guidelines (Applied Biosystems, Foster City, CA, USA). Controls of known DNA concentration were diluted from