European Journal of Cancer (2015) xxx, xxx–xxx

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Validation of methylation markers for diagnosis of oral cavity cancer

L.M.R.B. Arantes [a,b], A.C. de Carvalho [b], M.E. Melendez [b], C.C. Centrone [c], J.F. Góis-Filho [d], T.N. Toporcov [e], D.N. Caly [f], E.H. Tajara [a], E.M. Goloni-Bertollo [a], A.L. Carvalho [b,*], GENCAPO [g]

[a] *Department of Molecular Biology, São José do Rio Preto Medical School (FAMERP), São José do Rio Preto, SP, Brazil*
[b] *Molecular Oncology Research Center, Barretos Cancer Hospital – Pio XII, Barretos, SP, Brazil*
[c] *Virology Laboratory, Institute of Tropical Medicine of São Paulo, University of São Paulo, São Paulo, SP, Brazil*
[d] *Head and Neck Surgery Service, Arnaldo Vieira de Carvalho Cancer Institute (ICAVC), São Paulo, SP, Brazil*
[e] *Faculty of Public Health, University of São Paulo (FSP), São Paulo, SP, Brazil*
[f] *Laboratory of Clinical Pathology, Heliópolis Hospital, São Paulo, SP, Brazil*
[g] *GENCAPO – Head and Neck Genome Project*

Received 10 December 2014; received in revised form 14 January 2015; accepted 23 January 2015

KEYWORDS
OSCC
Methylation markers
CCNA1
DAPK
DCC
TIMP3

Abstract ***Purpose:*** Activation of proto-oncogenes and inactivation of tumour suppressor genes are the major genetic alterations involved in carcinogenesis. The increase in methylation at the promoter region of a tumour suppressor gene can lead to gene inactivation, selecting cells with proliferative advantage. Thus, promoter hypermethylation is considered a marker in a variety of malignant tumours, including oral cavity.

Experimental design: The methylation pattern of eight genes was evaluated in 40 oral cavity squamous cell carcinomas (OSCCs) and 40 saliva samples from healthy individuals by *Q-MSP*. Different combinations of genes were also assessed in order to identify gene panels that could better distinguish between OSCC and saliva samples.

Results: *CCNA1*, *DAPK*, *DCC* and *TIMP3* methylation were highly specific for being found in the OSCC samples. Moreover, the combination of these genes improved detection when compared with single markers, reaching values of 92.5% for sensitivity and specificity (when using the panel *CCNA1*, *DCC*, *TIMP3*). Moreover, *DAPK*, *DCC* and *TIMP3* were hypermethylated in nearly 90% of clinically T1 and T2 cases.

Conclusion: The pursuing of this panel of hypermethylated genes is an important tool for the detection of individuals with OSCC. Moreover, the identification of these markers in early stages of OSCC shows the feasibility of using the panel on saliva as possible biomarkers

* *Corresponding author at:* Molecular Oncology Research Center, Barretos Cancer Hospital – Pio XII, Rua Antenor Duarte Villela, 1331, Bairro Dr. Paulo, Zip code: 14784-400, Brazil. Tel.: +55 17 3221 6600.
E-mail address: alopescarvalho@uol.com.br (A.L. Carvalho).

http://dx.doi.org/10.1016/j.ejca.2015.01.060

for early diagnosis. The lack of association between the methylation status of these genes and clinical characteristics shows that they are able to distinguish OSCC cases irrespective of social and clinical factors (gender, age, human papillomavirus (HPV) status, clinical stage, vascular embolisation and perineural invasion).

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignant tumour type arising from the epithelial mucosal membranes of the upper-aerodigestive tract (oropharynx, hypopharynx and larynx) and the oral cavity, with a worldwide incidence of 685,000 patients annually [1–4]. Oral squamous cell carcinoma (OSCC) being the most common malignancy of the oral cavity poses a significant public health problem due its impact on the speech, mastication, taste, swallowing and aesthetics, with an annual incidence over than 300,000 cases [4].

Despite major progress, the overall survival of patients with oral cancer has slightly improved during the past 20 years, with 5-year survival rates around 60%. The poor survival rate has been ascribed to a high frequency of locoregional recurrences, and the occurrence of new tumours and deaths due to comorbidity; mostly to the fact that the majority of patients present advanced stages of OSCC at the time of diagnosis [5]. Only about one third of the patients present with early-stage disease (Union Internationale Contra le Cancer, UICC, stage I–II), whereas two thirds show already advanced disease (UICC stage III–IV) with poor outcome. The most important prognostic indicator for relapse of OSCC is the presence of metastatic spread to lymph nodes in the neck. In this case, the incidence of distant metastasis can be as high as 50% [6]. The presence of metastasis either regional or distant worsens the prognosis and reduces the survival rate in these patients. This makes it imperative to diagnose the disease at an early stage to facilitate appropriate therapeutic management to reduce the morbidity and mortality associated with this disease. Tobacco and alcohol consumption have been described as the most important risk factors associated with this carcinoma together with high-risk types of human papillomavirus (HPV) [7,8].

The use of molecular markers for tumour detection in body fluids has been explored with the intent to improve screening accuracy and cost-effectiveness. Body fluids can potentially carry whole cells as well as protein, DNA, and RNA that allow for detection of cellular alterations related to cancer. Examples of relevant body fluids used for detection include analysis of sputum for lung cancer diagnosis [9,10], urine for urologic tumours [11,12], saliva for HNSCC [13–16], breast fluid [17], as well as serum or plasma for almost all types of cancer [18–20]. The detection of DNA methylation in body fluids such as saliva is a non-invasive technique that can easily obtain epithelial cells shed from the mucosal lining of the mouth and throat.

Rosas et al. [21] demonstrated for the first time the possibility to detect hypermethylation in saliva. Likewise, Righini et al. [22] evaluated paired tumour and saliva samples collected at diagnosis and identified a panel of six genes with frequencies of hypermethylation of 82% and 78%, respectively. Carvalho et al. [16] were able to confirm an elevated rate of promoter hypermethylation detected in HNSCC patient salivary rinses by using a panel of gene promoters previously described as methylated in HNSCC but not in control subjects, by the same group [23]. Rettori et al. [14] in the analysis of salivary rinse samples taken at diagnosis of HNSCC patients, five genes (*CCNA1*, *DAPK*, *DCC*, *MGMT* and *TIMP3*) showed high specificity and sensitivity [24]. Thus, the detection of DNA methylation in body fluids opens the potential to develop biomarkers that can be useful for clinical use.

Epigenetic gene silencing is a molecular mechanism of gene silencing through the methylation of its promoter region, and plays a vital role in the development of several types of cancer, including HNSCC [25–27]. In HNSCC, aberrant promoter methylation of CpG islands may affect genes involved in DNA repair [22,28–32], cell-cycle control [22,28–31,33,34], apoptosis [15,22,23,28,30,34,35], cell differentiation [23], cell proliferation [23] and cell adhesion [22,29,36,37].

The search for biomarkers to evaluate and measure the status of normal and pathological processes in cell biology as well as treatment responses is of paramount importance. The pursuing of these biomarkers is important for the identification of individuals in the early stages of cancer, and to stratify patients according to tumour prognosis and response to therapy profiles. Assuming that cancer results from genetic and epigenetic alterations, analysis based on gene-methylation profiles in combination with the pathological diagnosis would be useful in predicting the behaviour of these tumours.

This study is a validation of previous studies published in this field. Carvalho et al. (2008) showed an elevated frequency of promoter hypermethylation in HNSCC in a panel of gene promoters previously described as methylated in HNSCC as well as other solid tumours [23]. Moreover, Rettori et al. (2013) found that

Please cite this article in press as: Arantes L.M.R.B. et al., Validation of methylation markers for diagnosis of oral cavity cancer, Eur J Cancer (2015), http://dx.doi.org/10.1016/j.ejca.2015.01.060