European Journal of Cancer (2014) **50**, 3262–3270

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Original Research

Rapid adherence to collagen IV enriches for tumour initiating cells in oral cancer

Xiao Liang [a], Tarig Al-Hadi Osman [a], Dipak Sapkota [a], Evelyn Neppelberg [b,c], Stein Lybak [c], Per Gunnar Liavaag [c], Anne Christine Johannessen [a,d], Hege Karine Jacobsen [e], Per Øyvind Enger [e,f], Daniela Elena Costea [a,d,e], Jian Wang [e,*]

[a] *The Gade Laboratory of Pathology, Department of Clinical Medicine, University of Bergen, Norway*
[b] *Department of Oral Surgery, Institute of Clinical Dentistry, University of Bergen, Norway*
[c] *Department of Ear-Nose-and-Throat, Haukeland University Hospital, Bergen, Norway*
[d] *Department of Pathology, Haukeland University Hospital, Bergen, Norway*
[e] *Department of Biomedicine, University of Bergen, Norway*
[f] *Department of Neurosurgery, Haukeland University Hospital, Bergen, Norway*

Received 22 May 2014; received in revised form 12 August 2014; accepted 15 September 2014
Available online 10 October 2014

KEYWORDS
Oral cancer
Stem cells
Collagen IV
Rapid adherent cells

Abstract ***Background:*** Although several approaches for identification and isolation of carcinoma cells with tumour initiating properties have been established, enrichment of a population of pure and viable tumour-initiating cells (TICs) is still problematic. This study investigated possibilities to isolate a population of cancer cells with tumour initiating properties based on their adherence properties, rather than expression of defined markers or clonogenicity.

Methods: Several human cell lines derived from oral dysplasia and oral squamous cell carcinoma (OSCC), as well as primary cells derived from patients with OSCC were allowed to adhere to collagen IV-coated dishes sequentially. Rapid adherent cells (RAC), middle adherent cells (MAC) and late adherent cells (LAC) were then harvested and further investigated for their morphology, stem cell-like properties and molecular profile while grown *in vitro* and tongue xenotransplantation in NOD-SCID mice at serial dilutions.

Results: RAC showed significantly higher colony forming efficiency ($p < 0.05$), sphere forming ability, greater migration ability ($p < 0.05$), exhibited longer G2 phase and displayed higher expression of integrin β1 and other stem-cell related molecules as compared to MAC and LAC. RAC induced tongue tumours in NOD-SCID mice with the highest incidence. These tumours were also bigger and metastasised more frequently in loco-regional lymph nodes than MAC and LAC.

* *Corresponding author:* Address: Department of Biomedicine, University of Bergen, N5009 Bergen, Norway. Tel.: +47 55586594.
E-mail address: jian.wang@biomed.uib.no (J. Wang).

http://dx.doi.org/10.1016/j.ejca.2014.09.010

Conclusions: These findings prove for the first time that OSCC cells with tumour initiating properties can be enriched based on their rapid adhesiveness to collagen IV. This separation procedure provides a potentially useful tool for isolating TICs in OSCC for further studies on understanding their characteristics and drug-resistant behaviour.

1. Introduction

Oral squamous cell carcinoma (OSCC) has poor prognosis due to therapy-resistant loco-regional recurrences and distant metastases [1]. The cancer stem cell theory, although controversial, seems to explain well the behaviour of this disease, and the existence of a sub-population of cells with tumour initiating properties and increased self-renewal has been shown in OSCC [2]. Understanding the biology of tumour initiating cells (TICs) is thus essential for better understanding and more specific targeting of OSCC. For the same reason, robust methods for isolation of TICs are in demand. Numerous strategies for enrichment of TICs have been previously described [2–5]. However, isolating a population of pure and viable putative TICs is still problematic. Fluorescence-activated cell sorting (FACS) is the most common technique to isolate TICs based on specific surface makers, and CD44 has been repeatedly reported to identify human oral cancer stem cells [2,4]. Nevertheless, there is no single marker that can be used for isolation of TICs for any type of cancer, including OSCC, because of tumour heterogeneity and lack of reproducibility [5]. Moreover, the FACS technique requires high-cost, high-speed sorters and high-quality antibodies. Sorting the side population (SP) on the basis of the ability to efflux the fluorescent DNA-binding dye Hoechst 33342, or sorting of cells based on the ALDH1 enzymatic activity has also been used for isolation of TICs, but sometimes only few cells are acquired by these methods, and thus they are hardly of any use for further investigations [5]. In addition, the method of sorting SP cells cannot be applied broadly since not all cell lines contain SP cells [6]. Recent studies that explored more functional, non-invasive alternative methods to FACS analysis, demonstrated that non-adherent sphere formation [3], *in vivo* serial tumour xenotransplantation [7], as well as chemotherapeutic drug resistance [7,8] could be used to enrich a subpopulation with TICs. However, these methods require a long time to yield TICs, and not all cell types and cell lines are able, for example, to grow as spheres. In this study we have assessed the potential of a functional approach for TIC isolation in OSCC, a method previously used for normal epithelium [9]. Although severely disturbed, OSCC has an organisation resembling the normal oral epithelium, including functionally different cell sub-populations: stem cells, transient amplifying cells and terminally differentiated cells [10]. Based on the ability of normal epidermal stem cells to rapidly adhere to basement membrane molecules such as collagen IV due to their differential expression of specific integrins [11], an adhesion assay for their isolation and enrichment was successfully developed in both human [11], murine [12], and rabbit normal epithelium [9]. We have adopted this method of isolating stem cells in normal epithelia for enrichment of TICs in OSCC, and determined whether a sub-population of cancer cells containing putative TICs could be separated over time according to adhesiveness to collagen IV. We report here, for the first time to our knowledge, that sub-populations of cells enriched for TICs, with increased self-renewal and higher tumourigenic potential could be isolated within 10 min using the method of rapid adhesiveness to collagen IV in OSCC.

2. Materials and methods

2.1. Cell culture and generation of primary cells from human samples

OSCC cell lines (Cal, H357 and CaLH3), human dysplastic oral keratinocyte (DOK) cell line and primary oral cancer keratinocytes isolated from patients with OSCC (P1 and P2) were used in this study. Written consent was obtained from all patients, and the project was approved by the Regional Committee for Ethics in Research in West Norway.

2.2. Adhesion to collagen IV

Tissue culture dishes (100 mm, Nunc, Denmark) were coated evenly with 10 μg/mL human collagen IV (Sigma, St. Louis, MO, United States of America (USA)) diluted in 10 mM acetic acid. Single cell suspensions in routine culture medium were allowed to attach to the collagen IV coated dishes in the incubator. Cells that attached within 10 min were collected after trypsinisation and referred to as rapid adherent cells (RAC). Cells that remained unattached within first 10 min were transferred to a new collagen IV-coated dish for additional 30 min in the incubator. Cells that adhered within this period were collected as middle adherent cells (MAC). Remaining unattached cells were then again transferred into a new collagen IV-coated dish for next 4 h. Adherent cells in this time period were collected and referred to as late adherent cells (LAC).