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Differences in the whole saliva baseline proteome profile associated with development of oral mucositis in head and neck cancer patients undergoing radiotherapy

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ABSTRACT

Oral mucositis (OM) is a common, painful and often treatment-limiting side effect of radiotherapy (RT) for head and neck cancer (HNC) patients. Unstimulated saliva was collected before the first radiotherapy application in 50 HNC patients. 41 out of 50 patients developed OM (grade III) during radiotherapy, of which 14 patients even displayed an early OM (grade III) at a low radiation dose of 30 Gy. Nine patients did not develop OM (grade III). Using an LC–MS/MS approach 5323 tryptic peptides were assigned to 487 distinct proteins (≥2 peptides) in the data set. The levels of 48 proteins differed significantly ($p < 0.05$) between patients developing OM or not. 17 proteins displayed increased levels (≥1.3-fold) and 31 proteins decreased in level in OM, respectively. Furthermore, using partial least square analysis protein patterns could be used to distinguish subjects which did not develop grade III OM even after 70 Gy total dose ($n = 9$) and those displaying early OM (grade III at <30 Gy total dose, $n = 14$). Using leave one out cross validation 37 of 41 patients (90%) developing OM could be correctly assigned indicating that prognostic proteome signatures may help identify patients that should be specifically monitored to increase overall effectiveness of RT treatment.

1. Introduction

Oral mucositis (OM) is a common and often dose-limiting side effect of radiation therapy with (CRT) or without chemotherapy (RT) for head and neck cancer patients (HNC) [1]. Up to 90% of the patients are diagnosed with acute OM, 60–70% of those suffer from severe OM l [1,2], characterized by ulceration and pseudomembranous formations. OM is associated with multiple different symptoms including intense pain, dysphagia and weight loss [3,4]. The severity of the symptoms or the fear that higher grade OM might progress to necrosis can lead to unplanned RT interruptions or reduced CRT compliance, resulting in poorer outcome [2], as well as in prolonged hospitalization, a need for feeding tubes and opiate medication [5,6]. Therefore, OM is associated with a negative impact on quality of life (QOL) and presents a major clinical and economic problem [7,8]. Although innovative new schedules and therapies such as Palifermin were tested, reliable and effective drugs reducing the risk of OM are lacking [1,9]. Risk factors of OM include the volume of the irradiated mucosa, treatment dose, concurrent radiochemotherapy agent(s) and the treatment schedule. Oral health, use of tobacco and alcohol, comorbidities, age, sex and genetic predisposition [6] as well as changes in salivary flow and oral bacterial flora [10] also influence the development of OM. However, despite its frequency and clinical importance of OM, the mechanisms of how risk factors impact development of OM in HNC patients have not been sufficiently defined [8].

Despite displaying similar risk factor profiles patients experience OM at different radiation doses. However, currently prediction of onset and severity of OM is not possible and thus early detection is not feasible, but limited to diagnosis and classification by its clinical manifestations [11]. Understanding the molecular details of the pathogenesis of OM will eventually allow the early identification of patients prone to develop early OM, but also facilitate close monitoring and characterization of this side effect [5]. Thus, novel prognostic and/or predictive markers that could easily be monitored and identify patients at risk of OM would be of high interest.

Human body fluids such as plasma and urine are routinely used in clinical diagnostics because they are in direct contact to tissues and organs and are therefore suitable for the investigation of local as well as systemic pathologies. Comparably, saliva may act as an indicator of oral disease status and therefore allow sensitive and/or specific protein biomarker discovery. Mass spectrometry-based proteomics approaches and the respective analysis pipelines now allow reproducible and

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reliable peptide-based quantification of protein levels even in large sample series [12]. But, analysis of protein composition of body fluids and particularly saliva is also strongly dependent on environmental factors such as nutrient uptake. However, recent data of a whole saliva proteome study indicate that the inter-subject variability is in part time-independent, allowing for meaningful screening of differences in the saliva proteome even in a cross-sectional design [13]. Examples of the successful application of whole saliva proteome screens include Sjögren's syndrome [14], diabetes mellitus [15], periodontitis [16] or oral cancer [17].

In the current study, we investigated the whole saliva protein pattern at baseline of radiotherapy treatment in patients undergoing RT (or CRT) for HNC to screen for differences related to the development of OM. The study was focused on human proteins and revealed 48 proteins differing in levels between patients developing OM or not. Furthermore, even with this limited number of patients a leave one out prediction facilitated identification of patients developing OM with 90% positive predictive value. Thus, expansion of the study in a follow-up may lead to the identification of signatures facilitating prediction of patients with enhanced risk of developing OM and thus help improve patient management and increase overall treatment effectiveness.

2. Material and methods

2.1. Patient selection

Patients with squamous cell carcinoma of the head and neck (oral cavity, oropharynx, hypopharynx, and larynx) who were to receive primary or adjuvant radiochemotherapy R(C)T at the Department of Radio Oncology of the University Medical Center Freiburg were eligible for this study. Patients were older than 18 years; and had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2; at least two of eight areas of the oral or the oropharyngeal mucosa (hard palate, soft palate, tongue, and bottom of the mouth, upper- and lower lip, cheek left and right) were planned to receive at least 50 Gy. Key exclusion criteria were as follows: tumor of unknown primary site, prior radiation to the head and neck region or prior chemotherapy.

2.2. Study design and radiochemotherapy treatment

This prospective study was conducted in Freiburg, Germany and followed the declaration of Helsinki principles. The protocol was accepted by the ethics committee of the University of Freiburg. All patients provided informed consent before any study-related procedure. Patients were enrolled between March 2010 and June 2012 and saliva analysis was performed in the Department of Functional Genomics, Interfaculty Institute for Genetics and Functional Genomics, Greifswald.

All patients received intensity-modulated radiation (IMRT). For patients with inoperable locally advanced squamous cell carcinoma 70 Gy were administered to gross tumor volume (GTV) and to regional lymph nodes >2 cm; 60 Gy were given to nodes >1 cm and ≤2 cm and 50 Gy to nodal areas suspected to be involved. Daily fractions of 2 Gy were administered five times weekly; patients in a good clinical condition received concomitant chemotherapy up to three doses cisplatin (100 mg/m^2 per day) every 21 days. Patients with prior surgery and a high risk for recurrence underwent RT with 60–66 Gy in total; concomitant chemotherapy of two doses cisplatin (100 mg/m^2 per day) every 21 days was given to patients with minimal residual disease (R1) or extracapsular spread [18].

The Oncentra Master-plan (V1.5/3.0 Nucletron B.V.) was used as 3D treatment planning system. 6–10 MV photons of a linear accelerator were used (Varian Clinac, Varian Medical Systems, USA and Elekta Synergy, Elekta Corporate, Sweden).

2.3. Clinical assessment of oral mucositis

Oral mucositis (OM) was assessed by region of the oral cavity including lips, tongue, right and left buccal mucosa, soft palate, hard palate and floor of mouth and eventual reactions were scored using the National Cancer Institute — Common Toxicity Criteria (NCI-CTC v3.0). Twice-weekly assessments (at least 3 +/− 1 days apart) continued throughout R(C)T till the end of therapy. Whenever possible the same evaluator assessed the patients throughout treatment. The exact time point and therefore irradiated total dose until the appearance of grade III mucositis (NCI-CTC vs. 3.0) was recorded.

2.4. Saliva sampling

Saliva was collected from patients at least three days before the radiotherapy started. The whole saliva collection was carried out between 9 am and 12 am. Patients were asked not to drink, to eat or to smoke at least 1 h before the collection. Five minutes after rinsing the mouth with water the unstimulated collection of saliva started. The patients were asked to sit head forward and to let saliva just float out of the mouth for 10 min into a funnel placed onto a 50 mL Falcon tube, kept in an ice cup. Collected saliva was immediately centrifuged at 3000 rpm, for 15 min and 4 °C to remove insoluble material. One mL of the supernatant was pipetted and mixed with 2 μL protease inhibitor cocktail as well as 3 μL of 1 mM sodium orthovanadate solution (both Sigma Aldrich, St. Louis, MO, USA)., aliquoted and stored at −80 °C until proteome analysis.

2.5. Protein composition of whole saliva and preparation for proteome characterization

The volume of whole saliva ranged from 0.2 to 1.7 mL (average 0.9 ± 0.3 mL, coefficient of variation (CV) = 0.39) and displayed high variability. Whole saliva proteins (aliquots of 0.5 mL) were precipitated using trichloroacetic acid (TCA) at a final concentration of 10% (v/v) and dithiothreitol (0.12% w/v) as described [13]. Protein pellets were resuspended in 8 M urea/2 M thiourea buffer and the protein concentrations were measured which resulted in values that varied from 0.4 to 10.4 μg/μL (average 3.3 ± 2.8 μg/μL). The amount of total protein available for further analysis ranged from 13 μg to 312 μg per subject, which was sufficient for processing for LC–MS analysis. In total, 4 μg of protein lysate was reduced (2.5 mM DTT for 1 h at 60 °C) and alkylated (10 mM iodacetamide for 30 min at 37 °C). Proteolysis was performed overnight using trypsin (Promega, Madison, WI, USA) using an enzyme/substrate ratio of 1:25 at 37 °C. Tryptic digestion was stopped by adding acetic acid to a final concentration of 1%, followed by desalting and purification using ZipTip-μC18 tips (Millipore, Billerica, MA, USA). Samples were measured in a randomized design to minimize systematic error during our analysis. Proteolytically cleaved peptides were separated prior to mass spectrometric analyses by reverse phase nanoHPLC on a 15 cm Acclaim PepMap100-column (C18, 3 μm, 100 Å) using an EASY-nLC Proxeon system (Thermo Scientific, Waltham, MA, USA) at a constant flow rate of 300 nL/min. Separation was achieved using a linear gradient of buffer B from 5% up to 25% within 63 min with 0.1% acetic acid, 2% acetonitrile in water (solvent A) and 0.1% acetic acid in 100% acetonitrile (solvent B). Separated peptides were monitored using an LTQ Orbitrap Velos MS (Thermo Scientific) equipped with a nanoelectrospray ion source operated with PicoTip Emitters (New Objective, Woburn, MA, USA). After a first survey scan (r = 30,000) MS/MS data were recorded for the 20 highest mass peaks in the linear ion trap at a collision induced energy of 35%. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 60 s and the minimal ion signal for MS/MS was 2000. Raw data from the Orbitrap Velos instrument was processed using the Refiner MS 7.5 module (Genedata, Basel,