Oral Oncology 68 (2017) 74–80

Contents lists available at ScienceDirect

Oral Oncology

journal homepage: www.elsevier.com/locate/oraloncology

Investigating a case of possible field cancerization in oral squamous cell carcinoma by the use of next-generation sequencing

Siavosh Tabatabaeifar [a,d,*], Martin J. Larsen [b,d], Stine R. Larsen [c,d], Torben A. Kruse [b,d], Mads Thomassen [b,d,1], Jens A. Sørensen [a,d,1]

[a] Department of Plastic Surgery, Odense University Hospital, Odense, Denmark
[b] Department of Clinical Genetics, Odense University Hospital, Odense, Denmark
[c] Department of Pathology, Odense University Hospital, Odense, Denmark
[d] University of Southern Denmark, Institute of Clinical Research, Odense, Denmark

ARTICLE INFO

Article history:
Received 7 February 2017
Received in revised form 3 March 2017
Accepted 28 March 2017

Keywords:
Ultra-deep sequencing
Oral squamous cell carcinoma
Field cancerization
Recurrence
Tumor heterogeneity
Subclonal structure
Second field tumor

ABSTRACT

Objectives: Local recurrence and the development of second primary tumors (SPT) are important factors that can influence the survival rate of oral squamous cell carcinoma (OSCC) patients. We investigate the concept of *field cancerization* which proposes that normal tissue adjacent to the primary tumor harbor pre-neoplastic alterations that can lead to the development of local recurrence and SPTs.
Materials and methods: To examine the concept of field cancerization, we applied whole-exome and targeted ultra-deep sequencing on 5 freshly frozen samples from a stage III OSCC patient from three tumor sites, lymph node metastasis and blood. Lastly, we sequenced one formalin-fixed paraffin-embedded recurrence biopsy that was collected approximately a year and half later located in the same area as before.
Results: Sequencing identified 126 somatic mutations. We identified 24 mutations in the recurrence biopsy and 14 mutations are shared by the primary tumor.
Conclusion: The low number of shared mutations indicates that either these mutations represent a very early clone in the primary tumor's evolution, or that these mutations represent a pre-neoplastic field, in which the primary tumor and recurrence are derived from. In both instances, the clinical recurrence is of a monoclonal origin which suggests either field cancerization by migration of mutated cells in the adjacent mucosa, or that the recurrence developed out of remaining tumor tissue.

Introduction

Oral cavity cancer, a subgroup of head and neck cancer, is primarily attributed to the exposure of carcinogens by the consumption of alcohol and use of tobacco [1]. More than 90% of tumors are oral squamous cell carcinomas (OSSC). Patients diagnosed with early stages of OSCC have a good prognosis; however, local recurrence and the development of second primary tumors (SPT) are important factors that can influence the survival rate. The incidence rate of local recurrence in head and neck cancer patients is about 10–30%, and for SPT, the rate varies between 2–30% dependent on the study [2–5]. These clinical observations led to the concept of *field cancerization,* first coined by Slaughter et al. [6] in a paper from 1953 in which it is proposed, that normal tissue adjacent to the primary tumor harbor pre-neoplastic alterations that can lead to the development of local recurrence and SPTs. Studies have suggested that in the initial phase, a cell acquires genetic alterations, divides and forms a patch, a clonal unit of daughter cells. Additional acquired alterations transforms the patch into a proliferating field that gradually displaces the normal mucosa, and from this field tumors develop [7,8]. The focus of head and neck squamous cell carcinoma (HNSCC) sequencing studies have primarily been on identifying somatic mutations in primary tumors by using a single tumor biopsy per patient [9]. The aim of this study is to examine the concept of field cancerization by analyzing a unique set of samples from one patient using whole-exome sequencing (WES) and targeted ultra-deep sequencing (UDS).

* Corresponding author at: Department of Plastic Surgery, Odense University Hospital, Sdr. Boulevard 29, 5000 Odense C, Denmark.
E-mail address: Siavosh.tabatabaeifar@rsyd.dk (S. Tabatabaeifar).
[1] Authors contributed equally to the study.

http://dx.doi.org/10.1016/j.oraloncology.2017.03.018
1368-8375/

Material and methods

Patient selection and sampling

Ethics approval was obtained from The Regional Scientific Ethical Committees for Southern Denmark and informed consent were acquired from the included patient. The study was carried out in accordance with the approved guidelines.

The patient, a 43 year old male of Caucasian descent with no history of tobacco use and no history of obsessive alcohol consumption, but with a history of lichen planus, was surgical treated for a buccal stage III T1N1M0 OSCC by unilateral neck dissection and tumor resection with a margin of 1 cm. The patient had not received treatment prior to the operation. The operation took place in late 2013 at the Department of Plastic Surgery, Odense University Hospital, which is the center for surgical treatment of oral cavity cancer for the Region of Southern Denmark's 1.2 million inhabitants. During the procedure, 4 tissue biopsies were collected: 3 from the primary tumor and 1 from the lymph node metastasis. A 10 mL sample of venous blood was drawn into a heparinized collection tube prior to the operation for use as the matched normal sample. All samples were freshly frozen and stored at -80° Celsius for later use. The primary tumor was tested positive for p16 protein overexpression, which is used as a prognostic marker and a marker for human papilloma virus infection (HPV) in oropharyngeal cancers. Additional radiation therapy was not given as the cancer did not to meet the criteria for adjuvant treatment. The patient was referred to the follow-up program at the department of Oncology which consists of an examination every 3 months for 2 years and every 6 months the following 3 years.

In the summer of 2015, the patient was referred to us from the Oncology department, with new suspicious alterations in the oral cavity in the same area as before. No suspicious alterations were present 3 months prior. A punch biopsy was taken which showed recurrence of squamous cell carcinoma. To investigate the recurrence further, 10 slices of the formalin-fixed paraffin-embedded (FFPE) punch biopsy were cut for later use in WES.

Pathology

The tissue biopsies were evaluated by a pathologist to confirm the presence of squamous tumor cells. One biopsy, tumor back, was evaluated not to contain any tumor tissue. To minimize the presence of normal tissue in the lymph node, the pathologist marked the areas that contained tumor before being macroscopically dissected.

DNA extraction

DNA from the 10 mL whole blood was extracted using the Gentra PureGene Blood kit (Qiagen) following the instructions provided by the manufacturer. DNA was extracted from approximately 30 mg of primary tumor and lymph node biopsies using the AllPrep DNA/RNA Mini kit (Qiagen). The DNA from the FFPE recurrence punch biopsy slices was extracted using 16 FFPE Plus LEV DNA Purification kit (Maxwell) following the instructions provided by the manufacturer.

Whole-exome sequencing

DNA extracted from the freshly frozen samples were subjected to sample preparation and exome capture by hybridization using TruSeq Exome Enrichment kit (Illumina) following the standard protocol provided by the manufacturer. The recurrence punch biopsy was subjected to sample preparation and exome capture by hybridization using SeqCap EZ Exome v3 kit (NimbleGen), as Illumina's kit was discontinued. To ensure that the findings between the enrichment platforms are comparable, the blood sample was also subjected to SeqCap EZ Exome v3 kit. Sequencing was carried out on the Illumina HiSeq1500 platform with paired-end 2×100 base-pair reads.

Validation

Somatic variants in the freshly frozen samples were filtered, and the same samples were enriched for all filtered variants using Agilent SureSelect XT. Validation by UDS was carried out on the Illumina HiSeq1500 platform with paired-end 2×100 base-pair reads.

B-allele frequency

The B-allele frequency (BAF) represents the fraction of alternative reads (B-allele) in the tumor biopsy related to the sum of the reference (A-allele) and alternative reads. BAF is calculated as

$$\mathrm{BAF} = \frac{B\text{-allele reads}}{A + B\text{-allele reads}}$$

BAF is calculated for somatic point mutations, but also for germline variants in the copy number analysis.

Bioinformatics

Raw reads were aligned to the hg19 reference genome using Novoalign v. 3.01 (Novocraft) and processed according to Genome Analysis ToolKit Best Practice pipeline v. 2.7 (Broad Institute), including duplicate removal, indel realignment and base quality score recalibration [10,11]. Calling of variants was performed using Varscan v. 2.3.4 [12], and Annovar (2013Aug23) [13] was used for annotation of variants. dbSNP build 138 [14] was used for filtering out known germline mutations. Only bases with a quality score of at least Q20 (corresponding to an error rate of 1:100) were considered. We used the following criteria to identify somatic mutations derived from the exome data:

1. A variant should only be identified if it had a BAF of at least 3% and had ≥ 3 alternative reads in one of the samples besides blood.
2. To ensure that the B-allele was not a germline variant, the blood sample should at least have 10x coverage at the same position and have 0 alternative reads.

The filtered variants were validated using UDS. Before analyzing the validated data, we used the following criteria to ensure a reliable analysis:

1. A variant should only be validated if it had a BAF of at least 3% and had ≥ 10 alternative reads in one of the samples besides blood.
2. To ensure that the B-allele was not a germline variant, the blood sample should at least have 50x coverage at the same position and have a BAF $\leq 1\%$.

If the criteria are met in one of the tissue biopsies, the variant will be reported. This consequently means that we are more certain of the existence of the same variant in the other biopsies, even if BAF is under 3%; however, alternative reads should comprise more than 0.5% to avoid false positive variants (error rate is under 0.2% as determined in UDS data; data not shown). It should be noted that in the final analysis, we use the exome criteria for the recurrence biopsy and validation criteria for the rest.