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# Clonality analysis in primary oral squamous cell carcinoma and related lymph-node metastasis revealed by *TP53* and mitochondrial DNA next generation sequencing analysis

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ABSTRACT

The chance of developing a neck nodal metastasis after initial treatment of oral squamous cell carcinoma varies from 12.4% to 62%. Despite being the main reason for cancer-related mortality, nodal metastases are still rarely subjected to molecular analyses, and our knowledge of the clonal heterogeneity of multiple lesions within the same patient is limited. The aim of the present study was to evaluate the relationship between primary oral cancer and lymph node metastasis in a series of patients with synchronous and metachronous metastases by 2 clonality tests: mt-DNA and *TP53* sequence analysis.

The study population consisted of 10 consecutive patients. Data identified in this study demonstrate that our assay based on next-generation analysis of *TP53* and mt-DNA is simple, is reliable, allows high throughput, and may be applied to retrospective cases. The combination of mt-DNA and *TP53* data analysis helped us to evaluate more precisely and consistently the genetic relationship among different tumor clones.



## 1. Introduction

Patients affected by oral squamous cell carcinoma (OSCC) can develop neck nodal metastases after initial treatment (Spector et al., 2001; Slootweg et al., 1996). The presence of nodal metastasis is the single most important predictor of survival (De Bree et al., 2000). Usually, these metastases are clearly related to the identified squamous cell carcinoma and synchronous with it. On the contrary, when a lymph node metastasis (LNM) is delayed with respect to the mucosal cancer diagnosis (metachronous metastasis), or when it appears without an obvious mucosal lesion (metastasis of unknown primary tumor), it can be difficult to find the related primary OSCC.

Some authors (Slootweg et al., 1996; De Bree et al., 2000) estimate that the incidence of cervical lymph node metastasis with no obvious primary site ranges from 3% to 9%, with squamous histology constituting 75% of these tumors.

Several molecular techniques have been applied to assess tumor heterogeneity and clonality between primary tumors and metastasis including karyotype analysis and cytogenetic techniques, X-chromosome inactivation, *TP53* mutations, loss of heterozygosity (LOH), microarray-based comparative genomic hybridization (aCGH) (Ha and Califano, 2003; Lydiatt et al., 1998; Pateromichelakis et al., 2005; Diaz-Cano et al., 2001), and, more recently, whole or exome next-generation sequencing (Mroz et al., 2013; Zhang et al., 2013).

A common problem in the molecular approach is the identification of early shared genetic alterations that should be unique to the lesions and not found in normal tissue. In addition, mutations are cumulative and can often change during cancer progression. A genetic change should be qualified as clonal marker when the

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following conditions are fulfilled: 1) the genetic change appears before the appearing of metastasis; 2) the same genetic change is maintained during neoplastic progression and metastasis; and 3) the early shared genetic alteration that is unique to the lesions should not be found elsewhere in normal tissue (Ha and Califano, 2003).

Among the genetic markers currently available in the literature, X-chromosome inactivation and wide genome CGH array are not suitable for routine clinical practice: X-chromosome inactivation is available only for female samples; and the majority of OSCC patients are males; and aCGH is expensive, is time consuming, and needs a large amount of intact DNA (Vekony et al., 2009; Diaz-Cano et al., 2001). A third possibility could be a combination of karyotyping and fluorescence in situ hybridization, but this approach includes tissue culture and therefore is less suitable for routine applications (Worsham et al., 1995).

*TP53* mutations analysis has been used as a clonal marker, considering that approximately 50–90% of tumors contain some forms of *TP53* mutation and these mutations show an huge variability (Kropveld et al., 1999; Boyle et al., 1993). Alterations in this gene are thought to be relatively early events in OSCC because they are present already in normal tissue distant from tumors and in premalignant lesions (Shin et al., 1994). The presence of an identical *TP53* mutation is usually understood to indicate a clonal relationship.

Tjebbes et al. (1999), Tabor et al. (2002), and Van Oijen et al. (2000) found identical *TP53* specific mutations in primary tumors and matched LNM in all analyzed samples, whereas Kropveld et al. (1996) and Zariwala et al. (1994) found different TP53 mutations between primary tumors and matched lymph node metastases.

Recently, mitochondrial DNA (mt-DNA) sequence analysis followed by neighbor-joining tree was introduced in head-and-neck oncology as a method for establishing the clonal relationship between 2 lesions or between a tumor and the loco-regional metastasis (Foschini et al., 2011; Montebugnoli et al., 2014). More recently, a case report described clonal relationship between primary OSCC and a LNM appeared 4 years later by using mt-DNA analysis (Tarsitano et al., 2014).

The aim of the present study was to evaluate the relationship between primary OSCC and LNM in a series of patients with synchronous and metachronous LNM by 2 clonality tests: mt-DNA and *TP53* sequence analysis.

## 2. Materials and methods

The study population consisted of 10 consecutive patients who were affected by OSCC and corresponding LNM and treated at the Maxillofacial Surgery Unit of University of Bologna between January 2012 and December 2013 (see Table 1 for clinical details).

Inclusion criteria were the following: 1) neck dissection with a pathological diagnosis of metastatic squamous cell carcinoma; 2) a complete medical history, physical examination, and diagnostic panendoscopy results available; and 3) imaging studies including head-and-neck computed tomography (CT) or magnetic resonance imaging, chest radiograph, and total body positron emission tomography–CT, when indicated.

Patient treatment consisted of composite resection, including excision of the primary tumor with ipsilateral or bilateral neck dissection. Neck dissection was monolateral in 8 cases and bilateral in 2 cases. Modified radical neck dissection (MRND) was performed in 8 patients and radical neck dissection (RND) in 1 patient. The remaining patient had an extended radical neck dissection (ERND). Metastases were defined as synchronous when detected at the time of surgical resection of the primary OSCC, and as metachronous when they appeared 1 month or more after surgery.

The work was approved by the ethical committee of the University of Bologna (mtDNA01, code 020/2013/U/Tess), and informed consent was obtained from all patients.

All tissue samples were sent for histological analysis to the Sections of Anatomic Pathology of the University of Bologna at Bellaria Hospital and of S. Orsola Hospital. All tissues were formalin fixed and paraffin embedded as is routine, and subsequently stained with hematoxylin and eosin. Histological diagnoses were made following the criteria proposed in the World Health Organization Blue Book (Gale et al., 2005).

### 2.1. Microdissection and DNA extraction

Sections 10 μm thick were carefully microdissected for DNA extraction by means of the laser-assisted SLμcut Microtest (MMI GmbH, distributed by Nikon, http://www.mmimicro.com) as previously described (Montebugnoli et al., 2014) to obtain morphologically homogeneous populations of tumor cells. Cells from normal mucosa were also selected and served as control reference DNA for mtDNA analysis. DNA was purified using the Quick Extract™ FFPE DNA extraction kit (Epicentre, Madison, WI) following the manufacturer's instructions.

### 2.2. Mitochondrial DNA and TP53 sequencing

DNA was sequenced for mtDNA D-loop region and for *TP53* by 454 platform (GSJunior, Roche, Branford, CT). In brief, the mtDNA D-loop sequence analysis was performed by amplifying 4 segments, covering the whole region from position 15,995 to position 700, according to Anderson (Anderson et al., 1981) as described in the human mitochondrial database (NC_012920 gi:251831106, MITOMAP: a Human Mitochondrial Genome Database; Center for

**Table 1**
Clinical and molecular profile from the case-series.

| Pt. | Age (years) | Gender | Smoking status | TN status | Primary tumor site | Neck dissection | LNM Synchronous/metachronous (time) | Follow-up |
|---|---|---|---|---|---|---|---|---|
| Case 1 | 53 | M | No smoker | T4N2a | Retro-molar region | MRND | Synchronous | DOD (10 mo) |
| Case 2 | 72 | F | No smoker | T2N1 | Alveolargingiva | MRND | Synchronous | A&W (18 mo) |
| Case 3 | 76 | F | No smoker | T2N1 | Cheek | MRND | Metachronous (5 months delayed) | A&W (14 mo) |
| Case 4 | 71 | F | No smoker | T4N2a | Floor of the mouth | MRND | Synchronous | A&W (11 mo) |
| Case 5 | 70 | M | Smoker | T4N2c | Tongue | MRND | 2 Synchronous LNM | DOD (8 mo) |
| Case 6 | 65 | M | Smoker | T2N2b | Tongue | ERND | Synchronous and metachronous (20 months delayed) | DOD (9 mo) |
| Case 7 | 53 | M | No smoker | T1N2b | Cheek | RND | Synchronous and metachronous (11 months delayed) | A&W (18 mo) |
| Case 8 | 73 | M | Smoker | T4N2a | Alveolargingiva | MRND | Synchronous | A&W (15 mo) |

RND: radical neck dissection; MRND: modified radical neck dissection; ERND: extended radical neck dissection; DOD: died of disease; A&W: free of disease; T: tumor; M: metastasis; WT: wild type.