Oral Oncology 51 (2015) 500–507

Contents lists available at ScienceDirect

Oral Oncology

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

Telomere shortening in mucosa surrounding the tumor: Biosensor of field cancerization and prognostic marker of mucosal failure in head and neck squamous cell carcinoma

Paolo Boscolo-Rizzo [a], Enrica Rampazzo [b], Egle Perissinotto [c], Maria Assunta Piano [b], Silvia Giunco [d], Lorena Baboci [b], Giacomo Spinato [e], Roberto Spinato [f], Giancarlo Tirelli [e], Maria Cristina Da Mosto [a], Annarosa Del Mistro [d], Anita De Rossi [b,d,*]

[a] Section of Otolaryngology and Regional Center for Head and Neck Cancer, Department of Neurosciences, University of Padova, Treviso, Italy
[b] Section of Oncology and Immunology, Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy
[c] Unit of Biostatistics, Epidemiology and Public Health, Department of Cardiological Thoracic and Vascular Sciences, University of Padova, Padova, Italy
[d] Immunology and Molecular Oncology Unit, Istituto Oncologico Veneto – IRCCS, Padova, Italy
[e] Head and Neck Department, University of Trieste, Trieste, Italy
[f] Otolaryngology Unit, Ospedale dell'Angelo, Venezia, Italy

ARTICLE INFO

Article history:
Received 22 October 2014
Received in revised form 1 January 2015
Accepted 24 February 2015
Available online 11 March 2015

Keywords:
Field cancerization
Head and neck cancer
Human papilloma virus
Local relapse
Molecular biology
Survival
Telomerase reverse transcriptase
Telomeres
TERT

SUMMARY

Objectives: The aim of the present study was to investigate the pattern of telomere length and telomerase expression in cancer tissues and the surrounding mucosa (SM), as markers of field cancerization and clinical outcome in patients successfully treated for with head and neck squamous cell carcinoma (HNSCC).
Materials and methods: This investigation was a prospective cohort study. Telomere length and levels of telomerase reverse transcriptase (TERT) transcripts were quantified by real-time PCR in cancer tissues and SM from 139 and 90 patients with HNSCC, respectively.
Results: No correlation was found between age and telomere length in SM. Patients with short telomeres in SM had a higher risk of mucosal failure (adjusted HR = 4.29). Patients with high TERT levels in cancer tissues had a higher risk of regional failure (HR = 2.88), distant failure (HR = 7.27), worse disease-specific survival (HR for related death = 2.62) but not mucosal failure. High-risk patients having both short telomeres in SM and high levels of TERT in cancer showed a significantly lower overall survival (HR = 2.46).
Conclusions: Overall these findings suggest that telomere shortening in SM is a marker of field cancerization and may precede reactivation of TERT. Short telomeres in SM are strongly prognostic of mucosal failure, whereas TERT levels in cancer tissues increase with the aggressiveness of the disease and are prognostic of tumor spread.

Introduction

Head and neck cancer was estimated to be the sixth most common malignancy in Europe in 2012, affecting approximately 140,000 new patients and resulting in nearly 65,000 deaths [1]. About 90% of head and neck cancers are squamous cell carcinomas (HNSCC), which develop in the epithelial lining of the upper aero-digestive tract (UADT) after exposure to tobacco, alcohol, or persistent oral infection by human papillomavirus (HPV) [2].

Despite improvements in functional outcomes due to developments in the field of radiotherapy, chemotherapy, surgical and imaging techniques, with the exception of the oral cavity and tonsillar cancers, survival has only marginally improved over the past two decades [3].

Patients with HNSCC who received successful curative treatment are in fact reported to have high propensity for mucosal failure [2,4]. Intensive investigations have demonstrated that mucosal failure may result from either outgrowth of histopathologically undetectable residual tumor cells or development of a second field tumor (SFT) following crucial genetic hits occurring in a pre-neoplastic field which may persist after treatment of both surgical and non-surgical treatment of the primary tumor [5–9]. For accurate risk assessment, disease monitoring and early

* Corresponding author at: Section of Oncology and Immunology, Department of Surgery, Oncology and Gastroenterology, University of Padova, Via Gattamelata 64, 35128 Padova, Italy.
E-mail address: anita.derossi@unipd.it (A. De Rossi).

http://dx.doi.org/10.1016/j.oraloncology.2015.02.100
1368-8375/

diagnosis, it is of particular interest to identify molecular markers of pre-neoplastic fields in tumors surrounding epithelium.

Telomeres are highly repetitive G-rich DNA sequences located at the end of chromosomes maintained by telomerase, a ribonucleoprotein complex containing an internal RNA component [telomerase RNA (TR)] and a catalytic protein with telomere-specific reverse transcriptase activity [telomerase reverse transcriptase (TERT)]. TERT, which synthesizes *de novo* telomere sequences by using TR as a template, is the rate-limiting component of the telomerase complex, and its expression is correlated with telomerase activity [10]. The telomere/telomerase complex is a key element in determining the replicative potential of cells. On one hand, maintenance of telomere length by telomerase is critical for preserving the replicative potential of cancer cells; on the other, telomere erosion may impair their function in protecting chromosome ends, resulting in genetic instability, a crucial step in the initiation of carcinogenesis [11]. Many studies, including ours on colorectal carcinomas [12,13], have demonstrated that neoplastic cells generally have shorter telomeres than their adjacent non-tumoral counterparts, suggesting that telomere erosion precedes the reactivation of telomerase. As telomere attrition is considered to be an early event in human carcinogenesis, and as telomerase activation is crucial for tumor formation [13–17], studying them as potential markers of field cancerization and disease outcome appears to be important.

Previous studies on HNSCC have shown that telomere attrition [18–25] and telomerase activation [18,20,22,26–35] are frequent events in this malignancy. However, several studies have analyzed only small numbers of patients, few studies have evaluated both telomere length and telomerase activation in the same samples [18–22,34], and only two research groups have examined matched tumoral and adjacent tissues [22,34]. Few studies have evaluated the relationship between telomere length/telomerase activation and clinical outcomes [20,31–33], and no studies have analyzed the significance of telomere and telomerase as markers of field cancerization in HNSCC.

The aim of the present study was to investigate the pattern of telomere length and telomerase expression in cancer tissues and the surrounding mucosa (SM) as markers of field cancerization and clinical outcome in patients successfully treated for HNSCC.

Materials and methods

Patients

In total, 139 untreated consecutive patients with histologically confirmed SCC of the UADT were included in this prospective cohort study (Supplementary Tables S1 and S2). Patients underwent treatment from 2009 to 2012. Patients with nasopharyngeal carcinoma were excluded from this study.

A multidisciplinary team decided on treatment planning, mainly according to TNM staging, irrespective of HPV status. Most T1 and T2 SCC were treated with functional-preserving surgery or definitive radiotherapy, while most patients with T3 or T4 SCC underwent radical surgery often followed by post-operative (chemo)-radiotherapy or concurrent chemoradiation. The local institutional review board approved the study protocol, and all patients gave their informed consent. Two samples were collected from each patient at the time of surgical resection or biopsy, one from a non-necrotic area of the carcinoma, and the other from SM.

Only patients who underwent complete surgical resection with clear margins (distance > 5 mm between carcinoma and margins) and achieved complete clinical and radiological response to (chemo)-radiotherapy were included in the study. The routine follow-up program consisted of locoregional examination at 2-month intervals during the first year, 3-month intervals in the second year, 4-month intervals between the third and fifth years, and every 6 months thereafter.

The median age of patients was 66 (range 27–95 years); 103 were men (74.1%) and 36 women (25.9%). The site of origin of the carcinoma was the larynx in 46 cases (33.1%), the oral cavity in 45 (32.4%), the oropharynx in 30 (21.6%) and the hypopharynx in 18 (12.9%). One hundred and three patients (74.1%) were ever smokers. According to the American Joint Committee on Cancer TNM 2002 [36], 91 patients (65.5%) had advanced disease (stage III or IV). All tumors were tested for HPV DNA sequences by PCR assays, as previously described [37], and nine (6.5%) were high-risk HPV-positive (HPV-16 in eight cases and HPV-58 in one). In all patients with HPV-positive tumors, the SM was HPV-negative.

Tissue samples

Surgeons were requested to biopsy the surrounding epithelium in the uninvolved mucosa at 4–8 cm from the tumor margins depending on anatomical condition. Precautions were taken not to contaminate the SM with tumor samples by changing surgical blades each time before cutting tissue. Both samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Cryostat sections, 6 μm thick, from each tissue sample were prepared using a 1720 Digital cryostat (Leitz, Germany). One section of each sample was stained with haematoxylin-eosin for histopathology. Surrounding mucosa was histologically assessed and in all cases no histopathological alterations were found. DNA was extracted by the standard phenol/chloroform method and RNA was extracted with Trizol reagent (Invitrogen).

Telomere length analysis

Telomere length was determined by real-time polymerase chain reaction (PCR), exactly as previously described [38,39]. Briefly, two PCRs were performed for each sample, one to determine the cycle threshold (Ct) value for telomere (*T*) amplification with the primer pair TEL1B and TEL2B [40], and the other to determine the Ct value for the amplification of a single-copy (*S*) control gene (acidic ribosomal protein P0, RPLP0) with the primer pair RPLP01 and RPLP02 [41]. Each sample was run in triplicate and each PCR reaction was performed using 10 μl of DNA sample (1 ng of DNA per μl) in 50 μl final reaction volume. A reference curve, consisting of fivefold serial dilutions of the reference DNA from the RAJI cell line, was generated at each PCR run, as previously described [38]. All PCR reactions were carried out in 96-well plates using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). Intra- and inter-assay reproducibility of both telomere and RPLPO PCR results have been evaluated using dilutions of the reference curve [38]. Mean Ct values were used to calculate the relative telomere length using the telomere/single copy gene ratio (*T*/*S*) according to the formula: $\Delta Ct_{sample} = Ct_{telomere} - Ct_{control}$, $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference\ curve}$ (where $\Delta Ct_{reference\ curve} = Ct_{telomere} - Ct_{control}$) and then $T/S = 2^{-\Delta\Delta Ct}$ [38,40].

Quantification of TERT transcripts

Sample sizes from both cancers and SM were sufficient to allow RNA extraction in 90 cases (Supplementary Tables S1 and S2). RNA samples were reverse transcribed on cDNA using the SuperScript TM III RNase reverse transcriptase assay (Invitrogen) [42]. The expression of TERT transcripts was quantified by real-time PCR using an ABI prism 7900 HT Sequence Detection System (Applied Biosystems). Absolute quantification was carried out with fivefold dilutions of TERT amplicon as a reference curve for TERT copies, and fivefold dilutions of housekeeping hypoxanthine–guanine