

## RESEARCH COMMUNICATION

# **hTR RNA Component as a Marker of Cellular Proliferation in Oral Lichen Planus**

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### **Abstract**

Previously, we have shown that the telomerase RNA component hTR is highly expressed in the epithelium of non-dysplastic Oral Lichen Planus (OLP) lesions (11). We concluded that it is possible that this high expression might be related to the increased cellular proliferation seen in OLP rather than being an indicator of potential malignant transformation. In the present study, and in order to confirm our finding in the previous study that hTR might be a marker for cellular proliferation in OLP, we analysed OLP biopsies known to be positive for RNA component of Telomerase (hTR) for the expression of Ki-67 as a marker for cellular proliferation. Fourteen OLP tissue biopsies known to be positive for telomerase RNA component hTR, were investigated using an immunohistochemical approach to determine the rate of cellular proliferation in OLP, looking at the expression of Ki-67 protein as a marker for cellular proliferation. A statistically significant increase was found between Ki-67 expression in OLP in comparison to normal control buccal mucosa samples. The expression of hTR component in OLP might thus be a marker for cellular proliferation.

**Key Words:** Oral Lichen Planus - telomerase RNA component - hTR - marker - cellular proliferation

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### **Introduction**

Oral lichen planus (OLP) is a common chronic inflammatory oral disorder. A number of studies have shown that OLP has a malignant potential: the rate of malignant transformation in OLP has been reported to be between 0.4 and 5.6% over ten years (Abbate et al., 2006). The WHO considers OLP to be a premalignant condition (Holmstrup, 1992).

Human telomeres are regions of tandem (TTAGGG)n repeats at chromosomal ends that protect chromosomes from degradation, fusion and recombination (Moyzis et al., 1988). Telomerase maintains telomere length during cell division (Vaziri and Benchimol, 1998). Increased telomerase activity is thought to be a key event in the immortalisation of cells and has been detected in many transformed cell lines, precancerous lesions and carcinomas (Kim et al., 1994). There are three major subunits comprising the human telomerase complex. The RNA component of human telomerase (hTR) (Feng et al., 1995), telomerase associated protein (TP1) (7) and the catalytic subunit, human telomerase reverse transcriptase (hTERT) (Harrington et al., 1997). Telomerase activity has been detected in immortalised and cancer cell lines, germ line cells and most human malignancies including head and neck squamous cell carcinomas (Kim et al., 1994;

Mutirangura et al., 1996; Chang et al., 1999).

Previously, we have shown that the telomerase RNA component hTR is found to be highly expressed in the epithelium of non-dysplastic OLP lesions (O'Flatharta et al., 2002). We concluded that it is possible that this high expression might be related to the increased cellular proliferation seen in OLP rather than being an indicator for potential malignant transformation. In the present study, and in order to confirm our finding in the previous study that hTR might be a marker for cellular proliferation in OLP as well as malignant transformation, we analysed OLP biopsies known to be positive for RNA component of telomerase (hTR) for the expression of Ki-67, a marker for cellular proliferation. The expression of Ki-67 in OLP biopsies samples was detected using an immunohistochemical technique. The expression patterns of Ki-67 in OLP tissue samples were compared to the expression of hTR in the OLP tissue samples.

### **Materials and Methods**

#### *OLP clinical data*

Fourteen OLP tissue biopsies known to be positive for hTR expression, were used to investigate the rate of proliferation in OLP. The relevant clinical data are shown in Table 1. Seven normal buccal mucosal tissue samples

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were used as controls. Ethical approval was obtained.

The OLP cases were selected on the basis of classical and uncontentious histopathological appearance. Patients from which the biopsies were taken are under regular clinical review. The mean follow up period is 4.4 years (range 4-6 years). At the time of publication, none of the patients have developed an oral malignancy.

#### *RNA in-situ hybridisation detection of hTR*

In a previous study, we developed a novel RNA *in-situ* hybridisation technique, in order to localise the expression of the RNA component (hTR) of the telomerase ribonucleoprotein in formalin-fixed paraffin-embedded OLP tissue sections (O'Flatharta et al., 2002). Briefly our *in situ* hybridization technique was carried in the following consecutive steps:

#### *Probe preparation*

The telomerase hTR gene fragment (gift from Dr. Nicole Keith, University of Glasgow) was subcloned into PCR script vector containing both T3 and T7 promoter. PCR amplification was performed using primers which were designed to amplify the fragment of human telomerase flanked by the T3 and T7 promoters as described in our previous report (O'Flatharta et al., 2002). Probe labelling was performed as described by the manufacturer (Boehringer Mannheim/ Roche Molecular Biochemicals, East Sussex, U.K.), using T3 RNA polymerase and T7 RNA polymerase, in order to create the antisense and sense probes respectively from the above PCR products. The labelled RNA was precipitated by the addition of 4M LiCl and was further purified by passing it through a Nick Column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as described by the manufacturer. The integrity of the RNA probe was verified by electrophoresis on a 1% BSSE gel and visualized by ethidium bromide staining (O'Flatharta et al., 2002).

#### *RNA in-situ hybridisation*

The RNA *in situ* hybridization was carried out as described in our previous report (O'Flatharta et al., 2002). Visualisation of digoxigenin labelled probes was carried out as described by the manufacturer using the Digoxigen Nucleic Acid Detection Kit™ (Boehringer Mannheim/ Roche Molecular Biochemicals) according to manufacturer's instructions, with one modification, which involved second one-hour incubation in the anti-digoxigenin (O'Flatharta et al., 2002).

#### *Immunohistochemical detection of Ki-67 expression*

The OLP and normal controls tissue sections were dewaxed in two changes of xylene, cleared in two changes of alcohol and brought to water. Endogenous peroxidase activity was blocked by incubating the sections in hydrogen peroxide solution for 15 min. Antigen retrieval was carried out using sodium citrate and a pressure cooking protocol. Briefly, tissue sections were placed in a 0.01 M sodium citrate pH6 solution (at boiling point) and placed in a pressure cooker for 90 secs. Next, the slides were cooled in H<sub>2</sub>O, the sections were washed twice in 1X PBS and blocked in normal horse serum (Vector

**Table 1. Clinical Data for OLP patients Analysed for the hTR RNA Component of Telomerase and Ki-67**

Sample	Clinical Variant	Biopsy Site	Age*	Sex	Follow-up*
1	Plaque	Tongue	37	F	4
2	Erosive	Buccal mucosa	63	F	5
3	Reticulopapular	Buccal mucosa	52	F	4
4	Reticulopapular	Buccal mucosa	62	F	4
5	Plaque	Buccal mucosa	42	F	5
6	Plaque	Tongue	39	F	5
7	Reticulopapular	Alveolar ridge	50	F	6
8	Plaque	Lower lip	55	F	6
9	Reticulopapular	Buccal mucosa	51	M	6
10	Desquamitive gingivitis	Gingiva	32	M	6
11	Reticulopapular	Buccal mucosa	34	M	4
12	Reticulopapular	Buccal mucosa	41	M	6
13	Erosive/Reticular	Buccal mucosa	40	M	4
14	Erosive	Buccal mucosa	35	M	6

Laboratories, Burlingame, CA) for 10 min. The blocking solution was then drained from the sections and a 1:150 dilution of the Ki-67 primary antibody (MIB-1 clone, Dako Corporation, Carpinteria, CA), diluted in 1X PBS, was placed on the sections for 30 min. The sections were then washed twice with PBS (4 min each wash). The PBS was then drained off, any excess wiped away and the sections were covered with Vector Elite™ secondary antibody and incubated for 30 min. The tissue was washed three times for 5 min each in PBS and incubated with the ABC complex from the kit for 30 min at room temperature according to the manufacturer's instructions. Following this, slides were washed as before and exposed to diaminobenzidine chromagen (DAB) (Sigma Chemical Company Ltd., Dorset, UK) for 5 min. Sections were counterstained with haematoxylin, washed briefly in tap water and cleared with xylene (3 separate washes). The slides were then mounted in DPX mounting media (BDH Laboratory Supplies, Poole, UK) and analysed microscopically. Negative control OLP tissue samples were included in each experiment in which the Ki-67 primary antibody was omitted, and human tonsillar tissue was included in each experiment as a positive control.

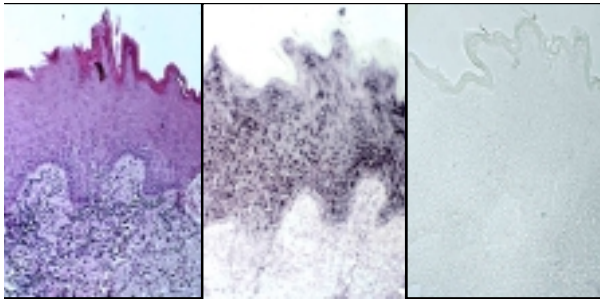
#### *Counting of Ki-67 positive cells and statistical analysis*

The level of Ki-67 expression was determined by counting the number of positive cells, with nuclear staining, in a 100 cell stretch of basal cells and one layer above the basal cell layer. All counts were done in triplicate. A 2-tailed Student's t-test was used to check for statistically significant Ki-67 expression in OLP tissues using the SPSS 11.0 analysis package (SPSS Inc., Chicago, IL).

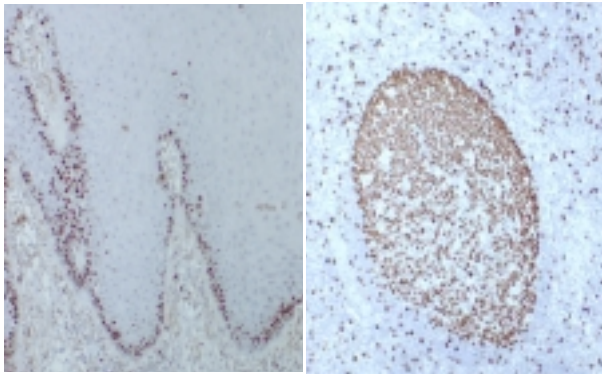
## Results

#### *Detection of hTR expression in test tissue*

All the OLP tissue samples used were confirmed histopathologically (Figure 1 A). The telomerase antisense RNA probe gave clear blue/purple staining in the epithelial cytoplasm of OLP tissue samples (Figure 1 B) and positive controls. Using the negative control telomerase sense RNA probe, no staining of any kind was seen in the



**Figure 1. In-situ Hybridisation Detection of Telomerase hTR RNA Expression.** (A) Haematoxylin and eosin stained OLP tissue biopsy. (B) hTR expression detected in the corresponding OLP biopsy using antisense hTR digoxigenin-labelled RNA probe with characteristic blue/purple staining in the basal and suprabasal layers. (C) No staining present with the hTR sense probe.



**Figure 2. Ki-67 Expression.** (A) Basal layer of an OLP lesion. (B) Germinal center of tonsillar tissue positive control epithelial cells of OLP (Figure 1 C).

#### *Immunohistochemical analysis of proliferation in OLP and normal control buccal mucosa tissue samples*

Ki-67 expression was detected in sequential sections in the basal and parabasal layers of OLP (Figure 2A). No staining was detected in all negative control OLP tissue samples, in which the Ki-67 primary antibody was omitted. Ki-67 expression was also detected in the active germinal centres of tonsillar tissue, which was used as a positive control in all experiments (Figure 2B). A few epithelial cells stained positive for Ki-67 in the basal layer of normal control buccal mucosa .

#### *Statistical analysis*

A statistically significant increase was found between Ki-67 expression in OLP in comparison to normal control buccal mucosa samples ( $p < 0.01$ ) using the 2-tailed Student's t-test.

## **Discussion**

The finding that telomerase activity is present in most malignant tumours while absent in most somatic cells has led to the hypothesis that re-expression of telomerase is critical for continued tumour cell growth (Rhyu, 1995).

A great deal of research has been conducted using the TRAP assay (Telomere Repeat Amplification Protocol), RT-PCR and in-situ hybridisation to elucidate the correlation between both telomerase activity with the

clinical and pathological findings of pre-cancerous and malignant lesions. The hTERT catalytic subunit of telomerase is also found to be over-expressed in many different types of cancer. Examples of these cancers include; colorectal cancer (Kammori et al., 2002), squamous cell carcinomas of the head and neck (Chang et al., 1999), cervical intraepithelial neoplasia (Jarobe et al., 2002), bladder cancer (Melissourgous et al., 2003), breast cancer (Kirkpatrick et al., 2004) and bronchial squamous cell carcinoma *in situ* (Snijders et al., 2004). Activation of telomerase is considered to be a critical step in the continuous proliferation of cancer cells and therefore telomerase may be considered as a marker for tumour progression (Kim et al., 1994; Snijders et al., 2004).

The Ki-67 antigen is present during all active phases of the cell cycle (G1, S, G2 and M-phases) but is absent in resting cells (G0). As such it is generally accepted to be a good marker for proliferating cells (Hitchcock, 1991).

In a previous study, we have shown that a high percentage of OLP expressed the telomerase RNA component, hTR (O'Flatharta et al., 2002). We hypothesized that hTR could be a marker for cellular proliferation in OLP as well as potential malignant transformation. In order to support the finding in our previous study, we looked at the expression of Ki-67 in those OLP tissue samples known to be positive for hTR by in situ hybridisation. We have shown, using the Ki-67 immunohistochemical marker, that OLP has an increased proliferation rate compared with normal control oral mucosa.

Although telomerase activity was thought to be associated mainly with the malignant phenotype, evidence has accumulated that its activity may also be related to cellular proliferation (Belair et al., 1998). Assessment of proliferative activity is possible by immunohistochemical staining with monoclonal antibodies against Ki-67 (Girod et al., 1998). Although variations in the site of Ki-67 staining have been reported in individual epithelial cells (both nuclear and cytoplasmic), all nuclei were scored as positive regardless of staining intensity because cytoplasmic staining may occur in normal cells and loss of antigenicity of the protein may be the result of tissue processing (Scott et al., 1988; Rijzewijk et al., 1989).

It is possible that the increased expression of the telomerase RNA component in OLP may be evidence of the hyperproliferative state as shown by the determination of growth fraction. A recent study investigated the proliferative activity of oral tissue specimens using Ki-67 immunohistochemistry and found that the Ki-67 labelling indices of dysplastic leukoplakia and oral squamous cell carcinoma specimens with high telomerase activity were significantly higher than those specimens with low or no telomerase activity (Liao et al., 2000). Ogoshi et al (1998) detected hTR expression amongst tumour cells, and proliferating cells. This finding supports our finding and the concept that proliferating and non-proliferating cells can express hTR. They also noted that cells at the periphery of squamous cell carcinoma clusters express more hTR than the more differentiated cells at the centre mimicking the pattern seen in non-malignant epithelium. A similar scenario was observed in our study

whereby hTR expression was noted in the basal, suprabasal and in some cases upper epithelial layers while expression of the Ki-67 proliferation marker was confined to more basal cells in general. This suggests that not only proliferation but also the differentiation state may play a role in hTR expression.

Studies on telomerase activity in cancers in tissues such as the stomach, liver, breast and prostate indicated the usefulness of telomerase activity as a prognostic marker and also proposed that the presence of telomerase activity could be used as an early detection marker to distinguish malignant and benign tissues (Hiyama et al., 1995; Tahara et al., 1995; Sommerfeld et al., 1996).

Although the sample number is relatively small, the detection of RNA component (hTR) in a high percentage of the OLP tissue samples and the high co-expression of Ki-67 in OLP suggests that hTR may reflect proliferation as well as potential malignant transformation.

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