Expression of novel p53 isoforms in oral lichen planus

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Summary Oral lichen planus (OLP) is a chronic inflammatory disease of unknown origin, showing little spontaneous regression. WHO classifies OLP as a premalignant condition, however, the underlying mechanisms initiating development of cancer in OLP lesions are not understood. The p53 tumour suppressor plays an important role in many tumours, and an increased expression of p53 protein has been seen in OLP lesions. Recently it was shown that the human TP53 gene encodes at least nine different isoforms. Another member of the p53 family, p63, comprises six different isoforms and plays a crucial role in the formation of oral mucosa, salivary glands, teeth and skin. It has also been suggested that p63 is involved in development of squamous cell carcinoma of the head and neck (SCHN). In contrast to p53, a decreased expression of p63 protein has been seen in OLP lesions. In this study, we mapped the expression of five novel p53 isoforms at RNA and protein levels in OLP and matched normal controls. In the same samples we also measured levels of p63 isoforms using quantitative RT–PCR. Results showed p53 to be expressed in all OLP lesions and normal tissues. The p53β and Δ133p53 isoforms were expressed in the majority of samples whereas the remaining three novel isoforms analysed were expressed in only a few samples. Levels of p63 isoforms were lower in OLP lesions compared with normal tissue, however, changes were not statistically significant.

Introduction Oral lichen planus (OLP) is a chronic inflammatory disease of skin and mucosa affecting approximately 1–2% of the adult population. The disease occurs more frequently in women.
than in men (1.4:1) and is more common in middle aged and elderly than in young people. In contrast to lichen of the skin, OLP seems to have a chronic cause with low tendency to spontaneous regression and high resistance to topical treatment. Despite numerous studies the origin remains unknown. However, an autoimmune cause has been suggested and we have recently demonstrated occurrence of auto antibodies against p63 and p73 in sera from patients diagnosed with OLP.

OLP lesions have a higher cell turnover than normal tissue and WHO classifies OLP as a premalignant condition making it a marker for oral malignancy, but the progression to oral cancer is unclear. The risk of developing squamous cell carcinoma of the head and neck (SCCHN) compared to a control group. The underlying mechanisms initiating development into SCCHN have not been clarified, however, an association between chronic inflammation and a variety of cancers is known.

Approximately two decades ago the p53 tumour suppressor was discovered. Activation of p53 after DNA damage or oncogenic signalling is an important protective mechanism enabling DNA repair, induction of cell cycle arrest or apoptosis. Mutation within the human TP53 gene is found in a majority of all human tumours studied and a high prevalence of TP53 mutations has been seen in patients diagnosed with OLP, particularly females. We and other investigators have previously shown an increased expression of p53 protein in OLP lesions compared with normal tissue.

Recently it was shown that human p53 encodes at least nine different isoforms through alternative splicing, alternative initiation sites for translation and the use of an internal promoter: p53, p53\(\alpha\), p53\(\gamma\), \(\Delta\)p53\(\alpha\), \(\Delta\)p53\(\beta\), \(\Delta\)p53\(\gamma\), \(\Delta\)p53\(\beta\), \(\Delta\)p53\(\gamma\) and \(\Delta\)p53\(\alpha\). In the first study of these novel p53 isoforms in SCCHN it was shown that p53 variant mRNAs were expressed in both normal oral epithelium and SCCHN. The most common of these isoforms being p53\(\alpha\), which was detected in the majority of samples. Of the other isoforms studied all but \(\Delta\)p53 could be detected in at least some samples from tumour and normal epithelium.

The p53 relative p63 is encoded by a gene located on chromosome 3q27–29. In humans this gene encodes six different isoforms: TAp63\(\alpha\), TAp63\(\beta\), TAp63\(\gamma\), \(\Delta\)Np63\(\alpha\), \(\Delta\)Np63\(\beta\) and \(\Delta\)Np63\(\gamma\). p63 is essential for development of ectodermal structures, and p63 \(-/-\) mice lack oral mucosa, skin, teeth, hair follicles, salivary and mammary glands. The exact function of each individual isoform is, however, not clear. It is known that each of the p63 proteins have different characteristics and functions, where some resemble its relative the tumour suppressor protein p53, while others have the opposite function. We have previously shown a decreased expression of the different p63 proteins in OLP lesions compared with normal oral mucosa. Concerning RNA levels of the different isoforms, no data is as of today available.

The aim of the present study was to clarify if the observed increased expression of p53 protein seen in OLP lesions represents a mixture of the different novel p53 isoforms or the original p53 isoform only. Furthermore we wanted to see in the same samples, if there was any correlation between RNA levels of the different p63 isoforms, and the decrease in protein expression that we have previously seen in these lesions. In order to judge whether OLP in this aspect resembles normal or tumour tissue, normal matched control tissue was analysed in parallel, and results compared with data from analysis of SCCHN.

**Materials and methods**

**Patients and specimens**

Two 4 mm punch biopsies were, after informed consent, taken from the buccal mucosa on 20 consecutive patients with OLP referred to the specialist clinic at the department of Odontology, Umeå University. All patients were clinically and histologically diagnosed with oral lichen planus. Fourteen were females with a mean age of 63.5 (range 42–78) and six were males with a mean age of 59.5 (range 48–75). Biopsies were also collected from twenty sex and age matched healthy controls. All samples were immediately frozen and stored at \(-80\)°C. Permission for the study had been granted by the Ethical Committee at Umeå University (dnr 05-010 M).

**RNA extraction**

One of the frozen biopsies was cut into pieces in a Petri dish placed on dry ice and transferred into a 1.5 ml tube containing 250 \(\mu\)l TRIzol reagent (Invitrogen). Samples were homogenized using a pellet mixer (MARCK Eurolab), a further 750 \(\mu\)l of TRIzol added and samples incubated at room temperature for 5 min. Mixtures were then centrifuged for 15 min at 13,000 rpm at 4°C. The upper liquid phase was moved to a new tube, diluted with an equal amount of isopropyl alcohol, incubated for 10 min and again centrifuged for 10 min at 4°C. The upper layer was carefully removed and pellets washed in 70% ethanol before drying and dissolved in DePc-H\(_2\)O by pipetting 10–20 times, heated to 60°C for 10 min and pipetted another 10–20 times. Extracted RNA was then kept at \(-80\)°C until use.

**cDNA synthesis and RT–PCR for analysis of p53 isoforms**

cDNA was synthesized using a Cloned AMW First-Stand cDNA Synthesis kit (Invitrogen) according to the manufacturer’s instructions. One microgram of RNA was added into a total reaction volume of 20 \(\mu\)l. The cDNA was amplified in two rounds of PCR (nested PCR) in order to detect the different isoforms of p53. The following p53-specific primer sets were used: for the first PCR-round of p53, p53\(\beta\) and p53\(\gamma\): e2/1/RT1. For \(\Delta\)p53\(\beta\) and \(\Delta\)p53\(\gamma\); and \(\Delta\)p53\(\gamma\): i4f1/RT2 were used. For the nested PCR the primers for p53 were: e2/RT2; for p53\(\beta\): e2/p53b; for p53\(\gamma\): e2/p53g; for \(\Delta\)p53\(\beta\): i4f2/RDNp53; for \(\Delta\)p53\(\gamma\): i4f2/p53b, and for \(\Delta\)p53\(\gamma\): i4f2/p53g.
The PCR programme comprised 35 cycles with 94 °C for 30 s, 60 °C for 45 s and 72 °C for 90 s. PCR products were loaded on a 1% agarose gel. All samples were done in duplicate and most of them in triplicate, and considered positive when positive in all replicates, or positive/variable when isoforms were detected in at least one PCR run.

Quantitative PCR for analysis of p63 isoforms

Quantitative RT–PCR for analysis of p63 isoforms was performed using a human p63/β-actin multi-parametric kit (Search LC, Heidelberg, Germany) and analysed on a LightcyclerTM from Roche. In brief, cDNA was diluted 12 times (Search LC, Heidelberg, Germany) and analysed on a LightcyclerTM with one denaturation cycle, 50 amplification cycles, one melting curve analysis cycle, and, finally, one cooling cycle. Reactions were run in duplicate, and a mean value of the two samples was calculated.

Statistical analysis

For analysis of p63 mRNA levels in OLP lesions and corresponding clinically normal oral mucosa an independent samples 7-test was performed using Computer software SPSS version 12.0.

Protein extraction

From one frozen biopsy protein was extracted using a Microdismembrator (B. Braun, Biotech International) for pulverizing the sample. One hundred microliter of lysis buffer containing 0.5% NP-40, 0.5% Na-doc, 0.1% SDS, 150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM NaF and protease inhibitor cocktail (Sigma-Aldrich Chemie, Steinheim, Germany) was added and the pulverized samples homogenized. Protein concentrations were determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Immunoblot analysis and quantification

Bio-Rad PROTEAN II xi | XL Vertical Electrophoresis system or vertical mini-gels were used according to the user manual. Thirty microgram of protein was mixed with 2× loading buffer, boiled for 10 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Little Chalton, NA, USA) and stained with ponceau red for evaluation of transfer efficiency and loading. Membranes were blocked in 5% milk with 0.4% Tween in 1X PBS, and antibodies diluted in the same blocking solution. Primary antibodies used in this study were DO-1 for detection of p53, p53β, p53γ,27, and DO-12 for detection of p53, p53β, p53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δ40p53, Δ40p53β, and Δ40p53γ isoforms.28

Results

Expression of p53 isoforms using RT–PCR

By the use of RT–PCR, six different p53 isoforms could be amplified from OLP tissue as well as normal tissue: p53, p53β, p53γ, Δ133p53, Δ133p53β, and Δ133p53γ. All samples were analysed as matched pairs, meaning that one OLP lesion and corresponding matched control were analysed in parallel, though coded, as to hide which sample was lesion and which was control. p53 mRNA could be detected in replicate analyses of all but 1 of the 20 OLP samples as well as all but 1 of the 20 controls. These two samples were positive in one analysis, but negative in the other. The majority of OLP and control samples also expressed the p53β and Δ133p53 isoforms in at least one of the replicate analyses. Expression of p53γ, Δ133p53β and Δ133p53γ was, however, limited to only a few samples. In general the novel p53 isoforms studied were detected in fewer OLP samples compared to matched controls (Fig. 1).

Expression of p63 isoforms

Using quantitative RT–PCR analysis of p63 showed that p63α had highest and ΔNp63 second highest level of all p63 mRNAs. TAp63 had lowest levels in both OLP and corresponding normal tissue. For all isoforms individual variations could be seen (results not shown). Results also showed a general decrease in levels of p63 isoforms in OLP compared to normal tissue. The most pronounced decrease seen in expression of p63γ, p63α and ΔNp63 (P = 0.116; 0.187 and 0.205, respectively). The level of TAp63 was unchanged while a slight reduction in p63β was found in OLP lesions compared to normal tissue. None of these changes were, however, statistically significant (Fig. 2).

Primer sequences : e2.1 (5'-GTCACTGCCATGGAGGAGGCCGCA-3')
e2 (5’-ATGGAGGACCGCAGTAGAT-3')
i4f1 (5’-CTGAGGTTGAGCGCAACTCTCTCTAG-3')
i4f2 (5’-GCTAGTGGGGTTGAGGAGTTACAC-3')
RT1 (5’ – GACGCAACCTATTGAAGCAGGATTTC-3')
RT2 (5’-AATGTGACTCTGAGTCCGCTTCGTC-3')
p53b (5’-TTTGAAAGCTGGTCTGGTCCTGA-3')
p53g (5’-TCGTAAGTCAAGTAGCATCTGAAGG-3')
RDNP3 (5’-CTCACGCCACGGATCTGA-3')
Immunoblot analysis

In order to detect different p53 isoforms also at protein levels, two antibodies were used. DO-1 recognising an epitope located at amino acids 20–25, and therefore not recognising the N-terminal truncated Δ133p53 or Δ40p53 isoforms and DO-12 recognising the p53, p53β, p53γ, Δ133p53, Δ133p53β, Δ133p53γ, Δ40p53, Δ40p53β, and Δ40p53γ isoforms. All samples were run in duplicate and results showed detectable levels of p53 protein in only one of the OLP samples. This protein had an identical molecular weight to p53 and bands that might represent p53-isoforms were not detected (data not shown).

Discussion

Increased expression of the p53 tumour suppressor protein has been seen in studies of oral lichen planus, simultaneously with a decreased expression of its sibling p63. With the discovery of several novel p53 isoforms we therefore wanted to see if this increased expression of p53 protein in fact represented different isoforms which could be important in OLP lesions. By using RT/PCR we could detect three of the isoforms in the majority of OLP and control samples, whereas the remaining three isoforms were detected in only a few samples. In a previous study of p53 isoforms in SCCHN we have shown that the method used,
nested RT/PCR, is very sensitive and identifies extremely low levels of mRNA. This was clearly shown when comparing RT/PCR results with just one single round of PCR. Using the single round PCR only the p53, p53β and Δ133p53 isoforms were detected.20 These three isoforms were also the ones that could be amplified in the majority of our samples. The inconsistencies seen between replicates of the same samples, using different batches of cDNA, is, as in the study of SCCHN, compatible with low levels of the novel p53 isoform mRNAs. Also, based on the comparatively higher inconsistency in amplification of the p53β and Δ133p53 isoforms seen in this study, we suggest that levels of these isoforms are in fact much lower in OLP lesions compared to tumour- and clinically normal tumour adjacent tissue in patients with SCCHN. This could in turn indicate a more tumour related role for these isoforms. The fact that we in the present study used three times as much RNA for cDNA synthesis, 1 μg of total RNA compared to 300 ng from the tumours, further supports the theory that levels of the novel p53 isoforms really are lower in OLP lesions compared to SCCHN.

By using immunoblotting and antibodies detecting p53/p53β/p53γ and all isoforms analysed respectively, only the p53 protein could be detected in one of the OLP samples, whereas around half of the tumours and a little fewer of the normal tumour adjacent tissue had detectable levels of p53 protein.20 However, none of the novel p53 proteins were expressed at detectable levels in the present or previous study. It should be kept in mind that wild type p53, and maybe also these novel isoforms, is predominantly regulated by post-translational mechanisms, and therefore a direct correlation to RNA levels cannot be expected.29 However, with the reagents available today, none of the novel isoforms could be detected. Other possible explanations could be short half life of the isoforms, in concordance with wild type p53, or tissue specific expression. In the latter case translation of RNA is dependent on tissue specific cofactors and with these absent, RNA is not translated into protein.

For the p53 sibling p63 we could see a down regulation of all isoforms at the RNA level in OLP lesions, though not significant. This is in clear contrast to SCCHN tumours where levels of all isoforms were upregulated compared to clinically normal tumour adjacent tissue. Levels of p63β and ΔNp63 further showed a statistically significant upregulation.20 In OLP lesions levels of the TaP63 isoforms were unchanged compared to the controls (0.0010 versus 0.0011). A discrepancy between RNA and protein levels for the TA isoform specifically has been shown previously, for example in psoriasis lesions.30

As chronic inflammation is known to cause DNA damage, the decreased levels of p63 seen in the chronic inflammatory condition OLP are comparable to changes seen in epithelia after exposure to UV light. In this DNA damaged epithelium decreased ΔNp63 expression is needed for induction of apoptosis.31 This may, as previously suggested, indicate a protective mechanism enabling removal of DNA damaged cells in OLP.18

In conclusion, we have been able to detect RNA of 3 p53 isoforms in the majority of OLP lesions and normal controls analysed. Two of these belong to the group of recently detected novel p53 isoforms. The function of these novel isoforms is, so far, not known but the same isoforms have also been amplified in samples of SCCHN, at seemingly higher levels. This could indicate a more tumour related role for these isoforms. None of them could, however, be detected at protein level with the reagents available today. Therefore, in order to clarify the role of these novel p53 isoforms in OLP lesions, further studies with preferably more isoform specific reagents are needed.

Conflict of interest statement

None declared.

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