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Detection of antibodies against p63 and p73 isoforms in sera from patients diagnosed with oral lichen planus

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BACKGROUND: Oral lichen planus (OLP) is a chronic inflammatory disease of oral mucosa. Despite numerous publications and intense research, the etiology of OLP is still unknown, however, autoimmunity as a possible causative factor has been discussed.

METHODS: In the present study sera from 20 patients clinically and histologically diagnosed with OLP were analyzed for antibodies directed toward p53, p63, and p73 using Western blot.

RESULTS: Sera from two patients reacted with all six p63 isoforms, and one also with p73. The strongest reaction was noted against the TAp63β protein, which is the most potent transactivator of all p63 proteins and is implicated in the differentiation of stratified epithelia.

CONCLUSIONS: This is the first demonstration of antibodies directed against all p63 and some p73 isoforms in sera from patients diagnosed with OLP.

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Introduction

Oral lichen planus (OLP) is a chronic inflammatory disease of the mucosa and skin, first described in 1869 (1). It is more common in females than in males (1.4:1) and affects approximately 1–2% of the adult population, mainly middle aged and elderly (2). In contrast to lichen of the skin, OLP has a tendency to show a chronic course with little spontaneous regression (3). The etiology of OLP is not known although an autoimmune cause has been suggested. In keeping with an autoimmune etiology, OLP can be associated with other autoimmune diseases such as alopecia areata, myasthenia gravis, ulcerative colitis, and vitiligo (4). In OLP, it has been suggested that $CD8^+$ cells induce apoptosis in epithelial cells within the lesion (5). Hepatitis C virus infection in OLP patients does, however, not give rise to development of autoantibodies (6). Parodi and Cardo demonstrated autoantibodies against a nuclear antigen in epithelial cells in two cases of erosive OLP supporting the theory of an autoimmune nature of OLP (7).

TP53 is a tumor suppressor gene that is mutated in more than 50% of human tumors. It is the most intensely studied gene in cancer etiology (8). Activated wild-type p53 protein induces apoptosis or cell cycle arrest of DNA-damaged cells and thus prevents formation of cancer (9). Until 1997, p53 was thought to be a unique protein but then two new family members were identified and named p63 and p73 (10–13). The *p63* gene is located on chromosome 3q27-29, and encodes six proteins with homology to p53 (11, 14). The structure of the different p63 proteins is similar to p53 with an N-terminal transactivation domain, a DNA-binding domain in the central part and an oligomerization domain close to the C-terminus (11). The three full-length proteins (TAp63α, TAp63β, and TAp63γ) contain a transactivation domain in the N-terminus whereas the other three proteins lack the N-terminus transactivational domain (ΔNp63α, ΔNp63β, and ΔNp63γ) (Fig. 1). Our previous data showed that the N-terminal-truncated ΔNp63 isoforms are restricted to the epithelium whereas the full-length TAp63 proteins are expressed in the epithelia and in other tissues such as the lymphoid and endothelial tissues (15, 16). The exact function of the different p63 isoforms is not completely understood, but it is clear that they play a crucial role in the development of normal oral epithelium, skin, etc (11). The third member of the p53 family is p73. The *TP73* gene has at least two distinct promoters (17) and like p63 produces multiple isoforms as a result of alternative splicing (8) (Fig. 1). Similar to p63, some p73 isoforms have functions similar to p53 (8).

The p63 proteins are crucial for formation of the oral mucosa. One of the isoforms, ΔNp63α, is also called chronic ulcerative stomatitis protein (CUSP) as autoimmunity against this protein has been seen in patients with chronic ulcerative stomatitis (CUS) (18). CUS is a

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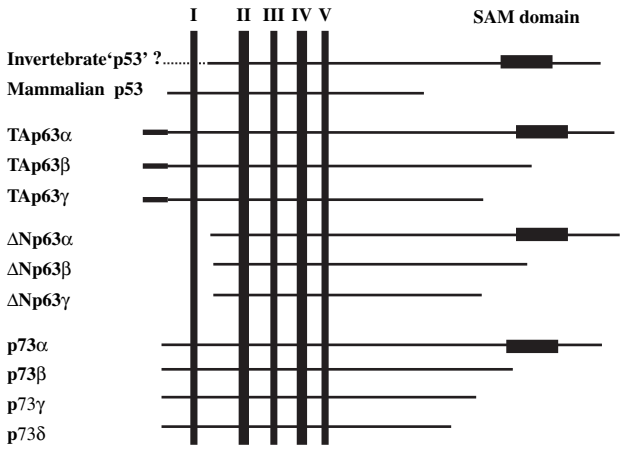

Figure 1 Schematic drawing of the p53 family proteins. Through alternative splicing at the C-terminus, and the presence of two promoters, six different p63 isoforms are formed. The p73 family consists of more than four proteins, but only p73α, p73β, and p73δ were included in the present study. Roman numbers denote highly conserved domains throughout evolution (27).

disease which clinically appears as desquamative gingivitis or as ulcerations of the tongue or buccal mucosa (19) resembling ulcerative lesions seen in erosive lichen planus (20). We have previously, in accordance with another study, shown a decreased expression of p63 protein in OLP and graft vs. host disease lesions (21, 22). This finding, together with the fact that one isoform, ΔNp63α, has been shown to be the target of autoantibodies in some patients suffering from a condition resembling OLP, made it interesting to investigate the role for p63 as a target for potential autoantibodies in a larger series of patients with OLP. We also mapped the particular isoforms of p63 that are recognized, and investigated for the first time the occurrence of autoantibodies against its siblings, p73 and p53, in patients with OLP.

Materials and methods

Material

Sera from 20 consecutive patients referred to the OLP specialist clinic at the Department of Odontology, Umeå University were collected after informed consent was obtained. All patients were clinically and histologically diagnosed with OLP. The clinical diagnosis was set by a clinician (Y.B.W.) with more than 15 years of experience. The same clinician also handled all patients. Sixteen patients were women with a mean age of 64 years (range 48–84) and four were men, with a mean age of 62 years (range 54–75). None of them smoked or used snuff. The majority used alcohol. Three women had immunosuppressive treatment (nos 3, 13, and 14). One (no. 3) after lung transplantation, and the other two (nos 13 and 14) suffering from cranial arteritis (polymyalgia rheumatica). One patient (no. 17) suffered from Mb Crohn and was treated with olsalazine (a sulfasalazine homolog), and one (no. 15) had Sjögren's syndrome and primary biliary cirrhosis, but had never received any treatment for her diseases.

At the time of referral, all patients clinically had white striations and atrophy. Ten patients had additional clinical symptoms: five desquamative gingivitis and ulcerations of the tongue and/or buccal mucosa, three desquamative gingivitis only, and two ulcerations of the buccal mucosa. Based on clinical appearance these 10 could thus be diagnosed as having CUS.

Sera were also collected from 20 controls, 14 women with a mean age of 57 years (range 24–78) and six men with a mean age of 62 years (range 42–77). Controls were healthy without known autoimmune disease and immunosuppressive treatment. There were problems finding healthy elderly female volunteers and the group thus comprised more men. The study was approved by the local Ethical Committee (dnr 05-010M).

Tissue culture

FaDu cells (ATCC), a human cell line originating from a squamous cell carcinoma of oropharynx were cultured in DMEM (Gibco Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Gibco Invitrogen) at 10% CO_2. For protein extraction, cells were lyzed in 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 mix (7 μl/3 ml) Sigma-Aldrich Chemie, Steinheim, Germany). Cells were shaken for 30 min on ice, sonicated for 15 s, shaken on ice for another 30 min, centrifuged for 30 min at 13 000 ***g*** and the supernatant collected and protein concentration measured.

In vitro *transcription/translation*

Vectors with cDNA for all p63 isoforms in pcDNA3, myc-tagged, were kindly provided by Dr. L Guerrini (Italy) and Dr. F McKeon (USA). cDNA for p73α, p73β, p73γ, p73Δ, HA-tagged, and p53 in pcDNA3 vector, was kindly provided by Dr. B Vojtesek (Czech Republic) and Dr. S Bray (Scotland). For *in vitro* transcription/translation (IVTT) the TNT® Quick Coupled System was used according to the manufacturer's recommendations (Promega Corporation, Madison, WI, USA). One microliter vector of cDNA was mixed with 40 μl TNT Quick Master Mix, 1 μl methionine, and 8 μl pH_20. The reaction was incubated at 30°C for 30–60 min.

Western blotting

Bio-Rad protean II xi/XL Vertical Electrophoresis System (Bio-Rad, Hercules, CA, USA) was used according to the user manual. Eighty micrograms of protein was mixed with 2X loading buffer, boiled for 10 min and electrophoresed on 10% polyacrylamide gels. When using IVTT proteins, 2 μl of each isoform was loaded into each lane. Protein was 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 incubated with sera from OLP patients and controls diluted 1:200, 1:500, or 1:1000 in 5% sheep serum (NeoMarkers, Fremont, CA, USA). The secondary antibody was HRP-conjugated sheep anti-human Ig antibodies diluted 1:20 000 and binding

was identified with chemiluminescence (Amersham Biosciences). After development, membranes were stripped and stained with mouse monoclonal antibodies for detection of myc diluted 1:500 (NeoMarkers); HA diluted 1:1000 (AbCam, ab1911, Cambridge, UK); and DO1 diluted 1:5000 to identify p53 (provided by Dr. B Vojtesek). Secondary antibody was peroxidase-conjugated rabbit anti-mouse Ig diluted 1:50 000 (Dako Cytomation, Glostrup, Denmark).

Results

Western blot on FaDu extracts

In order to test sera on squamous cells of head and neck origin also in Western blot, protein extract was made from FaDu cells. When incubating filters containing protein extracts from FaDu cells with sera from patients with OLP and control persons, a distinct band of approximately 70 kDa was detected in 4 out of 18 OLP patients analyzed (data not shown).

Western blot on p63, p73, and p53 proteins

The sera from both OLP patients (nos 14 and 20) reacted with p63 IVTT proteins (Fig. 2). Both of these patients reacted with all six p63 isoforms with varying intensity. Even at 1:1000 dilution serum 20 showed a strong staining reaction against all p63 proteins. On control staining of the same filters after stripping and staining with myc-antibody for detection of the myc-tagged p63 proteins, the TAp63α, ΔNp63β, TAp63γ, and ΔNp63γ proteins were most strongly expressed. The ΔNp63α protein also had detectable levels using the myc-antibody, whereas TAp63β only showed a very faint band. Despite this, serum from patient 20 showed a very strong reaction toward the TAp63 β protein even at 1:1000 dilution. Furthermore serum 14 detected the TAp63β protein at 1:500 and 1:1000 dilution, although levels of this protein were hardly detectable on the same gel after stripping using the myc-antibody (Fig. 2). Patient 20 had no additional disease whereas patient 14 also suffered from polymyalgia rheumatica.

Sera from one of these two OLP patients (patient 20) also reacted with the four p73 isoforms. No difference in staining intensity was seen between the p73 proteins after stripping the filters and staining with the HA antibody (Fig. 4). This patient had been referred to the clinic 5 years earlier. At that time she had lichen lesions on her lips, buccal mucosa, tongue, and on the right-hand side of the palate. A biopsy was taken from the palate. Histologic and clinical pictures of the palate on that occasion are shown in Fig. 3. At that time the patient was treated with topical cortisone. At present she has no lesions in the palate whereas lesions on the lips, buccal mucosa, and tongue persist.

None of the sera from OLP patients reacted with the p53 protein, although p53 was detected using the DO1 antibody (Fig. 4).

Sera from only 1 of 10 patients who fulfilled the clinical criteria of CUS reacted against the CUSP protein

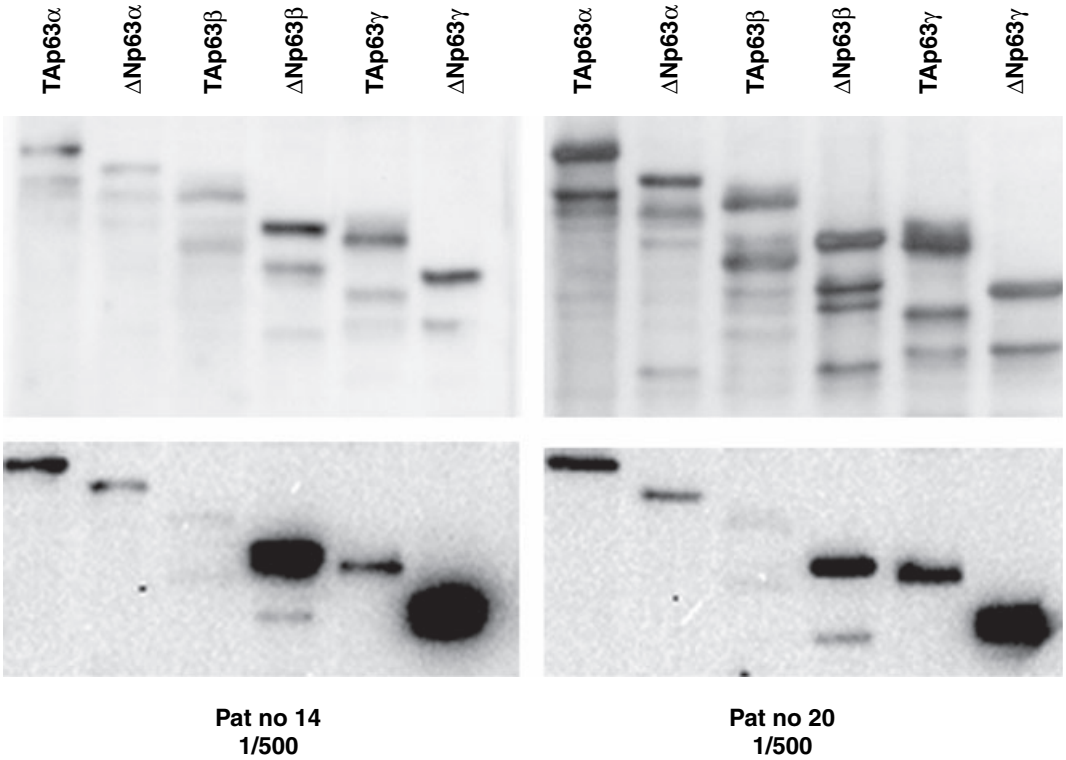

Figure 2 Sera from two patients (nos 14 and 20) with oral lichen planus reacted with p63 proteins. The first row shows *in vitro* transcription/translation (IVTT) proteins detected with patient sera, and the second row is the same filter stripped from patient sera and incubated with myc-antibody, recognizing all the myc-tagged p63 IVTT proteins. Despite very low levels of the TAp63β protein as judged by staining with the myc antibody, sera from both patients reacted strongly with this protein.

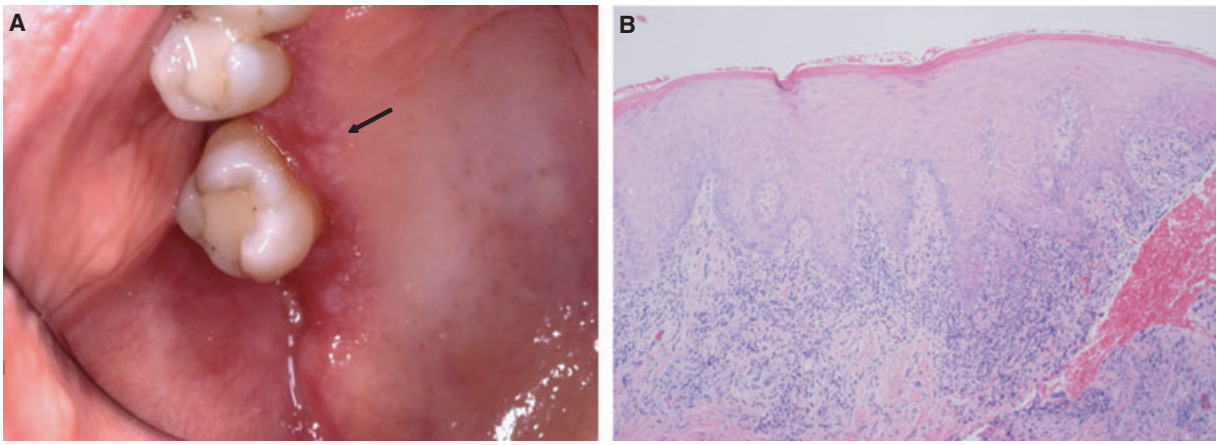

Figure 3 (A) Clinical picture of the palate of patient 20 at the time of referral to the clinic in 2000. The mucosa shows red and white areas. Arrow indicates site of biopsy taken on the same day. (B) Histologic picture of the palatal mucosa showing areas with slight hyperkeratosis, epithelial hyperplasia, areas with basal cell degeneration, and a subepithelial bandformed infiltrate of chronic inflammatory cells. This histologic picture fulfills the criteria for the diagnosis of oral lichen planus.

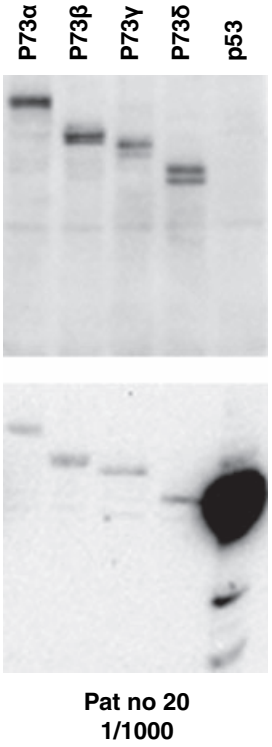

Figure 4 Serum from patient 20 reacting with the four p73 isoforms. The upper panel shows *in vitro* transcription/translation (IVTT) p73 proteins and IVTT p53 detected with patient sera. Note reactivity against all four p73 isoforms, but not against p53. The lower panel is the same filter as above, stripped from patient serum and incubated with anti-HA to detect HA-tagged p73 IVTT proteins, and DO1 for detection of p53.

(ΔNp63α), and one OLP patient who did not fulfill the criteria of CUS reacted against all p63 isoforms.

Discussion

Despite numerous publications and intense research within the field, the specific etiology of OLP is still unknown. Some investigators have focused on a role for exogenous agents such as drugs, trauma, infection, and stress in the pathogenesis of OLP (5). The potential involvement of such agents has led to the conclusion that OLP may represent more than one specific disease entity. Recently there has been growing evidence implicating autoimmunity in the formation of OLP lesions.

The p63 proteins are crucial for formation of the oral mucosa and autoantibodies against one of these six proteins, ΔNp63α, has previously been seen in patients with chronic oral ulcerations. By analyzing all six p63 isoforms, four p73 isoforms and p53 with sera from patients with OLP, we could detect antibodies directed against p63 and p73 in two out of twenty patients diagnosed with OLP. Sera from both patients reacted against all six p63 isoforms, and one of them also against the four p73 isoforms tested. However, sera from these patients did not react with all the IVTT proteins tested and the antibodies were thus specifically directed against p63 and p73 isoforms. Interestingly, only one out of the two patients demonstrated nuclear immunofluorescence on HEp-2 cells, indicating that this standard screening method is not efficient in identifying autoantibodies to p53-family members.

Sera from the patient who reacted against both p63 and p73 showed stronger reaction against the p63 isoforms compared with the other patient. Sera from both patients reacted strongly with TAp63 and ΔNp63, and also demonstrated a selectivity for the p63β isoforms. The reason for this is not clear, although it is known that TAp63β is one of the most potent transactivators of the p63 proteins (23). In the normal oral epithelium, the ΔNp63α isoform is the most prevalent of all isoforms, with the highest levels seen in the basal cell layers, whereas a significant upregulation of mRNA levels of the p63β isoforms is seen in squamous cell carcinoma of the head and neck (16). The TAp63β and ΔNp63β isoforms are thought to be involved in cell differentiation (24), suggesting that the reaction against these isoforms indicates a reaction

against the process of differentiation. Differentiation of epithelial cells starts as soon as cells leave the basal cell layer and start their journey toward the upper and superficial layers, until they finally reach the surface layers, die, and are shed. It has been speculated that the surface of epithelial cells changes in OLP lesions, and that this is the cause of the heavy immune response shown as a band of inflammatory cells in the connective tissue close to the epithelium. The strong reaction seen against the p63β isoforms in these patients could thus be taken as an indication that the epithelial cells in these lesions, due to blockage of these differentiation connected isoforms, could not differentiate normally, and thus are considered 'foreign' to the body and evoke an immune response. If this is true, why then, could this only be seen in 10% of the patients studied here, if this were to be an explanation for the establishment of OLP? It can be speculated that the severity and duration of the disease plays a role in its clinical appearance. Patients with a milder disease or disease of shorter duration thus could have antibodies against p63, and p73 as well, at levels too low to be detected by Western blotting. Both patients with antibodies directed against p63 had had their disease for more than 20 years, affecting oral as well as genital mucosa and skin. Patient 20, who showed a strong reaction against p63 and also p73, had an OLP that did not, in contrast to the majority of lichen planus lesions, respond very well to treatment. Autoantibodies against p73 have previously been detected in patients with cancer (25).

Based on the observations in the present study, the relevance and accuracy of the CUS diagnosis can be questioned, and it could be suggested that rather than being a diagnosis on its own, CUS covers a subgroup of OLP based on either clinical appearance and/or autoimmunity against the CUSP protein. This has previously been suggested (26). Moreover, the p63-positive patient that was definitively excluded from a diagnosis of CUS by the lack of ulceration or desquamation clearly demonstrates that autoimmunity against p63 is not specific for CUS.

Taken together, the most striking finding from this study was that sera from two patients, clinically and histologically diagnosed with OLP had detectable levels of antibodies against p73 and/or p63. The patient reacting with p63 proteins only also suffered from polymyalgia rheumatica, whereas, the patient showing the strongest reaction against p63 and also against p73 suffered from OLP 'only'. This emphasizes the need for further studies to map the importance of the different p63 and p73 proteins and autoantibodies against these in the pathogenesis and course of OLP.

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