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ORIGINAL ARTICLE

ELISA test for p63 antibodies in chronic ulcerative stomatitis

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OBJECTIVE: To develop a novel test for chronic ulcerative stomatitis (CUS), a chronic immunologically mediated condition that produces oral ulcerations. Current diagnostic methods require expensive and technically demanding *in situ* immunofluorescence (IF) studies.
DESIGN: An Enzyme-Linked ImmunoSorbent Assay (ELISA) was prepared and tested with serum samples from patients with CUS and negative controls.
MATERIALS AND METHODS: The N-terminal portion of the CUS autoantigen, ΔNp63α, was produced as a purified recombinant protein and used to coat ELISA plates. Sera from 25 patients with CUS and 16 negative controls were analyzed for reactive antibodies. The optimal cut-offs for positive and negative samples were determined.
MAIN OUTCOME MEASURES: The optimal cut-off of 0.236 resulted in a sensitivity and specificity of the ELISA of 0.80 and 0.75, respectively (exact 95% confidence intervals, *P*-value of <0.001).
RESULTS: The ELISA developed in this study provides a novel and reliable diagnostic assessment to distinguish CUS from other oral ulcerative diseases.
CONCLUSIONS: Immunoassay will allow the true incidence and prevalence of CUS to be determined in future studies. When combined with clinical correlations, the ELISA results will facilitate the evaluation of the prognostic utility of antibody titers and allow correlation with treatment responses in individual CUS cases.
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Keywords: mucosa; autoimmune diseases; pathology; chronic ulcerative stomatitis; oral lichen planus; ΔNp63α; autoantibodies; ELISA

Introduction

Chronic ulcerative stomatitis (CUS) is a painful, debilitating mucocutaneous condition that primarily affects the oral mucosa with chronic, exacerbating, and remitting ulcerations. Women are the most commonly affected and the average age of CUS patients is 59 years. CUS is a recently described condition and only 39 cases have been reported in the literature (Islam *et al*, 2007). The actual number of cases is likely to be higher than this statistic indicates, as many patients are likely undiagnosed or misdiagnosed.

The clinical and histopathologic findings are nonspecific. CUS is currently diagnosed by direct immunofluorescence (IF) studies of surgically biopsied tissues, which show *in vivo* autoantibody deposition in a unique, stratified epithelial specific-antinuclear antibody (SESANA) pattern (Jaremko *et al*, 1990). These antibodies target a transcription factor, ΔNp63α, which is normally present in nuclei of basal and parabasal cells of stratified squamous epithelia (Lee *et al*, 1999).

Chronic ulcerative stomatitis is one of the several immunologically mediated conditions, which affect the oral cavity with chronic erosions and ulcerations, e.g., erosive lichen planus (LP), mucous membrane pemphigoid, pemphigus vulgaris, and linear IgA disease (Scully and Porter, 1997). These conditions are often successfully managed with corticosteroid pharmacotherapy (Gonzalez-Moles and Scully, 2005a) and treatment failures may be the result of incorrect diagnosis (Gonzalez-Moles and Scully, 2005b). In CUS, corticosteroids are less effective than other therapeutics such as hydroxychloroquine (Islam *et al*, 2007), underscoring the importance of accurate diagnosis to institute appropriate management.

The purpose of this study was to develop an Enzyme-linked Immunosorbent Assay (ELISA) for the detection of IgG antibodies to the N-terminal immunogenic portion of ΔNp63α (ΔNp63) in a cohort of CUS sera, including a control group. There is a need to develop new diagnostic tools to identify CUS patients because the currently available methods require specialized and costly testing. Our data demonstrate that IgG antibodies reactive with ΔNp63 are present in all CUS sera tested and that ELISA is a more sensitive method than immunoblotting to detect these antibodies.

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Materials and methods

Serum samples

Serum samples were obtained from 25 CUS patients, diagnosed by clinical findings, histopathology with hematoxylin & eosin staining, and IF studies. Control sera were obtained from IMMCO Diagnostics and include 16 samples from patients previously diagnosed with dermatologic or rheumatic clinical conditions. Six control sera were from patients with LP and negative for SES-ANAs on esophagus substrates; five control sera were from patients with positive ANAs on indirect IF using mouse kidney or HEp-2 cells, but negative on esophagus substrates; five sera were from patients negative for ANAs on indirect IF using mouse kidney, HEp-2 cells and esophagus substrates (Solomon, 2004).

All of the sera were existing diagnostic samples and exempt from human subjects' review. This exemption is provided for human subjects' research involving 'the collection or study of existing diagnostic specimens, if the information is recorded by the investigator in a manner that the subjects cannot be identified, directly or through identifiers linked to the subjects.' Additional information is available on the US Department of Health & Human Services website http://www.hhs.gov/ohrp/humansubjects/guidance/45cfr46.htm#46.101.

Primer design and PCR amplification and sequencing of ΔNp63α

Cloned ΔNp63α was provided by the Center for Biologics Evaluation and Research, FDA. The cDNA has a 1761-bp open reading frame, GenBank accession No. AF091627, National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/. Primer sets for cloning the ΔNp63α N-terminal nucleotides 1–825 (corresponding to amino acids 1–275) with incorporation of a His tag were designed as follows:

F(5′-ATTAATATGTTGTACTGGAAAACAATGC CCAGACTCAATTTAGTGAGCCACAGTACACG-3′)

R(5′-AAGCTTCACTTTGTACTGTCCGAAACTTG CTGC-3′)

Gel purified primers (Integrated DNA Technologies, Coralville, IA, USA) and the AccuPrime™ Taq DNA polymerase PCR system (Invitrogen, Carlsbad, CA, USA) were used in a GeneAmp® PCR System 2400 thermal-cycler (PE Applied Biosystems, Foster City, CA, USA) as per the manufacturer's instructions under standard conditions (Saiki, 1990). PCR products were gel purified and cloned into a Zero Blunt® TOPO® vector (Invitrogen). DNA was purified using the Wizard® *Plus* Megaprep DNA purification System (Promega, Madison, WI, USA), and sequenced at Roswell Park Cancer Institute, http://biopolymer.roswellpark.org/ (Buffalo, NY, USA).

Production of recombinant protein

The partial ΔNp63α cDNA obtained after PCR was digested with restriction enzymes AseI and HindIII, and ligated into the pET28c plasmid, prepared by restriction enzyme digestion with NdeI and HindIII, to generate a bacterial expression vector. DNA for pET28c-ΔNp63 was purified using the Wizard® *Plus* Miniprep DNA purification System (Promega), and subsequently used to transform BLR(DE3) cells. After induction with isopropyl-D-1-thiogalactopyranoside (IPTG), His-tagged protein was recovered in the supernatant and purified using nickel affinity chromatography to >90% purity. A yield of 3.8 mg protein was recovered per gram of cell pellet.

Immunoreactivity of recombinant protein-Western blot analysis

Recombinant His-tagged ΔNp63 protein was subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. Membranes were incubated with monoclonal anti-human p63 antibody A4A (1:500 dilution) (Oncogene Research Products, San Diego, CA, USA) and horseradish peroxidase (HRP) conjugated, goat anti-mouse IgG (KPL, Inc., Gaithersburg, MD, USA) secondary antibody was used (1:10 000 dilution), with SuperSignal® West Pico Chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA). The membrane was exposed to X-ray film (X-OMAT, Kodak, Rochester, NY, USA) for 1 s and developed in an automated processor (Figure 1).

Nitrocellulose membranes were immunoblotted (Targoff *et al*, 1993) with 16 CUS serum samples (1:100 dilution). Alkaline phosphatase conjugated, goat anti-human IgG, Fcγ fragment-specific (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) secondary antibody (1:3 000 dilution) was used to demonstrate immunologic reactivity. Western Blue® Substrate for Alkaline Phosphatase (Promega Corporation) was used to develop color.

Detection of ΔNp63-specific IgG in CUS sera by ELISA

Microtiter plates were coated with purified ΔNp63 protein solution (2 μg protein/ml bicarbonate ELISA coating buffer). Before each ELISA, the microtiter plate wells were washed three times with phosphate buffered

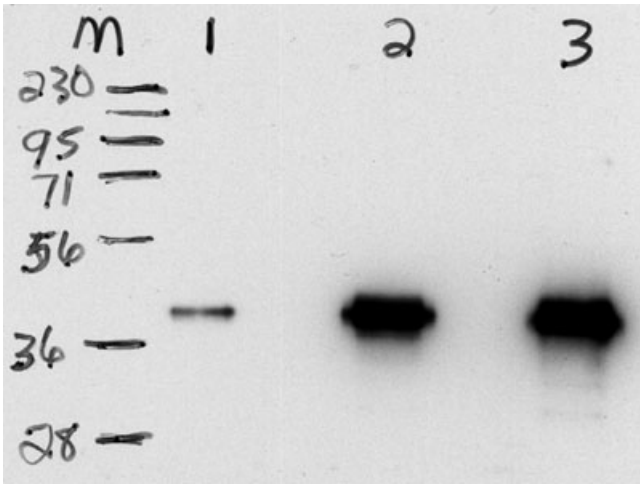

Figure 1 Immunoblot of His-tagged recombinant Np63 protein with monoclonal anti-human p63 antibody A4A. Monoclonal antibody is reactive with the protein, which consistently ran at a higher weight (about 42 kDa) than the calculated molecular weight of 32.9 kDa. Each lane has a different amount of Np63 protein: lane 1–0.15 g, lane 2–0.3 g, lane 3–0.6 g

saline Tween-20 (PBST), incubated with assay diluent (5% BSA), and then washed again with PBST three times. Triplicate wells in prepared microtiter plates were incubated with human sera (1:100 dilution in 5% BSA). The wells were washed three times with PBST, and then incubated with goat anti-human IgG HRP conjugated secondary antibodies (1:10 000 dilution in 5% BSA) (Jackson ImmunoResearch Laboratories, Inc.). After removal of secondary antibodies and washing 5× with PBST, color was developed with tetramethylbenzidine (TMB) peroxidase substrate (KPL, Inc.). The color development reaction was stopped with TMB Stop solution (KPL, Inc.). Results were read at 450 nm with a BioRad Model 680 microplate reader with Microplate Manager 5.2 PC software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis of ELISA

Samples were tested in triplicate and results averaged. A receiver operator characteristic (ROC) curve was constructed for all ELISA values, to determine the optimal cut-off for classifying disease state. The cut-off value that maximized the Youden Index was selected as the optimal cut-off. Sensitivity, specificity, positive and negative predictive value, and area under the ROC curve were all calculated.

Results

Western immunoblot

Sixteen of the 19 CUS sera tested by immunoblotting were immunologically reactive (12 strongly and four weakly) with recombinant ΔNp63; three were negative (Figure 2). None of the control sera was immunologically reactive with recombinant ΔNp63. Positive results were identified based on visual detection of a reactive band of the expected size, 42 kDa.

ELISA

Specific IgG immunoreactive with recombinant ΔNp63 was detected in all CUS sera samples tested by ELISA. Three of the CUS samples that were negative on immunoblot were positive by ELISA. Based on the maximization of the Youden index, the cut-off point for the ELISA with recombinant ΔNp63 was set at 0.236 optical density units, which corresponds to a Youden index of 0.55 (Figure 3). Sensitivity, specificity, positive and negative predictive value, and area under the ROC curve were all calculated. (Table 1). The sera from CUS cases had higher optical densities than those from the controls. The mean optical density for the cases was 0.37 (0.11) and for the controls the mean was 0.18 (0.09), resulting in a highly statistically significant difference between the two groups ($P < 0.001$) (Figure 4).

Discussion

Chronic ulcerative stomatitis was first described as a clinical and immunopathologically distinct condition in 1989 (Beutner *et al*, 1989) and since then, only 39 cases have been described in the literature (Islam *et al*, 2007).

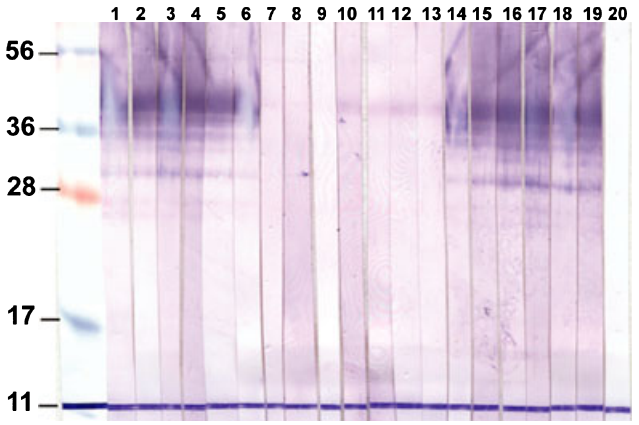

Figure 2 Immunoblot of His-tagged recombinant Np63 protein with chronic ulcerative stomatitis (CUS) patient sera. Sixteen of the 19 CUS sera tested were immunologically reactive with recombinant Np63. Lanes 1, 2, 3, 4, 5, 6, 14, 15, 16, 17, 18 and 19 were strongly reactive, lanes 10, 11, 12 and 13 were weakly reactive and lanes 7, 8 and 9, were negative. Lane 20 is a negative control without a serum sample

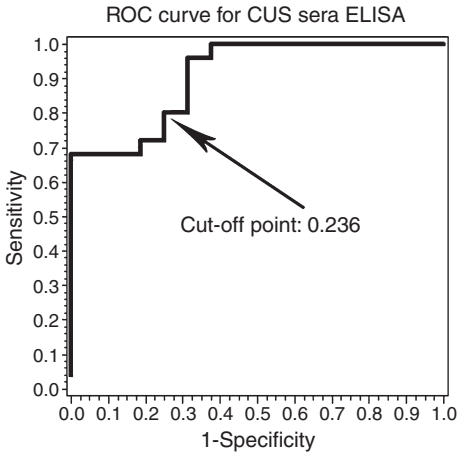

Figure 3 Sensitivity and specificity of the Np63 ELISA. Sera of patients with disease ($n = 25$) and controls ($n = 16$) were screened for Np63 IgG reactivity. The diagnostic properties of the ELISA test are depicted as a receiver operating characteristic curve, when the cutpoint is set at 0.236. The area under the receiver operator characteristic curve is 0.908

Table 1 Sensitivity and specificity of the Np63 ELISA. Calculations show properties of the ELISA cutoff value and an excellent sensitivity (80%) and specificity (75%).

	CUS sera (n = *25*)
Cut-off point	>0.236 O.D. units
Positive sera	25
Sensitivity (exact 95% CI)	0.80 (0.59, 0.92)
Specificity (exact 95% CI)	0.75 (0.47, 0.92)
Youden index	0.55
Diagnostic odds ratio (95% CI)	12 (2.69, 53.62)
Positive predictive value (exact 95% CI)	0.83 (0.62, 0.95)
Negative predictive value (exact 95% CI)	0.71 (0.44, 0.89)

CUS, Chronic ulcerative stomatitis.

CUS is a benign condition that requires sophisticated and expensive testing to diagnose, thus the true incidence and prevalence have not been determined. It may

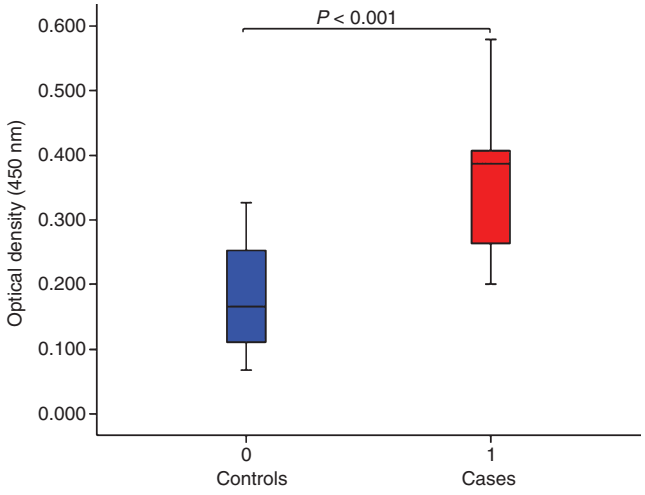

Figure 4 Distribution of results. Boxplots showing the results of a pairwise test of the results of IgG reactivity to Np63 on ELISA testing. There is a highly significant ($P < 0.001$) difference between the CUS cases (red box) and control sera (blue box). On the x-axis, 0 represents samples that were negative on direct IF (controls); and 1 represents samples that were positive on direct IF (cases). Top of the S.D. bar is the maximum. Top of the colored bar is 75th percentile. Line in colored bar is the median. Bottom of the colored bar is 25th percentile. Bottom of the S.D. bar is the minimum

be possible that the paucity of published cases means that CUS is a rare condition, or it may be that the lack of a convenient and inexpensive means of diagnosis results in many undiagnosed cases being treated empirically.

Non-specific immunoglobulins that recognize epithelial cell proteins are often produced in reactive inflammatory processes, e.g., the complement fixing autoantibodies to keratin intermediate filaments in normal adult sera, which mediate apoptotic removal of damaged keratinocytes (Beutner *et al*, 1987a,b). However, on direct IF, these autoantibodies are cytoplasmic, unlike CUS autoantibodies, which are nuclear. However, certain connective tissue diseases may demonstrate an ANA pattern of autoantibodies in epithelia, although *in vivo* these reactions are in the stratum spinosum, not the basal layers (Koelle *et al*, 1991; Solomon, 2008).

The autoantibodies produced in CUS are specific circulating antibodies that are identified on indirect IF testing using esophagus substrates, as well as bound to nuclear antigen in epithelial tissues where the antibodies are shown by direct IF. The specificity of these antibodies allowed the CUS antigen, ΔNp63α, to be initially identified in a pull-down assay of keratinocyte extracts (Lee *et al*, 1999). The ΔNp63α autoantigen is a nuclear transcription factor that is normally present in nuclei of basal and parabasal cells of stratified squamous epithelia (Lee *et al*, 1999). As the initial identification of ΔNp63α as the autoantigen in CUS, several other laboratories independently identified ΔNp63α and numerous studies have explored its biological role as a member of the p53, p63, and p73 family of proteins (Solomon *et al*, 2007).

Transcription of the *p63* gene leads to distinct N-terminal isoforms, and alternative mRNA splicing results in α, *β*, and *γ* isoforms of p63 that are unique at their C-terminus (Yang *et al*, 2002). These p63 isoforms are expressed in a developmentally regulated, tissue-specific manner (Dellavalle *et al*, 2001). ΔNp63α is the major p63 isoform, which is preferentially expressed in stratified squamous epithelia where it is essential for differentiation (Koster and Roop, 2004), maintenance of proliferative potential and epithelial integrity (Ihrie *et al*, 2005), and for normal epithelial cell-matrix adhesion and survival (Carroll *et al*, 2007). It is highly likely that the CUS autoantibodies interfere with the normal function of ΔNp63α and cause epithelial breakdown and detachment, which present clinically as erosions and ulcerations. *In vivo* experiments to examine the pathogenicity of the CUS antibodies have not been reported.

Chronic ulcerative stomatitis is immunopathologically distinguished from LP by the positive SES-ANA reaction on direct IF studies of biopsied tissues from CUS patients, whereas these autoantibodies are not found on direct IF studies of biopsied tissues from LP patients. Some authors describe cases of clinical oral erosive LP with serum antibodies to a 70-kDa keratinocyte antigen that they claim is the CUS antigen, although the protein was not sequenced or identified with a control monoclonal antibody to ΔNp63α. Thus, these authors claim the SES-ANA pattern of autoantibodies is not exclusive to CUS (Parodi *et al*, 2007). Logically, however, if a patient has what is clinically erosive LP, and they have a positive SES-ANA reaction on direct IF, then the accurate diagnosis should be CUS, not oral erosive LP (Solomon, 2008). A clinical distinction between CUS and oral erosive LP is that CUS is often not responsive to glucocorticoid pharmacotherapy, which is often an effective treatment for LP.

The current gold standard test for CUS diagnosis is direct IF. The cost of a direct IF panel in a commercial laboratory ranges from $480.00 to $1 141.00. Aside from expense, there are other challenges to CUS diagnosis (Islam *et al*, 2007), e.g., (1) biopsy from perilesional mucosa is preferred, as tissue from an ulcerated area lacks epithelium for diagnosis; (2) requirement for specimen transport in Michel's media (Michel *et al*, 1972) not 10% neutral buffered formalin; (3) requirement for specialized processing equipment, the specimen must be frozen at the laboratory and sectioned with a cryostat; and (4) requirement for skilled technical processing. *En face* specimen orientation may result in an inconclusive diagnosis; it may not be possible to distinguish CUS from other entities that demonstrate ANAs in the stratum spinosum, i.e., systemic lupus erythematosus, systemic sclerosis, and mixed connective tissue disease.

In the event of an inconclusive direct IF test for CUS, indirect IF is used as an adjunctive test. Indirect IF requires a serum sample from the patient and the proper substrate must be utilized. To demonstrate the SES-ANA pattern with CUS patient serum, it is necessary to use guinea pig or monkey esophagus substrates. Indirect IF using mouse kidney or HEp2 cell (a cell line derived from a human laryngeal carcinoma) substrates gives a negative or a low titer with CUS sera.

The challenges of the current methods of CUS diagnosis led us to investigate a novel diagnostic test

in this study. In 1999, the pathobiology of CUS was partially elucidated with the discovery and cloning of the CUS antigen, ΔNp63α (Lee *et al*, 1999). Our previous studies showed that CUS patient sera were immunologically reactive with ΔNp63α produced by *in vitro* transcription/translation in rabbit reticulocyte lysates (Solomon *et al*, 2003) and that the most consistently immunogenic portions of ΔNp63α were the N-terminal amino acids 1–275 (Solomon *et al*, 2007).

The current study produced the immunogenic N-terminal portion, ΔNp63, as a recombinant protein. By immunoblotting, this protein was immunoreactive with most of the CUS sera tested. However, ELISA is shown to be a more sensitive assay, as all sera were immunologically reactive by this method. ELISA requires only a serum sample from the patient and is a technically straightforward test to perform. ELISA readers are widely available at many diagnostic laboratories, and ELISA testing does not involve the specialized equipment and technical skills that IF studies require.

These results have importance when considering that direct IF is currently the gold standard in the diagnosis of immunologically mediated conditions. The expense and limited availability of IF testing result in empirical treatment of many oral ulcerative conditions, without a diagnosis. These preliminary data have demonstrated excellent sensitivity and specificity; however, the lack of a positive control is a limitation that we acknowledge. In future studies, it would be desirable to construct a standardized curve using serum from immunized animals as a positive control. In addition, many more samples will need to be analyzed to validate this ELISA-based diagnostic test for CUS. Future studies with clinical correlations will establish the incidence and prevalence of CUS and facilitate the evaluation of antibody titer correlation with disease prognosis and treatment response.

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