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Cell cycle regulation and proliferation in lichen sclerosus

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ABSTRACT

Introduction: Genital lichen sclerosus (LS) is considered a potential precursor lesion of squamous cell carcinoma. We aimed to investigate the expression pattern of cell cycle regulators, tumour suppressor proteins and proliferation markers in genital LS as compared to extragenital LS (ELS) and healthy controls (HC).

Methods: In order to assess the expression of minichromosome maintenance protein 3 (MCM3), MCM7, Ki-67, cyclin D1, cyclin E, p16, p21, and p53, immunohistochemistry and immunofluorescence were performed on skin specimens obtained from the genital region of LS patients (short-standing LS, n = 19; long-standing LS, n = 15), patients with ELS (n = 10), and HC (n = 8).

Results: Median protein expression of MCM3 and Ki-67 was significantly higher in LS when compared to ELS and HC. In patients with long-standing LS, the expression profiles of MCM3 and Ki-67 significantly correlated. Moreover, long-standing LS lesions showed significantly increased expression of p53 when compared to short-standing LS, ELS, and HS. Immunoreactivity of MCM7, p16, p21, cyclin D1 and cyclin E did not significantly differ between the groups.

Conclusions: Tumour suppressor proteins such as p53 are significantly overexpressed in genital LS when compared to extragenital disease and healthy skin. The significant p53 overexpression, particularly in long-standing genital lesions, may reflect the increased risk of malignant transformation and/or oxidative stress associated with LS. Moreover, we have demonstrated that proliferation markers such as Ki-67 and MCM3 are significantly up-regulated in genital LS as compared to controls. With regard to cell cycle regulation and proliferation rates, ELS significantly differs from its genital counterpart.

1. Introduction

Lichen sclerosus (LS) is a chronic inflammatory dermatosis that mostly affects the genital and perianal skin. The disease is most common in postmenopausal Caucasian women. LS has a characteristic clinical presentation with ivory or pink macules or papules that eventually coalesce into thin, grey, parchment-like areas. Microscopically, LS shows epidermal atrophy, hyperkeratosis, follicular plugging, interface lichenoid dermatitis, subepidermal oedema, telangiectasia, and homogenization of collagen in the papillary and upper dermis. The aetiology of LS remains unclear; hormonal factors, autoimmunity and oxidative stress have been implicated. Extragenital involvement alone is relatively rare and has been reported in about 6% of all women with LS [1–4]. Genital LS is associated with a 5% risk of malignant transformation into squamous cell carcinoma (SCC), making long-term follow-up essential in these patients. Similar to penile SCC, vulvar SCC can be divided into two patterns. The first is predominantly found in younger women often presents as multifocal lesions and is related to oncogenic viruses (HPV) such as HPV16 and HPV18. There is no association with LS in these cases. The second is found in older women, and is unifocal, not related to HPV, and can originate from differentiated vulvar intraepithelial neoplasia grade 3. In this condition, LS is considered a predisposing factor [5–7]. The cell cycle is the orchestrated series of molecular events, which coordinate the production of two daughter cells. It is divided into four distinct phases: G1, S, G2 and M. Progression through the successive stages of the cell cycle is accompanied by the alternate expression and lack of expression of specific regulatory proteins. Dysregulation of the cell cycle is known to play an important role in the oncogenic transformation of cells [8]. In this study, we have analysed the expression patterns of several cell cycle regulatory proteins, tumour suppressor proteins and proliferation markers across the spectrum of short-standing genital LS (SS–LS), long-standing genital LS (LS–LS), extragenital LS (ELS), and healthy controls (HC).

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2. Materials and methods

2.1. Patients and controls

This study was performed in the outpatient clinic for connective tissue diseases of the Department of Dermatology, Ruhr-University Bochum (Germany). The investigation comprised 19 patients (13 women; 6 men; mean ± SD age: 50.2 ± 19.7 years) with SS–LS (≤1 year duration of disease) and 15 patients (11 women; 4 men; mean ± SD age 55 ± 17.1 years) with LS–LS (≥5 years duration of disease). As controls, we recruited 10 female patients with ELS (mean ± SD age: 67 ± 6.3 years) and 8 HC (6 females; 2 males; mean ± SD age: 69 ± 14 years). Four millimetre punch biopsies were obtained from vulvar or penile skin (LS and HC) and extragenital sites (ELS). Each specimen was analysed by two dermatohistopathologists confirming the diagnosis of LS in accordance with previously suggested diagnostic criteria [1,9]. Control samples taken from healthy subjects were histologically confirmed as normal tissue. The protocol of the study was approved by the ethics review board of the Ruhr-University Bochum. The study was conducted according to Declaration of Helsinki principles. Informed consent was obtained from every subject included.

2.2. Immunohistochemical analysis of protein expression

Four micrometer paraffin-embedded sections were mounted on silanized slides and stored for 1 h in a humid chamber at 60 °C. Sections were deparaffinized in xylene and washed with 100%, 96%, 70% and 50% ethanol for 5 min each and rinsed with demineralized water. Immunohistochemical staining was eventually performed for p16, p21, p53, cyclin D1, cyclin E, MCM3, and MCM7 as previously described in detail (Table 1) [10].

2.3. Microscopic evaluation of immunohistochemistry

Immunohistochemical specimens were independently analysed by two observers under a light microscope at 200× magnification. Five randomly chosen fields of view were assessed in the centre of LS and control specimens. Briefly, 100 consecutive basal cells were identified and all cells above the basement membrane (epidermis) in these fields were scored for p16, p21, p53, Ki-67, cyclin D1, cyclin E, MCM3, and MCM7. Quantitative results were expressed as the percentage of positively stained epidermal cells per field on total epidermis cell count. Mean values of immunoreactivity scores as assessed by the two observers were used for statistical analysis. Staining intensity was semiquantitatively evaluated in the epidermis using a simple score: none (0), slight (1), moderate (2), strong (3), and very strong (4). In addition, the distribution of staining from basal layer to the surface layer of the epithelium was assessed.

Table 1
Overview on performed immunohistochemistry (alkaline phosphatase anti-alkaline phosphatase technique using the labelled streptavidin-biotin method) in patients with lichen sclerosus and controls.

Antibody	Source	Pre-treatment*	Dilution
P16	mtm laboratories AG, Heidelberg, Germany	H	1:40
P21	Dako, Hamburg, Germany	H	1:200
P53	Dako, Hamburg, Germany	H	1:50
Cyclin D1	Dako, Hamburg, Germany	H	1:40
Cyclin E	Santa Cruz Biotechnology, Inc., California, USA	H	1:50
MCM3	Dako, Hamburg, Germany	H	1:50
MCM7	Santa Cruz Biotechnology, Inc., California, USA	H	1:100

* H = heat (microwave-3-step-technique); incubation for primary antibodies 30 min or overnight.

2.4. Double-labelled immunofluorescence for Ki-67 and MCM3

Immunofluorescence was performed in short- and longstanding LS lesions. After heating at 58 °C for 30 min, tissue sections were deparaffinized in two subsequent 10-minute xylene-incubations and then hydrated through graded alcohol-series (100%, 95%, 70%, water). Antigen-retrieval was performed in DakoCytomation Target Retrieval Solution pH 6 (Dako, Glostrup, Denmark Code S3308) by heating for 30 min. After cooling of the samples to room temperature and a brief rinse in distilled water, sections were blocked for unspecific protein binding with TBS containing 3% BSA and 0,2% Triton ×100 for 10 min. Then, the tissues were incubated in a solution containing mouse-primary antibody against human Ki67 (Dako, Glostrup, Denmark Code M7240) and rabbit-primary antibody against human MCM3 (Cell signalling, Code #4012), both in a dilution of 1:50 in background reducing antibody diluent (Dako, Code S3002) for 1 h. After three 5-minute washes in PBS, 2% Tween, a Dylight 549 (red) labelled goat-anti-rabbit antibody (Jackson immuno research, Code 111-505-205) and a Cy2 (green) labelled donkey- anti-rabbit antibody (Jackson Immuno Research, Code 711-225-152) were simultaneously applied in a dilution of 1:100 each in background reducing antibody diluent (Dako, Code S3002) for 30 min, again followed by three 5-minute-washes in PBS-Tween. Finally, the nuclei were stained with DAPI (blue) by incubation in a 0.33 μg/ml DAPI (Invitrogen, Paisley, UK, Code SKU#1306) solution in PBS for 5 min. Following 5-minute-incubations in 3 changes of distilled water, the tissue sections were mounted with Dako Fluorescence Mounting Medium (Glostrup, Denmark, Code S-3023) and stored at 4–8 °C in the dark. Omission of one or both primary antibodies yielded no staining signal.

2.5. Statistical analysis

Data analysis was performed using the statistical package MedCalc Software (Mariakerke, Belgium). Distribution of data was assessed by the D'Agostino-Pearson test. Non-normally distributed immunohistology data were expressed in medians including the range. Data were analysed using the Kruskal–Wallis-ANOVA and the Mann–Whitney test for independent samples. Correlation studies were performed using the Spearman's coefficient of rank correlation. No α adjustment for multiple testing was performed, since this was an explorative study. P values <0.05 were considered significant (2-sided).

3. Results

No cancerous lesions or clinical evidence of human papillomavirus infection was found in any of the patients. Medians and range of protein expression are described in detail in Table 2. Almost all LS samples showed linear basal and suprabasal keratinocyte p53 staining (Fig. 1). Median percentage immunoreactivity of p53 was significantly higher in LS–LS (46%) when compared to SS–LS (12.5%; $P=0.0027$), ELS (16%; $P=0.017$), and HC (12%; $P=0.005$). Ki-67 and MCM3 immunoreactivity was restricted to the lower one-third of the epithelium in LS. Both SS–LS (23.5%) and LS–LS (18%) lesions showed significantly increased percentage immunoreactivity for Ki-67 as compared to ELS (12%; $P=0.017$ and $P=0.021$, respectively) and HC (13%; $P=0.026$ and $P=0.0020$, respectively, Fig. 2). In LS–LS (Fig. 3), median percentage immunoreactivity of MCM3 (51%) was significantly increased compared to SS–LS (45%; $P=0.032$), ELS (26%; $P=0.001$), and HC (28.5%, $P=0.012$). In LS–LS, MCM3 immunoreactivity did significantly correlate with Ki-67 immunoreactivity ($r=0.54$; $P=0.043$). Moreover, double-labelled immunofluorescence for Ki-67 and MCM3 revealed in part co-expression of both markers in the nuclei of keratinocytes (Fig. 4). In SS–LS lesions,