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Gene expression profiling in male genital lichen sclerosus

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Summary

Male genital lichen sclerosus (MGLSc) has a bimodal distribution in boys and men. It is associated with squamous cell carcinoma (SCC). The pathogenesis of MGLSc is unknown. HPV and autoimmune mechanisms have been mooted. Anti extracellular matrix protein (ECM)1 antibodies have been identified in women with GLSc. The gene expression pattern of LSc is unknown. Using DNA microarrays we studied differences in gene expression in healthy and diseased prepuces obtained at circumcision in adult males with MGLSc (n = 4), paediatric LSc (n = 2) and normal healthy paediatric foreskin (n = 4). In adult samples 51 genes with significantly increased expression and 87 genes with significantly reduced expression were identified; paediatric samples revealed 190 genes with significantly increased expression and 148 genes with significantly reduced expression. Concordance of expression profiles between adult and paediatric samples indicates the same disease process. Functional analysis revealed increased expression in the adult and child MGLSc samples in the immune response/cellular defence gene ontology (GO) category and reduced expression in other categories including genes related to squamous cancer. No specific HPV, autoimmune or squamous carcinogenesis-associated gene expression patterns were found. ECM1 and CABLES1 expression were significantly reduced in paediatric and adult samples respectively.

Keywords

gene expression profiling, male genital lichen sclerosus, squamous carcinoma

Lichen sclerosus (LSc) is an acquired, chronic, inflammatory, fibrotic and atrophic cutaneous disorder associated with a predilection for genital skin where there is a risk of developing squamous cell carcinoma (SCC). Genital (G) LSc shows a bimodal pattern of incidence in men and women. Genital lichen sclerosus is responsible for serious urinary and sexual dysfunction, the latter amounting to dyspareunia. But, it is also associated with the development of SCC in both sexes (Bunker & Neill 2010).

Although LSc exhibits inflammatory, fibrotic and neoplastic characteristics, the underlying pathogenesis of the disease is obscure (Bunker & Neill 2010). It is not known whether the pathogenesis of GLSc is the same in women as it is in

men, nor whether the disease in men and boys represents the same entity. Humoral and cell-mediated autoimmune mechanisms have been proposed (Bunker & Neill 2010; Neill *et al.* 2010). Antibodies to extracellular matrix 1 protein (ECM1) have been found in GLSc (Oyama *et al.*, 2003; Edmonds *et al.* 2011).

Human papilloma virus (HPV) has been cited as a causative agent as it has been found in association with GLSc (Drut *et al.* 1998; Bunker & Neill 2010), but GLSc does not possess the attributes of a communicable sexually transmitted disease, not least because it is virtually never seen in sexual partners.

Data are emerging from studies of other inflammatory, neoplastic and fibrosing conditions that are delineating char-

acteristic mRNA expression patterns in these fundamental pathological processes as well as distinctive patterns in specific diseases. The *molecular* patterns of inflammation, fibrosis and carcinogenesis in male genital lichen sclerosis (MGLSc) have not been established. Clinically and histologically, MGLSc can exhibit all three characteristics.

Our hypotheses are that MGLSc is a chronic inflammatory process and that MGLSc in boys and men, paediatric and adult disease represent the same disease entity; that neither infection with HPV nor autoimmunity are aetiopathogenic factors; and that MGLSc is *not* essentially a premalignant condition.

Our aims were to refine the characterisation of the pathology of MGLSc by seeking differences in the levels of gene expression between normal and diseased genital skin through microarray analysis, to correlate any such differences with important disease characteristics, to identify genes known to be associated with squamous carcinogenesis, autoimmunity, autoimmune disease, fibrosis and inflammation and, additionally, to compare expression profiles between adult and paediatric disease, to determine whether they represent the same entity or have different pathogenic pathways.

Therefore, we have used whole genome microarrays to study the expression profile of healthy and diseased prepuces in adult men and boys.

Subjects

Local ethical committee approval was obtained. Adult patients with ascertained MGLSc ($n = 4$) were identified (26, 37, 62, 74 years). Areas of diseased and normal skin of the prepuce in the same patient were sampled. Patients undergoing circumcision were directly observed by Dr Edmonds during the procedure.

Clinically diseased and normal samples were divided and a portion submitted for histological analysis and another snap frozen in liquid nitrogen. Once the histological analysis confirmed both the presence of MGLSc in the diseased prepuce and entirely normal prepuce from the same patient, RNA extraction was performed from commensurate tissue according to Qiagen protocols.

Two boys with MGLSc (4, 9 years) and four boys undergoing circumcision for religious and cultural reasons (<6 weeks) were identified. Samples were divided and portions submitted both for histological corroboration of the clinical diagnosis and subsequent nucleic acid extraction. Once histological analysis demonstrated normal or MGLSc, RNA extraction was performed.

Microarray data generation

(i) Total RNA was extracted using conventional published methodology (RNeasy; Affymetrix and Imperial College Microarray Centre (<http://microarray.csc.mrc.ac.uk>) standardised protocols. cRNA was then hybridised on Affymetrix Human U133Plus 2.0 GeneChips.

Data processing and analysis

Data were analysed using GeneSpring version 7 (Agilent Technologies, Santa Clara, CA, USA). The data were preprocessed using the Robust Multi-array Analysis (RMA) algorithm (Bolstad *et al.* 2003) and further normalised using a per chip normalisation, to the 50th percentile and a per gene normalisation, to the median value. The data were quality assessed using box plots, condition tree plots and principal component analysis plots on the conditions.

Significantly, differentially expressed genes were determined between the various groups of samples using a *t*-test ($P = 0.01$). As there was only a limited number of biological replicates available, no multiple testing correction was applied to the *P*-value and a Cross-Gene Error Model based on replicates was used to determine deviation for each gene in the *t*-test. The gene list was filtered to remove any genes that had a ratio of <1.5-fold difference between the normal and diseased samples. Based on the relative gene expression values, genes were described as having 'low'/'moderate'/'high' expression if their unlogged normalised value was '<100'/'between 100 and 100'/'>1000' respectively.

The Database for Annotation, Visualization and Integrated Discovery (DAVID), (Dennis *et al.* 2003) was used for functional annotation and enrichment analysis.

The differentially expressed gene lists were also analysed for Gene Ontology (GO) category enrichment using the Gene Ontology Tree Machine (GOTM) web-based tool (Zhang *et al.* 2004).

Results

The full data set is available from Dr Edmonds on request by email (emmaedmonds@hotmail.com).

Statistical analysis of differences in gene expression between normal and MGLSc samples

Differentially expressed genes were determined between the normal ($n = 4$) and MGLSc ($n = 4$) adult samples. The differential expression analysis of the adult samples showed 51 genes with increased expression in the MGLSc tissue and 87 genes with reduced expression in MGLSc tissue compared with normal tissue.

To compare MGLSc gene expression patterns in paediatric samples with those adults, differential expression analysis was performed separately on the adult samples (disease, $n = 4$; normal, $n = 4$; $P = 0.01$) and the paediatric samples (disease, $n = 2$; normal, $n = 4$; $P = 0.01$). Genes expressed differentially between the two age groups of normal tissue samples (adult $n = 4$; child $n = 4$; $P = 0.01$) were also determined and filtered from the two previous gene list, to remove those genes that are differentially expressed between adult and child samples, but not because of differences caused by the disease.

The differential expression analysis of the paediatric samples resulted in 190 genes with *increased* expression in the MGLSc tissue and 148 genes with *reduced* expression in MGLSc tissue compared with normal tissue.

In the analysis of statistically significant differentially expressed genes ($P < 0.01$) between normal and diseased in both adult and paediatric samples, only five genes overlapped with *increased* expression in MGLSc in both adults and children. Six genes overlapped with *reduced* expression in MGLSc. Although not statistically significant ($P > 0.01$), the trend of genes found to be increased in the disease state in the adult and child samples had a very high correlation ($R = 0.96$) with a high proportion of the genes ($n = 116$ genes) having very similar expression. The reason for the relatively small overlap of numbers of significant genes being detected could be because of small sample size and relatively large amount of variance between biological samples.

There were a small number of genes that had converse expression patterns between the adult and child samples. That is, there were 30 genes that were found to have significantly increased expression profiles in the child MGLSc samples. These were found also to have reduced expression, although not significantly so, in adult MGLSc compared with adult normal. Similarly, there were 21 genes that had significantly reduced expression profiles in the child MGLSc samples that were shown to have had an increase in expression (although not significantly so) in the adult normal samples compared to the adult MGLSc samples.

The analysis of all the normal ($n = 8$) and all the MGLSc ($n = 6$) samples revealed 228 differentially expressed genes, with 56 of these genes having reduced expression in the disease samples compared with the normal sample and 172 having increased expression in the disease samples compared with the normal samples.

SCC carcinogenesis-associated genes analysis

A list of genes associated with SCC carcinogenesis was compared with the lists of differentially expressed genes derived from the previous analysis. Only probe set 209295 at (TNFRSF10B) had significant differential expression, being increased in MGLSc samples in both adults and children. The probe sets associated with CDKN2A had very low or no expression levels. The p53 and TLE1 probe sets had low to moderate gene expression levels. The PTEN probe set had low to high expression levels and gave consistent results between all sample types. TGFBR2 had two probe sets with low and one high expression levels. TNFRSF6 probe set had moderate expression levels.

Biological annotation and functional enrichment analysis of differentially expressed genes

The 228 significantly differentially expressed genes were uploaded into DAVID. The resulting top 20 most significant

'annotation clusters' all had terms associated with immune responses although none were associated specifically with autoimmune disease.

The differentially expressed genes were then divided into the two groups of increased expression and reduced expression and again uploaded into DAVID. The majority of annotation clusters for the up-regulated gene list again had terms associated with immune response, including, antigen processing, lymphocyte activation, chemokine activity, regulation of T-cell activation and apoptosis. Genes from this list were also significantly overrepresented in the 'actin cytoskeleton organization' and 'biogenesis' categories.

The DAVID annotation clusters that were significantly overrepresented by the down-regulated gene list involved transcription regulation, cell cycle regulation, leucine-rich repeats, cytoskeleton, cell adhesion and cell communication.

The differentially expressed gene lists were also analysed for GO category using GOTM. Four different lists were uploaded to GOTM – genes increased in expression in adult MGLSc ($n = 51$), genes reduced in expression in adult MGLSc ($n = 87$), genes increased in expression in paediatric MGLSc ($n = 190$) and genes reduced in expression in paediatric MGLSc ($n = 148$).

Analysis of the genes with increased expression in adult MGLSc samples again resulted in the majority of significantly enriched GO categories involved in immune response with the most significant of the overrepresented GO groups being 'immune system' and 'antigen processing'.

The genes with reduced expression in adult MGLSc had no significantly enriched terms at level 4 of the GO hierarchy, although there were several terms that had a higher than expected observed value, including 'signal transduction', 'cellular metabolism', 'regulation of cellular metabolism', 'regulation of physiological processes' and 'gametogenesis'. The most significant of the overrepresented GO groups being 'biogenic amine metabolism', 'development', 'male gamete generation' and 'spermatogenesis'.

Analysis of the genes that showed increased expression in child MGLSc samples again resulted in the majority of significantly enriched GO categories involved in immune response with the most significant of the overrepresented GO groups being 'immune response', 'T cell differentiation', 'cellular defense response', 'immune system', 'response to biotic stimulus', 'response to other organism' and 'process antigen processing'.

Analysis of the genes that showed reduced expression in child MGLSc had no significantly enriched terms at level 4 of the GO hierarchy, although there were several terms which had a higher observed value than expected, including 'cell cycle', 'regulation of cellular physiological processes', 'regulation of metabolism' and 'negative regulation of physiological processes', 'response to DNA damage stimulus'. The most significant of the overrepresented GO groups were 'actin binding', 'development', 'RNA polymerase II transcription factor activity' and 'intracellular non-membrane-bound organelle'.

There were several genes of potential interest identified amongst the list of differentially expressed genes that had significantly reduced expression in MGLSc; diminution of their expression has been found to be associated with other disorders: Smad5, Id4, HOX10, MYOPODIN and CABLES1; the former were down-regulated in both adult and paediatric cases; the last was found to be significantly down-regulated only in adult cases; although the same trend in expression profile was seen in the paediatric cases, it was not significant.

Discussion

Genital lichen sclerosis is a skin disease with a cryptic pathogenesis. It fascinates because not only because of its consequences, being sexual dysfunction and cancer, but also because it involves the three fundamental processes in pathology namely inflammation, fibrosis and cancer. Our results have proved to be illuminating.

We studied men because that reflects the exclusive practice of CBB who instituted the multidisciplinary Male Genital Dermatoses Clinic at the Chelsea & Westminster Hospital nearly 20 years ago. We decided to investigate both adults and boys because it has not been clear that the disease is the same in these age groups; although clinically and histopathologically adult and paediatric MGLSc represent the same disease, this has not previously been confirmed at a molecular level.

In our work, there was very good concordance between profiles of genes increased in expression in disease tissue between adult and paediatric and little evidence for genes having a converse expression profile between adult and paediatric cases samples indicating that adult and paediatric disease most likely represent the same disease entity.

The association of Female Genital LSc (FGLSc) with organ and non-organ-specific autoimmune disorders has been well described in women (Baldo *et al.* 2010a,b; Bunker & Neill 2010; Gambichler *et al.* 2010; Gambichler *et al.* 2011) but this relationship is not so distinct in men (Azurdia *et al.* 1999). We have demonstrated that most of the genes that show increased expression in adult and child MGLSc are involved in 'immune response/cellular defence', at first glance indicating that there might be a relationship. However, genes associated with autoimmunity were initially sought from a database that did not differentiate between generic immune responses and autoimmunity. Genes specifically associated with autoimmune diseases were, therefore, investigated and cross-referenced with the gene profiles from our MGLSc samples. None was found. Recently, Gambichler *et al.* (2009) have shown up-regulation of some antimicrobial peptides and proteins in *female* GLSc and speculate that this represents either an innate immune defence response or may be of direct pathogenic relevance. These moieties were not found to be differentially up-regulated in our study.

Extragenital LSc shares several cutaneous characteristics with lipoid proteinosis (Hamada, 2002). In 2002, lipoid

proteinosis was mapped to chromosome 1q21 and pathogenic loss of function mutations were delineated in the gene for extracellular matrix protein 1 gene (ECM1) on that chromosome (Hamada *et al.* 2003). Autoantibodies to ECM1 have been identified in women with GLSc (Oyama *et al.* 2003) and recently in MGLSc by our group (Edmonds *et al.* 2011). Expression of the ECM1 gene was found to be significantly reduced in the paediatric samples. The implication of these findings in MGLSc will need further study.

The concept that some, if not all, cases of LSc might be caused by an infectious agent is longstanding. *Borrelia burgdorferi* has been incriminated (Bunker & Neill 2010) but is unlikely to be culpable (Edmonds *et al.* 2009). There is only one reported case of GLSc occurring between sexual partners, and in this instance, there was an interval of 10 years (Zapolski-Downar *et al.* 1987). The fact that GLSc does not affect sexual partners means that any putative infectious process must be acquired in a non-sexually communicable manner. Yet HPV has frequently been incriminated in the pathogenesis of GLSc (Drut *et al.* 1998; Nasca *et al.* 2006). There is no denying its involvement in penile SCC. Up to 50% of penile SCC is associated with HPV and 50% with MGLSc but curiously with little overlap: (Campus *et al.* 1992; Perceau *et al.* 2003; Prowse *et al.* 2008). Analogously with vulvar carcinoma, penile SCC has been shown to occur in association with two types of penile lesions: *undifferentiated* (or bowenoid) carcinoma *in situ* (CIS) and LSc-related, *differentiated* CIS and/or squamous hyperplasia, and furthermore it is apparent that the subtype of the SCC is directly related to nature of the adjacent lesions (Renaud-Vilmer *et al.* 2010). We were unable to identify significant over or under expression of any genes associated with HPV infection.

Squamous cell carcinoma of the penis is the most serious complication of MGLSc. The carcinogenic pathway is likely to be HPV-independent (*vide supra*) but has not yet been established. Meffert *et al.* (1995) postulated that GLSc itself is not a premalignant condition but that local factors associated with LSc, such as phimosis, poor hygiene and HPV predispose to malignancy. It is not known whether there is a specific precancerous attribute to GLSc or whether it is a non-specific manifestation of the well-recognised phenomenon whereby SCC complicates chronic inflammation and scarring as is seen in chronic venous ulceration. The pathogenesis of SCC of the skin remains uncertain but is thought to be multifactorial (Quinn & Perkins, 2010). There is a genetic basis, and HPV infection surely plays a role in some instances. Other risk factors include chronic inflammation, immunosuppression and ultra violet (Hussein, 2005) and ionising radiation exposure (Hussein, 2005; Yoshinaga *et al.*, 2005; Quinn & Perkins, 2010).

Work that partially illuminates the pathomechanisms of HPV-independent squamous carcinogenesis complicating GLSc has largely been performed in women and FGLSc. Scurry *et al.* (2001) found that LSc adjacent to vulvar carcinoma showed exaggerated epidermal thickness, basal atypia and loss of the oedematous-hyaline layer. Pinto *et al.* (2000)

suggested that allelic imbalance may predispose to atypia and cancer in FGLSc. Sander *et al.* (2004) postulated oxidative stress as a possible mechanism. The role of p53 in the progression of FGLSc to vulval SCC has also been studied. Tapp *et al.* (2007) proposed that oncogenic point mutations in p53 occur in normal genital skin and are selected for in LSc. Recently, Gambichler *et al.* (2011) have shown significant p53 expression (by immunohistochemistry) in longstanding GLSc. We specifically sought genes implicated in SCC carcinogenesis including p53. No significant expression patterns were found.

We identified several genes that showed reduced expression in MGLSc: Smad5, Id4, HOX10, MYOPODIN and CABLES1. A literature search suggested that of these only MYOPODIN and CABLES1 might be relevant to MGLSc and SCC. MYOPODIN deletion and inactivation has been associated with prostate cancer (Lin *et al.* 2001; Jing *et al.* 2004; Yu *et al.* 2006; De Ganck *et al.* 2009). Loss of CABLES1 expression has been found in approximately 50–60% of primary human colon and head and neck SCC (Wu *et al.* 2001; Dong *et al.* 2003; Zhang *et al.* 2005).

Our findings strongly support the notion that adult and paediatric disease MGLSc represent the same entity. The overall trend of gene expression is interpreted as showing a non-specific inflammatory tissue response. This provides support for the hypothesis that MGLSc is a chronic non-specific inflammatory disease and has no *specific* precancerous properties, although the relationship with the biology of ECM1 and CABLES1 warrants further study.

Conflicts of interest

None.

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