CLINICAL SCIENCE

Minichromosome maintenance 2 and 5 expressions are increased in the epithelium of hereditary gingival fibromatosis associated with dental abnormalities

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INTRODUCTION: Gingiva fibromatosis is a relatively rare condition characterized by diffuse enlargement of the gingiva, which is caused by expansion and accumulation of the connective tissue.

OBJECTIVE: The aim of the present study was to investigate proliferative and apoptotic biomarker expression in normal gingiva and two forms of gingival fibromatosis.

METHODS: Archived tissue specimens of hereditary gingival fibromatosis, gingival fibromatosis and dental abnormality syndrome and normal gingiva were subject to morphological analysis and immunohistochemical staining. The results were analyzed statistically.

RESULTS: Proteins associated with proliferation were found in the nuclei of epithelial cells from the basal and suprabasal layers, whereas apoptotic proteins were detected in the cytoplasm of the upper layers of the epithelium. Increased expressions of minichromosome maintenance proteins 2 and 5 were observed in the gingival fibromatosis and dental abnormality syndrome samples. In contrast, geminin expression was higher in normal gingiva samples. No difference in the expression of apoptotic proteins was observed among the groups.

CONCLUSION: Our findings support a role for augmented proliferation of epithelial cells within the overgrown tissues associated with gingival fibromatosis or dental abnormality syndrome. However, our data suggest that different biological mechanisms may account for the pathogenesis of different types of gingival fibromatosis.

KEYWORDS: Gingival Fibromatosis; Proliferation; Apoptosis; Minichromosome Maintenance; Geminin.

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INTRODUCTION

Gingival fibromatosis (GF) is an oral condition clinically manifested by a generalized and fibrotic enlargement of the gingiva. It may be induced as a side-effect of systemic drugs, or it may be inherited.[1,2] As an inherited disorder, GF may be isolated and referred to as hereditary gingival fibromatosis (HGF), or it may be part of a genetic syndrome.[3] The GF feature has been described as a syndrome associated with hyperthricosis and/or mental retardation syndrome, Zimmermann-Laband syndrome, Murray-Puretic-Drescher syndrome (juvenile hyaline fibromatosis), Rutherfurd syndrome, GF with distinctive facies syndrome, Ramon syndrome, Cross syndrome, Jones syndrome, and prune belly syndrome.[2,4] Recently, GF in association with dental abnormalities (DA) was reported. In this case, thin generalized hypoplastic amelogenesis imperfecta was indicated as the main dental feature.[4]

The minichromosome maintenance (Mcm) protein family is essential for the initiation and elongation of DNA replication and is comprised of six members (Mcm-2 to Mcm-7), which are highly conserved in all eukaryotes.[5] Mcm proteins have been demonstrated to move quickly from the cytoplasm into the nucleus as mitosis is completed where they persist until the next round of division is initiated.[6] Therefore, Mcm proteins have been considered a novel class of proliferation markers, and their expression has been used in the prognosis of different disorders.[7,8] Geminin is a negative regulator of DNA replication that acts as a cell proliferation inhibitor.[9] As geminin is only expressed in the S and M phases of the cell cycle, it has been proposed that geminin could also be a novel proliferation marker.[10] The balance between cell division and cell death is essential for an organism. Apoptosis is a

highly regulated form of cell death and is defined by distinct morphological and biochemical features.[11] Abnormal apoptosis has been implicated in various diseases, including cancer, AIDS, periodontal diseases and gingival overgrowth.[11-15] Apoptosis is regulated by an interplay of gene products that can either act as inducers or inhibitors of the process. Following the discovery of the Bcl-2 gene,[16] several mammalian and viral homologs have been identified. The Bcl-2 family consists of anti-apoptotic genes, such as Bcl-2, and pro-apoptotic genes, including Bax.[15]

The aim of this study was to determine the expression of proliferative (Mcm-2, Mcm-5 and geminin) and apoptotic (Bcl-2 and Bax) proteins in samples from two distinct families affected by an isolated form of GF and one family affected by GF and DA syndrome.

MATERIALS AND METHODS

Tissue samples. This cross-sectional study included gingival samples from members of two families with HGF and one family with GF and DA syndrome. Clinical, histological and genetic data from these three families were previously reported. Six tissue samples were selected from the first HGF family[17] (designated GF Family 1 for the purposes of this study). Four tissue samples were used from the second HGF family[18] (designated GF Family 2). Four samples were also used from the family affected by GF and DA syndrome[4] (designated GF Family 3). Ten samples of normal gingiva (NG) were used as controls. New sections from paraffin-embedded blocks were stained with hematoxylin and eosin (H&E) or used for immunohistochemical analysis. This study was carried out in accordance with the World Medical Association Declaration of Helsinki and with approval of the Human Research Ethics Committee of the University. All of the patients gave consent for this study.

Immunohistochemical analysis. Immunohistochemistry was performed using the streptavidin-biotin method. Briefly, after de-waxing and hydration in graded alcohol solutions, sections were treated with 3% H_2O_2, followed by incubation with 10 mM citric acid pH 6.0 in an electric pressure cooker to allow for antigen retrieval. After washing with phosphate-buffered saline (PBS), sections were incubated with primary antibodies (anti-human Mcm-2 and -5, diluted 1:20, Novocastra; anti-human geminin, diluted 1:40, Novocastra, Newcastle Upton, Tyne, UK; anti-human Bax and Bcl-2, diluted 1:50, Dako Corp., Carpenteria, CA, USA). Following incubation with Mcm-2, Mcm-5 or geminin antibody, slides were washed in PBS and incubated with the secondary antibody (StreptABComplex/HRP, Dako Corp., Carpenteria, CA, USA, diluted 1:20) for 30 min at 37℃. Slides were then incubated with the streptavidin-biotin complex (StreptABComplex/HRP, Dako, diluted 1:300) for 30 min. Sections incubated with antibodies against Bcl-2 and Bax were incubated with the LSAB system (Labeled Streptavidin Biotin, Dako, diluted 1:100) for 30 min at 37℃. Reactions were developed by incubating the sections with 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) containing 0.01% H_2O_2. Negative and positive controls were performed for each antibody. For immunohistochemical assessment, the method previously described by Kantarci et al.[14] was used. The orientation of each sample and the identification of tissue sites were determined at 100x and 400x magnification. Five sites were defined and used to determine the labeling index (LI), which is expressed as the percentage of positive cells at 1000x magnification with the aid of the image computer analyzer (Nikon NIS-Elements-2.35, Nikon Corporation, Melville, USA) for quantification. The sites were chosen to represent basal and suprabasal cell layers of the epithelium. The absence of staining was considered negative.

Statistical analysis. Data are presented as the mean ± SD for each group. The Kruskal-Wallis multiple comparison test was used to test group effects, and Spearman's correlation test was performed to determine the correlations between immunohistochemical markers. In our comparisons, $p<0.05$ was considered indicative of statistical significance.

RESULTS

Histological examination of gingival overgrowth tissues from both GF Families 1 and 2 stained with H&E revealed very similar findings. The gingival tissues included a well-structured epithelium with elongated and thin papillae inserted into deep fibrous connective tissue with collagen fiber bundles running in all directions (Fig. 1A-1C). In GF Family 3, a large number of cementicles and odontogenic epithelium rests were observed in the connective tissue

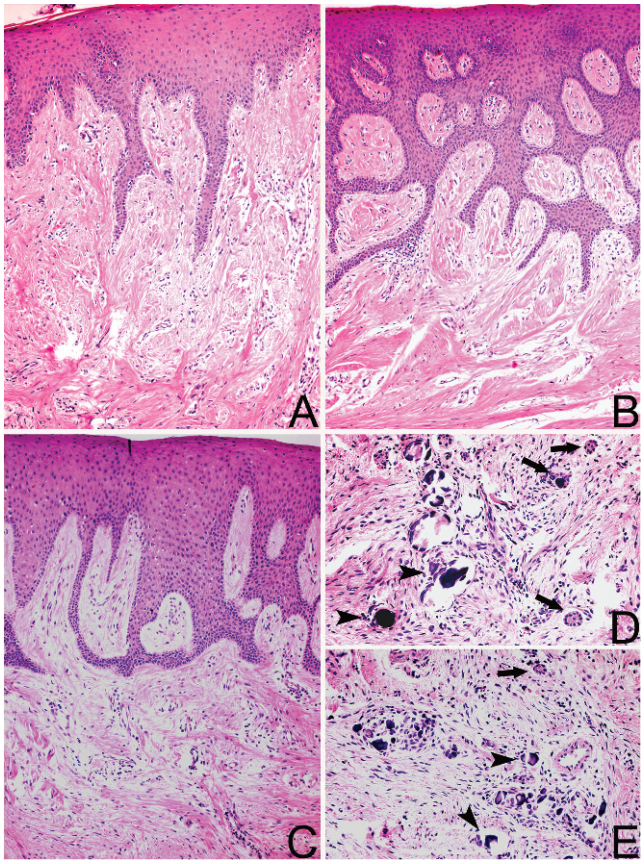

Figure 1 - Histological features of the different GF forms in this study. Shown here are representative samples of GF Family 1 (A), GF Family 2 (B), and GF Family 3 (C-E). GF samples revealed a well-structured epithelium with elongated and thin papillae inserted into deep fibrous connective tissue with collagen fiber bundles running in all directions. Additionally, GF tissues from Family 3 demonstrated calcifications and odontogenic epithelial rests in the dense fibrous connective tissue (D and E) (original magnification 100x).

in addition to the previously mentioned features (Fig. 1D and 1E).

Nuclear immunoreactivity for Mcm-2, Mcm-5 and geminin was identified in the basal and suprabasal cell layers of the epithelium, whereas immunoreactivity for Bcl-2 and Bax was detected in the cytoplasm of the upper layers of the epithelium (Fig. 2). The staining pattern of the apoptotic markers was fine and granular with reactivity scattered throughout the cytoplasm. Some inflammatory cells demonstrated immunoreactivity for Bcl-2. Normal mesenchymal cells were not stained by any of the antibodies tested. Both Mcm-2 and Mcm-5 LIs were significantly higher in GF Family 3 as compared with NG or GF Family 1, whereas the LI of geminin was significantly higher in NG as compared with GF Family 2. A strong and positive correlation between the LIs of Mcm-2 and Mcm-5 was found ($r_s = 0.54$; $p = 0.006$). No statistical differences for Bcl-2 or Bax LIs were observed in this study. Table 1 summarizes the immunohistochemical results obtained in the present study.

DISCUSSION

The exact causal mechanism resulting in the accumulation of excessive gingival tissue in GF remains unclear, and inquiries of GF pathogenesis have largely focused on the connective tissue alterations. It has been proposed that GF develops through activation or selection of the resident tissue fibroblasts, which is phenotypically characterized by increased proliferation, low levels of extracellular matrix-degrading metalloproteinases (MMP-1 and MMP-2) and abnormally high collagen production.[19,20] Histologically, it has been determined that GF gingiva demonstrates epithelial alterations such as higher and deeper epithelial papillae. Previous studies have demonstrated that GF tissues have a significantly higher number of Ki-67-positive cells as compared with NG epithelia,[18] and the expression of epidermal growth factor (EGF) and its receptor (EGFr) is positively correlated with the proliferative potential of the cells in the papilla tip area.[21] Data from the present study revealed that all gingival samples were positive for Mcm-2, Mcm-5 and geminin expression in the epithelial tissue, although significant differences in their expression levels were observed. A positive correlation between Mcm-2 and Mcm-5 immunohistochemical expression was observed, and both markers were more highly expressed in samples from GF Family 3 as compared with samples from either the NG group or the GF Family 1 group. The anti-geminin antibody reacted with a lower percentage of epithelial cells, although its levels were significantly higher in the NG group as compared with the GF Family 2 group. Additionally, Mcm-2 exhibited higher levels of expression than Mcm-5 and geminin. Finally, no differences in the levels of apoptotic markers Bcl-2 and Bax were observed among the groups.

Although an increase in the expression of proliferation markers in gingival overgrowth may be expected when compared with NG samples, we did not observe this result in all groups. Likewise, one might expect fewer apoptotic cells to be present in proliferating tissues. Nonetheless, these results may be explained by the intense clinical, genetic and biological heterogeneity of GFs. Previous studies have demonstrated that differences often occur in these diseases. For instance, although the disease manifested as an isolated finding in both GF Families 1 and 2, it was more severe and frequently associated with both aesthetic and functional

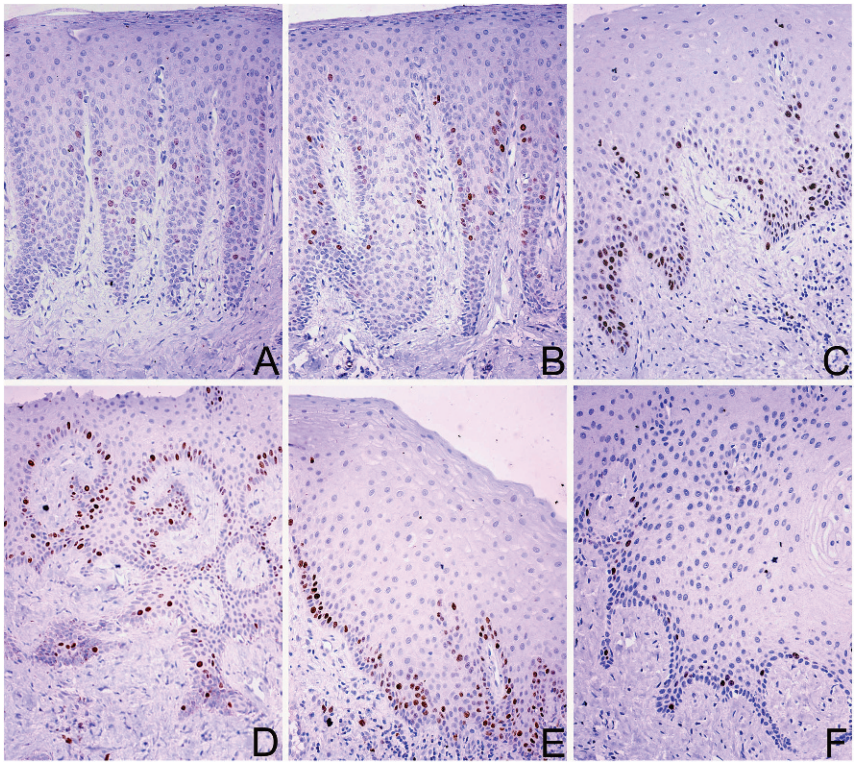

Figure 2 - Pattern of Mcm-2, Mcm-5 and geminin immunohistochemical staining in NG (top panel; A-C) and GF Family 3 (bottom panel; D-F) samples. Note that Mcm-2-positive cells (A and D) and Mcm-5-positive cells (B and E) were increased in number in GF Family 3, and that geminin-positive cells (C and F) were abundant in the NG sample (original magnification 200x).

Table 1 - LIs of Mcm-2, Mcm-5, geminin, Bax and Bcl-2 in NG and GF tissues.

Tissues	Mcm-2	Mcm-5	Geminin	Bax	Bcl-2
NG	39.52±7.68	21.51±15.99	24.06±6.49***	6.09±2.00	3.18±3.41
GF Family 1	45.03±8,39	30.71±29.34	20.01±4.61	9.61±2.45	2.73±4.40
GF Family 2	51.97±5.56	45.97±20.53	12.24±4.29	2.24±3.00	3.06±5.39
GF Family 3	58.44±4.86*	60.73±6.47**	20.96±1.96	3.79±3.00	3.99±5.39

*LI in GF Family 3 was significantly higher than in NG ($p<0.01$) and GF Family 1 ($p<0.05$).
**LI in GF Family 3 was significantly higher than in NG ($p<0.01$) and GF Family 1 ($p<0.05$).
***LI in NG was significantly higher than in GF Family 2 ($p<0.05$).

problems in GF Family 1. Associated problems included prominent lips and open lip posture, prolonged retention of primary dentition due to a delay in the eruption of permanent teeth, diastemas, malpositioning of the teeth, and cross or open bites.[22] Moreover, disease recurrence was more frequent in patients from GF Family 1 than it was in patients from GF Family 2, and the unaffected individuals in GF Family 2 transmitted the disease to their offspring in an autosomal dominant pattern despite being clinically unaffected themselves.[22] On the other hand, members of GF Family 3 transmitted the disease in an autosomal recessive mode of inheritance in association with generalized thin hypoplastic amelogenesis imperfecta, pulpal calcifications, root dilacerations, hypodontia, delay of tooth eruption and pericoronal radiolucencies in unerupted teeth.[4] Moreover, myofibroblasts, the main cell type involved in extracellular matrix deposition in fibrotic diseases, were found in abundance in the gingival tissues of patients from GF Families 2 and 3 but not GF Family 1.[22] Another uncommon finding, which supports the heterogeneity of GF, was that a broad distribution of calcified psammomatous structures associated with odontogenic epithelial remnants were present in the overgrown gingiva in GF Family 3. Together, these findings suggest that GF is associated with distinct biological mechanisms, which may be associated with the genetic defect.

The results presented here also revealed that there were no apoptotic or proliferating fibroblasts in the connective tissue of either NG or GF samples. This result is in agreement with a study by Buduneli et al.,[23] which showed that no Ki-67- or TUNEL-positive fibroblasts were found in the connective tissue of cyclosporin A (CsA)-induced gingival overgrowth. However, the authors demonstrated a significantly lower number of apoptotic keratinocytes in the CsA group as compared with gingivitis or healthy control groups, which suggests that decreased apoptosis may play a prominent role in the pathogenesis of CsA-induced gingival overgrowth. Handajani et al.[24] showed that nifedipine treatment resulted in the increased expression of Bcl-2 by gingival keratinocytes in a dose- and time-dependent manner. Another study suggested that c-Myc and Bcl-2 may be associated with the pathogenesis of the gingival overgrowth induced by nifedipine and phenytoin, particularly with the morphogenesis of the hyperplastic gingival epithelia that exhibited increased cell mitotic activity.[25] In contrast, Kantarci et al.[14] identified both PCNA-positive and TUNEL-positive fibroblasts in the connective tissue of phenytoin-, CsA- and nifedipine-induced gingival overgrowth as well as in GF. These authors demonstrated that, independently of the gingival overgrowth tissue, the number of fibroblasts in apoptosis was significantly reduced as compared with the NG control, while the proliferative potential of these cells was significantly higher than in the control samples. These differences may have been related to the methodology as both the PCNA antibody and TUNEL assays are low specificity methods.

In conclusion, our results confirmed the heterogeneity of GF and demonstrated that gingival epithelial cells from GF and DA syndrome have an elevated proliferative potential. Although no difference in Bcl-2 or Bax expression among GF and NG groups was observed, future studies are necessary to determine the exact role of apoptosis in the various forms of GF and whether it contributes to GF pathogenesis.

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