GENETIC COUNSELING, Vol. 19, No 2, 2008, pp 183-192

TUMORAL CALCINOSIS DUE TO GALNT3 c.516-2A >T MUTATION IN A BLACK AFRICAN FAMILY

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Summary: *Tumoral Calcinosis due to GALNT3 C.516-2A >T mutation in a black African family:* Familial Tumoral Calcinosis (FTC) is a rare autosomal recessive disorder of the phosphocalcic metabolism caused by mutations in the FGF23 or GALNT3 genes. We have identified a Beninese family in which two brothers present FTC caused by a homozygous A>T transversion at the acceptor splice site in intron 1 of GALNT3 gene. We report on the clinical, biochemical, histopathological and molecular spectrum of the disorder in this family. The particularly severe phenotype, the amelogenesis imperfecta, and the carbapatite deposit observed in these patients, seem to be characteristic of our observations.

Key-words: Familial Tumoral Calcinosis – Phosphocalcic metabolism disorder – GALNT3 mutation

INTRODUCTION

Familial Tumoral Calcinosis (FTC) [OMIM 2119000] is a rare autosomal recessive disorder of the phosphocalcic metabolism (14). Patients present periarticular calcium deposits in skin and subcutaneous tissues which often result in local tumefactions on the level of the hips, shoulders and knees. Hyperphosphataemia, secondary to increased renal phosphate retention, is the major metabolic abnormality associated with FTC and is accompanied by normal or elevated 1,25 $(OH)_2$ Vit D3, and normal serum levels of calcium and parathyroid hormone (PTH).

Mutations in the FGF23 and UDP-N-acetyl-alpha-D-galactosaminyl-transferase 3 (GALNT3) genes which code respectively for a phosphaturic protein and a Golgi's glycosyltransferase responsible for the O-glycosylation of proteins, account for the majority of FTC cases (4, 16, 20). O-glycolylation by normal GALNT3 regulates intact FGF23 secretion which is required in renal clearance of phosphates (7, 8, 11). This condition, although well described by most authors in melanoderm people, has rarely been documented in sub-Saharan Africans. Here we report on a documented case of FTC due to a GALNT3 c.516-2A >T mutation in a black African family.

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CASE REPORT

Two brothers, 19 and 17 years old respectively, were referred because of swellings around the hip and shoulder joints. The painless masses appeared during the second year of life and progressively caused limitation of movements because of their increasing size. Sometimes, skin ulcerations occurred with drainage of a chalky milk-like fluid. This condition was recurrent after surgical excision. The clinical examination revealed that tumours were renitent, multiple, and localised near articulations. The articulations affected in the older patient were shoulders, hips and the left knee while those affected in his younger brother were the left shoulder, hips and the right elbow. They showed a generalised gingivitis, multiple tooth decay and many tooth hypoplasia. Moreover, a loss of vision and a light divergent strabismus was noted in the older patient.

Blood sampling, X-ray examination, biochemical analyses and tumour biopsies were performed on the patients. From these index-cases, other members of the family were found and examined. Patients' photographs and X-rays are shown in figure 1; their biochemical data are summarised in Table 1.

In the radiological view, the affected joints showed a hypercondensation in soft tissue with no bony structures involvement (Fig.1c). The matrix of this condensation was made up of small lodges like honeycombs which is traditional in the FTC.

Moreover, in addition to the generalized gingivitis and the multiple teeth decay, an attack of enamel on the vestibular face of the occlusal edge of the higher central incisors in one of the patients and the side incisors in the other was noted (Fig.1d). These morphological aspects evoked a punctual amelogenesis imperfecta in a band limited to the vestibular face of the central higher incisors. Some teeth roots appeared short and bulbous with partial obliteration of the pulp. (Fig.1e). Moreover, tooth resorption was visible, in particular on teeth number 12 and 22, thus giving a "baby teeth" aspect. (Fig.1f).

The microscopy of the tumour biopsies showed large basophilic calcic areas observed with von Kossa stain within a fibrous conjunctive tissue. These damages occurred in conjunctive tissue which is surrounded by chronic inflammatory reaction with giant cells, macrophages and foamy histiocytes (Fig. 2). The infra-red spectrometry of the tumours' content disclosed phosphate of carbonated calcium (Fig. 3).

Genomic DNA was extracted from blood using standard techniques and sequencing of the ten exons of the GALNT3 gene was performed (ABI 3130XL Genetic Analyser, Applied Biosystems). A homozygous A>T transversion at the acceptor splice site in intron 1 was identified

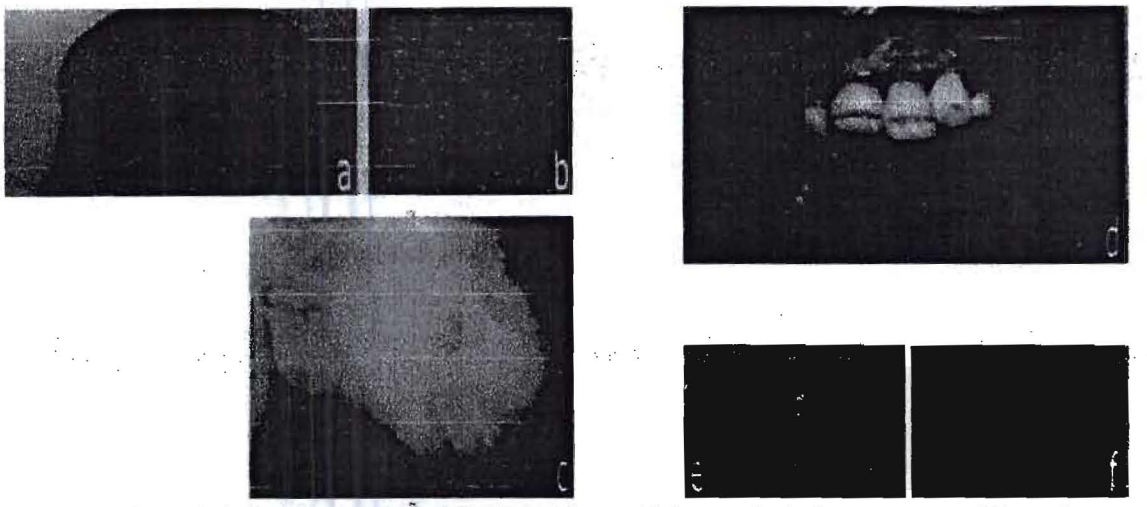


Figure 1: FTC morphological features: (a) and (b) show patients with large periarticular tumors around hips and shoulders; (c) Radiographic film of the right hip showing calcification in the soft tissues around the joint; (d) dental alteration: amelogenenesis imperfecta; (e) and (f) important rhyzalysa on certain teeth and partial calcification on pulpar cavities showing bulbous and short racines.

Table I: Biological feature of the FTC patients

Biological parameters	Patient 1	Patient 2	Reference values
Calcium	2.04 mmol/L	2.09 mmol/L	2.10-2.65 mmol/L
Phosphore	**2.48 mmol/L**	**2.91 mmol/L**	**0.80-1.40 mmol/L**
Alkalin phosphatise	109 U/L	184U/L	40-130 U/L
Creatinin	52 µmol/L	55 µmol/L	50-120µmol/L
Uricemia	455 µmol/L	345 µmol/L	113-547 µmol/L
Calciuria	3.41 mmol/L	0.13 mmom/L	
Phosphaturia	41.87 mmol/L	13.33 mmol/L	
Parathormone	**14.4 ng/L**	**37.4 ng/L**	**10.0-69.0 ng/L**
25-OH vitamine D	17.6 ng/L	12.5 ng/L	10-40 ng/L
1.25-$(OH)_2$ Vitamine D	**152.4 pg/mL**	**169.2 pg/mL**	**20-67pg/mL**

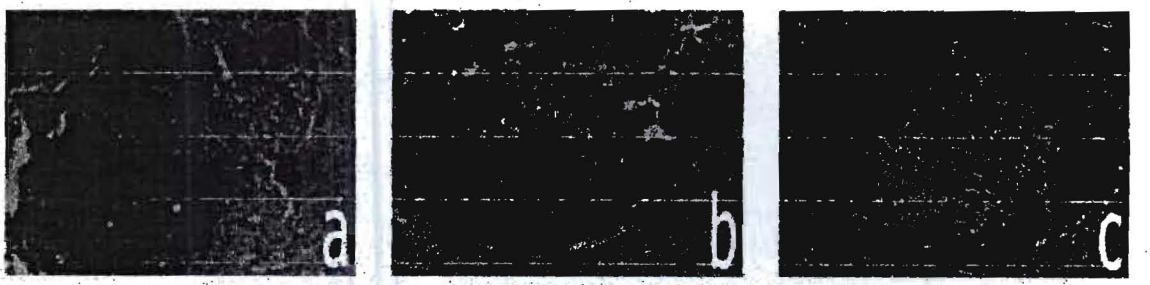


Figure 2: Histological findings within surgical biopsy of the masses fixed in 10% formol and paraffin embedded: (a and b) haematoxylin-eosin (H & E) staining showing fibrosis and chronic inflammatory cells; (c) Von Cosa staining presenting with irregular calcified deposit.

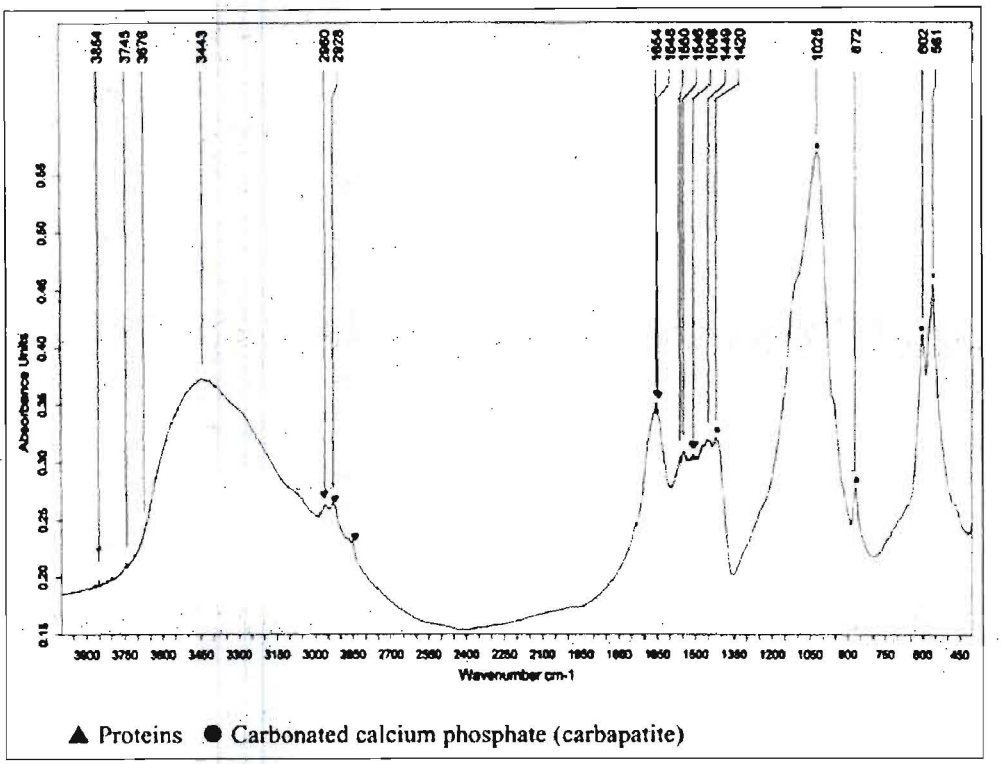


Figure 3: Infrared spectrometry of the tumor's content discloses carbapatite bands.

in both patients: c.516-2A>T. Mutation analysis performed in the patients' family showed that both parents, as well as 3 of their sisters carried the mutation in the heterozygous status, whereas one sister was homozygous for the wild type allele. All heterozygous individuals were asymptomatic. The family pedigree is shown in figure 4.

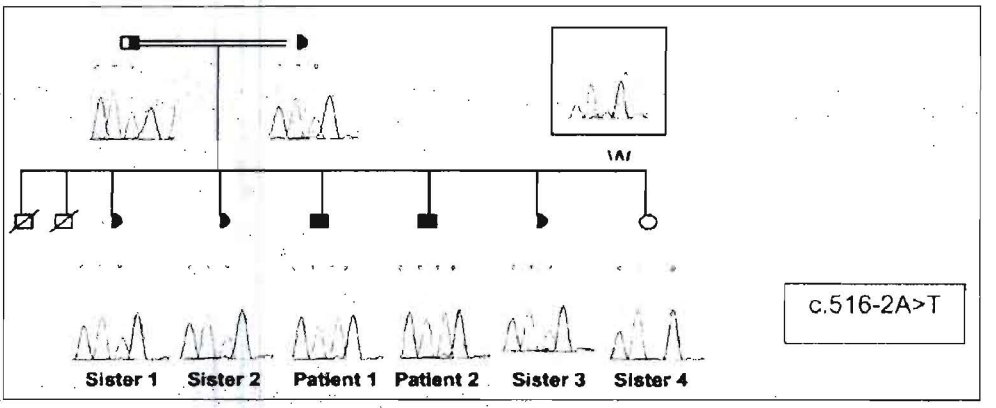


Figure 4: Pedigree of the family and mutation analysis: Segregation of the GALNT3 c.516-2A>T transversion within the family. The affected individuals are homozygous for the mutation; the parents and 3 sisters are heterozygous. c.516-2A>T is a splice site mutation occuring in intron 1.

In order to investigate the effect of the mutation by reverse transcription PCR (RT-PCR) analysis, total RNA was purified from fibroblast cultures of both patients and a control individual using the Trizol reagent system (Invitrogen) according to standard techniques.
RT-PCR using primers located in exons 1 and 3 showed that whereas a 404 bp PCR product was seen in the control individual, both patients displayed a 231 bp PCR product, suggesting that it lacked exon 2 which is 173 bp long (Fig. 5a). Sequencing of this smaller RT-PCR product (Fig. 5b) showed that exon 1 was directly followed by exon 3, thus demonstrating that exon 2 was skipped (r.516_587del). The lack of exon 2 produced a frame shift in exon 3. Therefore the expected effect of the mutation was the appearance of a STOP codon after four amino acids into exon 3 (p.Cys173ValfsX4), resulting in a truncated protein that lacked not only the amino acids encoded by exon 2, but also the part of the protein encoded by exons 3 to 10. This was likely to lead to a total loss of function of the protein.

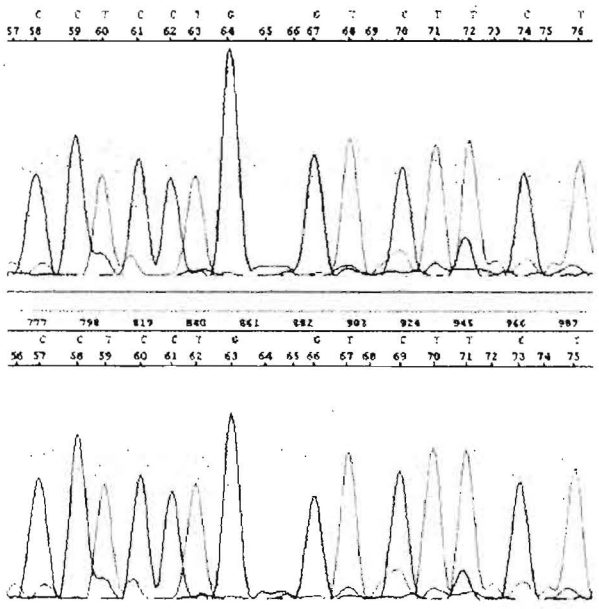
DISCUSSION

Familial Tumoral Calcinosis (FTC) is a well-described rare autosomal recessive disorder of the phosphocalcic metabolism. Until this report, close to 100 cases have been reported (15), mostly in blacks, but rarely in sub-Saharan Africans. This disease is also rare in Europe and in North America (12).We now have documented an African family affected by FTC due to a GALNT3 c.516-2A >T mutation.
The two cases described in this family fulfil the traditional features of tumoral calcinosis, but show some particularities. In most cases the tumours are rarely symmetrical (2); but this is seen in one of our patients.
Odontological anomalies like dental hypoplasia, short and bulbous dental roots and pulp cavities' occlusion are typical signs of familial tumoral calcinosis (9), whereas an enamel growth anomaly, such as the punctual amelogenesis imperfecta, seen in our patients is seldom reported.
The dental abnormalities observed in the patients suggest that *GALNT3* mutations play a role in dental embryogenesis. The variety of the damages is probably due to differences in the embryological stages at which they occur. This aspect should be studied in more depth.
Although the cream-like aspect of the tumour content after surgical ablation could wrongly lead to the suspicion of tuberculosis, the infra red spectrometric analysis has revealed that the tumours are made of phosphate carbonated calcium. According to some authors, the tumour

Figure 5: Analysis of the GALNT3 mRNA in the two affected patients.
5a: RT-PCR analysis of GALNT3 exons 1-3 in the patients' fibroblasts.
1) RT-PCR of control fibroblasts; 2) 100 bp Ladder ; 3) RT-PCR of Patient 1 fibroblasts; 4) RT-PCR of Patient 2 fibroblasts ; 5) Blank.
5b: Sequencing of mRNA extracted from the patients fibroblasts. The wild type sequence is shown at the top of the Figure. The sequence of exon 2 that is skipped in the patients'mRNA, is underlined. In orange is shown the end of exon 1, and in pink the beginning of exon 3.

Wild type mARN

CTTGGACCAGACACTCGACCTCCTGAATGTATTGAACAAAAATTTAAGCGCTGCCCTCCCCTGCCCACCACCAGTGTCAT
AATAGTTTTTCATAATGAAGCGTGGTCCACGTTGCTTAGAACTGTCCACAGTGTGCTCTATTCTTCACCTGCAATACTGCT
GAAGGAAATCATTTTGGTGGATGATGCTAGTGTAGATGAGTACTTACATGATAAACTAGATGAATATGTAAAACAATTTTC
TATAGT



content would be hydroxyapatite deposit (9) or a mixture of carbonated calcium, phosphate calcium and hydroxyapatite crystals (5). This structural difference could be the cause of the variety in the tumour's consistency which can be soft or solid. In fact, hydroxyapatite crystals could lead to the content's ossification while carbapatite could not.

The pathological process which leads to the calcic deposit in soft tissue could be related to micro-haemorrhages due to vascular damages, followed by foamy histiocytes' aggregation.

For Crook and Silver (6), it is a collagen necrobiosis with formation of cysts and granulomatosis reaction. The cavitary lesions are secondarily occupied by calcic material around which a fibrosis and sometimes a bony metaplasia occur (13).

One distinguishes two types of tumoral calcinosis: (1) the sporadic form, observed in some renal complications, chronic dialysis or systemic diseases (primary parathyroid disease, hypervitaminosis D, neoplasic syndrome and sarcoidosis) and (2) the familial form or tumoral calcinosis with hyperphosphataemia.

For a long time, the familial form of tumoral calcinosis was regarded as an autosomal dominant disease (12). Recent works show that the disease is rather autosomal recessive. It realizes a mirror image of some well known syndromes such as X linked hypophosphataemia (XLH) and autosomal dominant hypophosphataemia rickets (ADHR). These diseases are due to function mutations in genes regulating phosphates homeostasis: PHEX (phosphates regulating gene with homology to endopeptidases on the X chromosome) and the fibroblastic growth factor 23 (FGF23) (3, 18).

Mutations in the FGF23 and UDP-N-acetyl-alpha-D-galactosaminyl-transferase 3 (GALNT3) genes which code respectively for a phosphaturic protein and a Golgi's glycosyltransferase responsible for the O-glycosylation of proteins, have been identified in families with familial tumoral calcinosis (4, 16, 20). Very recently, mutations of a third gene, SAMD9, coding for the sterile alpha motif domain-containing-9 protein, were identified in the normophosphatemic form of the disease (17).

GALNT3 encodes a glycosyltransferase belonging to a large family of Golgi-associated biosynthetic enzymes that transfer GalNac from the sugar donor UDP-GalNac to serine and threonine residues and are thereby responsible for initiating O-glycan synthesis, a prevalent form of post-translational modification.

In the present study, we have analyzed two brothers with FTC and found that they are homozygous for the c.516-2A>T mutation in the GALNT3 gene. According to Ichikawa *et al.* (10), based on a software analysis, this mutation would lead to GALNT3 truncated protein by

frameshift with loss of the exon 2 translation. This leads to glycosylation defect. Functional studies performed by RT-PCR on RNA extracted from our patients' fibroblasts showed that the mutation not only leads to exon 2 skipping as suggested by Ichikawa *et al.* (10), but has actually a more profound effect, because it leads to the production of a truncated protein that lacks all domains encoded by exons 2 to 10. Indeed, the skipping of exon 2 leads to an out of frame translation that results in the occurrence of a STOP codon after four amino acids into exon 3 (p.Cys173ValfsX4; r.516_587del). The protein, if it is produced at all, is therefore expected to lack the following domains: 1) a polypeptide N-acetylgalactosaminyltransferase domain; 2) a Golgi stack domain found in proteins involved in the maintenance of the Golgi apparatus integrity, including fragmentation of the Golgi apparatus during apoptosis and docking of transport vesicles with the Golgi membranes; 3) a Glycosyl transferase 2 domain found in a diverse family of glycosyl transferases that transfer the sugar from UDP-glucose, UDP-N-acetylgalactosamine, GDP-mannose or CDP-abequose to a range of substrates including cellulose, dolichol phosphate and teichoic acids, and 4) a Ricin B lectin that has been shown to bind simple sugars, such as galactose or lactose domain.

This c.516-2A>T mutation in GALNT3 leading to a probable total loss of function of the truncated protein confirms that defective expression of this transferase is the molecular cause of FTC in this family. The total loss of GALNT3 function probably accounts for the severe phenotype observed in the two patients studied here. We believe that this is due to the lack of activation of FGF23 which is necessary for the renal clearance of calcium and phosphates. This strengthens the hypothesis that defective O-glycosylation mediated by GALNT3 is the molecular mechanism underlying the aetiology of FTC.

Other changes in the *GALNT3* and *FGF23* genes have been identified with familial tumoral calcinosis by several authors (1, 19, 20).

In summary, our case report illustrates the clinical and biological aspects of the familial tumoral calcinosis in a black sub-Saharan family and the phenotype heterogeneity in this disease that is not well known. The c.516-2A>T mutation in GALNT3 gene, the amelogenesis imperfecta and the carbapatite deposit underline the particularity of this report.

ACKNOWLEDGEMENTS

Sequence analysis was performed on the Genotyping and Sequencing Platform of Bordeaux. This platform was constituted thanks to grants from the Conseil Régional d'Aquitaine (n°20030304002FA and n°20040305003FA) and from the FEDER (n°2003227).
The authors are grateful to:
- Dr. Denise Higueret for spectrometric analysis of tumours,
- Dr. Didier Griffiths for advice in dental XRay interpretation
- Belgian Air Forces for their logistic assistance
A. Laleye was recipient of a grant from the Ministry of Foreign Affairs of France (Bourse Charcot).

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