

Thr300Ala ATG16L1 Polymorphisms and Bone Strength in Crohn's Disease Patients

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Abstract

Introduction: Studies on the effect of deletion of ATG5 and ATG7 proteins on bone cell function and bone strength in laboratory mice have revealed an association between autophagy and osteoporosis. The effect on bone strength of the Thr300Ala variant (rs2241880 polymorphism) of the ATG16L1 gene, a Crohn's disease susceptibility gene essential in macro-autophagy, has not yet been explored.

Methods: 101 Crohn's disease patients underwent DEXA bone density scanning. Real time PCR, high resolution melt (HRM) and restriction fragment length polymorphism (RFLP) were made use of as to assess for the rs2241880 polymorphism of the ATG16L1 gene in these patients.

Results: HRM and RFLP demonstrated that 39.6% had the wild type rs2241880 (Thr300Ala) polymorphism while 7.9% were homozygous and 52.5% were heterozygous for the polymorphism. Mean DEXA bone mineral density scores in these patients showed lower T scores at the hip (-1.74) among patients with the homozygous polymorphism than among patients with the heterozygous polymorphism (mean T score hip: -1.29). The highest mean T scores were found in patients with the wild type polymorphism (-1.04).

Discussion: This study demonstrates the first evidence that polymorphisms in the ATG16L1 gene may play a role in bone metabolism.

Keywords

Osteoporosis; Autophagy; Crohn's Disease; ATG16L1

Introduction:

The ATG16L1 (autophagy-related 16-like) gene, essential in macro-autophagy, is located on chromosome 2q37.1.¹ The Thr300Ala variant (rs2241880 polymorphism) of this gene, located in the c-terminal WD40 domain,¹ is an important

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Crohn's disease (CD) susceptibility gene. Osteoporosis is common among CD patients^{2,3} being associated with an increased risk of fracture.

The autophagy pathway appears to play an important role in bone metabolism and bone diseases, by having a direct effect on osteoclast, osteoblast and osteocyte function.⁴ Autophagy maintains cellular homeostasis and allows longer-surviving cells like osteocytes, to function normally. In conditions of increased stress, autophagy is increased, resulting in recycling of intracellular components like amino acids and prolonging cell survival.⁵ Autophagy also allows osteocytes to adjust to a more hypoxic and nutrient poor environment. Osteocytes play a key role in bone remodelling by synthesizing sclerostin, receptor activator of nuclear factor kappa B-ligand (RANKL), fibroblast growth factor-23 and collagens. Osteocyte autophagy plays an important role in age-related bone loss.⁶ Deletion of the autophagy protein Atg7 from osteocytes resulted in reduced bone mass in mice and suppressed autophagy in osteocytes appears to lead to age-related bone loss.

Autophagy is also important in osteoclasts, as has been demonstrated using conditional knockout mice. Deletion of key proteins involved in autophagosome formation, like Atg5, resulted in increased bone volume in vivo and protection from ovariectomy-induced bone loss. Deletion of ATG7 was associated with reduced resorptive capacity of osteoclasts. This data seems to suggest that inhibition of autophagy in osteoclasts may provide a potential therapeutic mechanism for various bone disorders. Osteoclast formation and size is increased by hypoxia⁷⁻⁸ and upregulation of autophagy has been shown to increase this effect.⁹ However,

Rapamycin, an autophagy inducer, has been shown to decrease the number of osteoclasts and to be associated with reduced bone resorption in laboratory rats.¹⁰ More in vivo and in vitro studies are needed to better understand the role of autophagy in regulating osteoclast function.

Osteoblasts are also under autophagic control.¹¹ Animal models have shown that impaired autophagy is associated with severe osteopenia due to reduced bone formation.¹² Rapamycin appears to promote osteoblast differentiation therefore suggesting that autophagy increases bone formation while impaired autophagy may be associated with an increased risk of osteoporosis.¹³

Studies analysing the association between autophagy and osteoporosis have mainly investigated the effect of deletion of ATG5 and ATG7 proteins on bone cell function and bone strength in laboratory mice. No studies on the effect of impaired autophagy secondary to polymorphisms in the ATG16L1 gene on bone strength in humans have been carried out to date.

Impaired autophagy and CD have both been shown to be associated with an increased risk for osteoporosis and osteopenia. Impaired autophagy has also been shown to be one of the pathways involved in mucosal inflammation in CD, with a higher overall risk of CD in patients with the rs2241880 ATG16L1 polymorphism. However, this polymorphism has never been studied as a potential risk factor for osteoporosis. We therefore hypothesized that CD patients with impaired autophagy secondary to the rs2241880 ATG16L1 polymorphism have lower bone mineral density dual energy X-Ray absorptiometry (DEXA) scores than patients not exhibiting this polymorphism.

Methodology

Ethical approval was obtained through the University of Malta Research and Ethics Committee. Maltese CD patients diagnosed through standard clinical, histo-pathological and endoscopic findings were recruited prospectively through the gastroenterology clinic at Mater dei Hospital (MDH), Malta.¹⁴ All CD patients seen at medical out-patients between September 2012 and June 2014 were invited to participate. Patients with indeterminate colitis and individuals who did not have Maltese ancestry were not included in the research. CD diagnosis was defined according to the Copenhagen Diagnostic Criteria.¹⁵ Written informed consent was obtained from each patient. Each patient was asked questions related to his duration, type, location and severity of Crohn's disease, ongoing and previous treatments and the duration of this treatment, history of previous fractures and the aetiology behind such fractures. Where possible, this information was corroborated with information from the patients' files. All data was entered into a tailor-made database. All patients underwent a DEXA bone mineral density scan at the MDH using a Hologic DEXA scanner. T score (comparison of bone density with peak bone mass at around age 30) was used to assess risk of fracture with T scores <-2.5 being indicative of osteoporosis and T scores between -1 and -2.5 being suggestive of osteopenia. However, since a section of the population included young, premenopausal women and men younger than 50 years of age, the Z score was also used as a marker of bone density.¹⁶

Genotyping for the common coding variant rs2241880 (Thr300Ala) of the ATG1611 gene was carried out on peripheral venous blood extracted from the CD patients. Three millilitres of whole blood

was extracted from each patient and collected in an ethylenediamine tetraacetic acid (EDTA) tube. Deoxyribonucleic acid (DNA) extraction from whole blood of these CD patients was carried out using the DNA Mini Kit (Qiagen, Hilden, Germany).¹⁷ Gradient polymerase chain reaction (PCR) was then carried out to establish the optimal annealing temperature for this variant. Real-time PCR and high resolution melt (HRM) were subsequently carried out at annealing temperatures of 54°C (optimal temperature found at gradient PCR). The reaction mixture for real time-PCR and HRM consisted of 4.0µl of 5x Hot FirePol® EvaGreen® qPCR Mix Plus (*Solis BioDyne™*), 0.5µl of 10µM primers F and R, 14.0µl of distilled water and 1µl of template DNA. The samples were then run through qRT-PCR and HRM under the following conditions: initiation at 95°C for 5 minutes, denaturation at 95°C for 10 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 10 seconds (denaturation, annealing and extension were repeated for 40 cycles) and HRM at 75–95 °C. The results obtained using HRM were then compared with the results of restriction enzyme digest of the PCR product. Restriction fragment length polymorphism (RFLP) was carried out using the SfaNI/LweI enzyme (*NEB Inc*). The reagent mixture consisted of 2µL of NE Buffer 4 (X10), 1µL of the SfaNI/LweI enzyme (2,000 units/mL), 9µL of DNase and RNase free water, and 8µL of the DNA under study (0.5-1µg/µL). 5µl of this mixture was added to 2µl of 6X DNA loading dye buffer double blue and then loaded on 2.5% agarose gel. 5µl of 100 base pair DNA Ladder were also loaded on the same gel. The electrophoresis was run for 1 hour and 30 minutes at 100 Volts and then the gel was analysed to identify the

mutant and wild type samples.

Kruskall-Wallis test and χ^2 test were used to analyse for any statistical differences in the phenotypic characteristics of patients with the wild type, heterozygous and homozygous rs2241880 polymorphisms. Kruskal-Wallis test was also used to assess whether there were any statistically significant differences in bone mineral density T scores and Z scores in patients with the wild type, heterozygous and homozygous rs2241880 polymorphisms.

Results

Demographic and Phenotypic Characteristics

One hundred and one (101) patients with CD were recruited. This represents approximately 25% of the Maltese CD population and should therefore be a truly representative sample of the Maltese CD cohort. Table 1 demonstrates the phenotypic characteristics of these patients. The mean duration of CD was 8.2 years (range: 5 months to 32 years). Table 2 describes the relevant drug history of the CD patients in the study population.

Table 1: Phenotypic characteristics of CD cohort

Characteristics	
Current Age, mean, (years) [range]	39.9 [18-83]
Male	51 patients
Postmenopausal Women	13 %
Patients with family history of IBD	4 %
Current Smokers	20 %
ex- smokers	6 %
Documented fractures	7 patients
Hip	1 patient
Spine	1patient
Others	5 patients
Montreal Classification:	
A1	12.8%
A2	64.4%
A3	22.8%
L1	23.8%
L2	33.7%
L3	42.5%
B1	69.3%
B2	23.8%
B3	6.9%
Perianal disease	5%
Extra-intestinal manifestations :	21%
H/o IBD related abdominal surgery	25%

A1 - age at diagnosis <17 years; A2 – age at diagnosis 17-40 years; A3 – age at diagnosis >40 years; L1 – ileal disease only; L2 – colonic disease only; L3 – ileocolonic disease; B1 – non-stricturing, non-penetrating disease; B2 – stricturing disease; B3 – penetrating disease

Table 2: Medical Treatment

Treatment	Number of patients (mean dose)
5-Amino- Salicylates	82 %
Thiopurines	55 %
Current steroid use	7 %
Previously or currently on steroids	69%
Previously on anti-TNF -alpha	7%
Currently on anti-TNF-alpha	37%
5 mg/kg every 8 weeks	31%
10 mg/kg every 8 weeks	5%
5 mg/kg every 4 weeks	1%
Dual Immunosuppressant Use (anti-TNF-alpha and Thiopurine)	28%
Previous Elemental Diet	3%
Methotrexate	6%
Calcium and Vitamin D replacement	11%
Bisphosphonates	2%

TNF : Tumour necrosis factor; mg – milligrams; kg – kilograms

Table 3: Mean T and Z scores at the Hip and Spine

	Mean T Score (Normal: -1.0 to 1.0)	Mean Z score
Hip	-1.22 (Range:-5.2 to 1.2)	0.55 (Range:-3.6 to 1.68)
Spine	-0.80 (Range:-5.1 to 1.6)	-0.41 (Range:-2.2 to 1.9)

Bone Densitometry Results

Table 3 describes the mean T and Z scores at the hip and spine. Eleven percent (11%) of patients had osteoporosis at the hip (T score <-1.5) and 6% had osteoporosis at the spine while 46% had osteopenia at the hip (T score -1.0 to -2.5) and 34% had osteopenia at the spine. Seven patients had documented fractures. Two patients had rib

fractures and humeral fractures. These were all related to major trauma (motor vehicle accidents). Another 3 individuals had Colles' fractures and rib fractures related to minor trauma (fall from low height).

The mean T score (hip) among these 5 patients was -1.0 (Z score: -0.5) and mean T score (spine) was -1.2 (Z score: -1.0). One patient had a history of vertebral fracture

with no documented trauma (DEXA bone mineral density T score spine: -2.86, Z score spine: -2.07) and one patient had a hip fracture following a fall from her own height (T score hip: -2.3, Z score hip: -1.7).

Two patients with a previous history of fractures (one had a hip fracture and the other patient had a vertebral fracture) were being administered oral bisphosphonates. Both patients had a T score less than -2.5 (mean T score: -3.6). All patients on Vitamin D and calcium replacement treatment (11%) had a T score less than -1.0 (mean T score -1.9).

Genotype Analysis

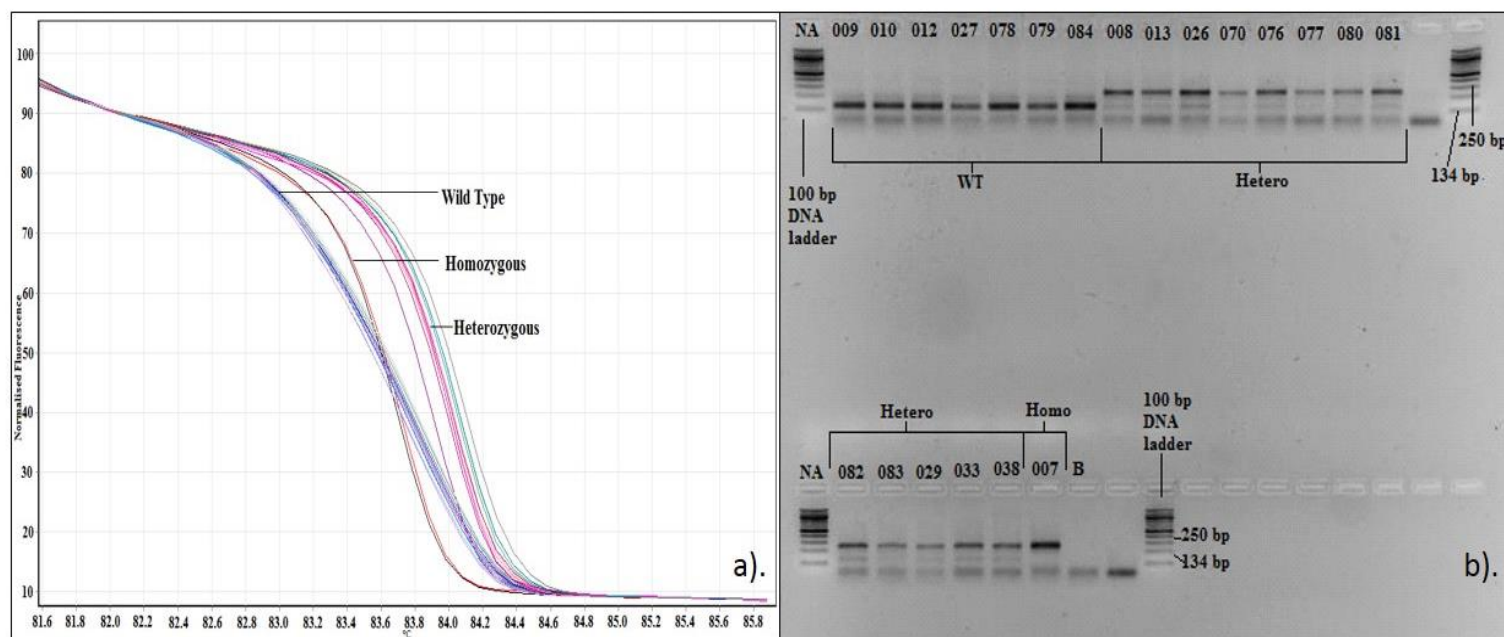
HRM (sample on Figure 1a) on the 101 DNA samples from the study population revealed that 39.6% of the CD patients (40 patients) did not have the rs2241880 (Thr300Ala) polymorphism (wild type) while 7.9% (8 patients) were homozygous. The rest, 52.5% (53 patients) were heterozygous for the polymorphism. These results were confirmed by RFLP (Figure 1b).

Phenotypic differences between patients with wild type, homozygous and heterozygous rs2241880 polymorphisms were then analysed (Table 4). Using the chi-squared (χ^2) test, there were no significant differences in the age at diagnosis (p : 0.21) of CD, disease location (p : 0.73), disease behaviour (p : 0.36), history of surgical intervention (p : 0.46) and smoking history (p : 0.26) between patients with the wild type, heterozygous and homozygous rs2241880 polymorphism. There were also no significant differences in the use of thiopurine therapy (χ^2 test p : 0.12), corticosteroids (p : 0.83), anti-TNF α therapy (p : 0.78) and use of dual immunosuppression therapy (thiopurine and anti-TNF α) (p : 0.82) between patients

with the wild type, heterozygous and homozygous rs2241880 polymorphism. There was no significant differences in disease duration (Kruskal –Wallis test p : 0.22) and body mass indices (p : 0.52) between patients with the wild type, heterozygous and homozygous rs2241880 polymorphism.

The mean T and Z scores at the hip and spine of patients with the wild type and mutant genotypes are shown in Table 1. There was no statistically significant difference in the T score at the hip between patients with the wild type, heterozygous and homozygous ATG16L1 rs2241880 variants (Kruskal-Wallis test p : 0.09). Similarly, there were no significant differences in the mean T score spine (p : 0.48), Z score hip (p : 0.23) and Z score spine (p : 0.73) between patients with the wild type and mutant ATG16L1 variants. However, a trend in DEXA T scores was observed with patients with the homozygous rs2241880 polymorphism (T300A variant) having lower T scores than patients with the heterozygous and wild type polymorphisms. Subgroup analysis on 13 postmenopausal CD women in our population revealed that 3 patients exhibited the wild type polymorphism, 2 had the homozygous and 8 had the heterozygous polymorphism. Mean T scores (hip) among postmenopausal women demonstrated a similar trend to that seen in the CD population (mean T score wild type: -0.1, heterozygotes: -1.19, homozygotes: -2.58). A similar trend was also seen at the spine (mean T score wild type: -0.8, heterozygotes: -0.96, homozygotes: -1.89).

Figure 1: a). High Resolution Melt using 5x Hot FirePol® EvaGreen® qPCR Mix Plus for Exon 9 (Thr300Ala) of the ATG16L1 gene with rs2241880 primers using DNA from Crohn's disease patient samples b). Agarose gel analysing Restriction Fragment Length Polymorphism for the Thr300Ala variant (rs2241880 polymorphism) using the SfaNI/LweI enzyme on DNA extracted from Crohn's disease study patient samples (bp: base pairs, B: blank sample, NA: code for Crohn's disease samples, WT: Wild Type allele, Hetero: Heterozygous allele, Homo: Homozygous allele for rs2241880 variant)



Discussion

An association between autophagy and bone metabolism has recently been established.^{5-13, 18} However, the effect of impaired autophagy secondary to ATG16L1 polymorphisms on bone strength has never been studied. In this study, we have analysed whether individuals with impaired autophagy secondary to the rs2241880 ATG16L1 polymorphism have lower bone mineral densities than individuals not exhibiting this polymorphism. This analysis was carried out on a population of CD patients since this polymorphism has been shown to be an important CD susceptibility gene in several population studies.¹⁹⁻²⁰ In addition, CD is associated with lower bone mineral densities and higher risk of osteoporosis. While many phenotypic characteristics have been identified as

possible risk factors for osteoporosis in CD (age at onset, history of surgical intervention, male gender, corticosteroid use), studies linking CD susceptibility genes with risk for osteoporosis have not been carried out. Further evidence to the validity of our cohort is that the clinical characteristics of our cohort was very similar to that reported in other European countries.²¹⁻²²

Genotyping of the CD study population was carried out using both RFLP and HRM. The results from both techniques were identical, allowing us to confirm the ATG16L1 variant genotypes of our population with two different techniques. Statistical analysis did not show any significant differences in the phenotypic and clinical characteristics of patients with wild type, heterozygous and homozygous

ATG16L1 polymorphisms. Statistical analysis also did not reveal any significant differences in DEXA bone mineral density T scores and Z scores between patients with the wild type, heterozygous and homozygous polymorphisms. However, a trend in the mean DEXA T scores at the hip

and spine may be observed with lower T scores in patients with the heterozygous allele and with the lowest scores in patients with the homozygous allele (Table 4). A significant value might have been obtained if a larger cohort was studied.

Table 4: Phenotypic characteristics and mean DEXA bone mineral density scores of patients with wild type, heterozygous and homozygous rs2241880 polymorphism

	Wild Type (n=40)	Heterozygous (n=53)	Homozygous (n=8)
Gender (male:female)	22:18	26:27	3:5
Smoking	28%)	28%)	0
Mean body mass index (kg/m ²)	25.6	25.9	24.5
Montreal Classification			
A1	17.5%	11%	0
A2	67.5%	64%	50%
A3	15%	25%	50%
L1	27.5%	21%	25%
L2	32.5%	32%	50%
L3	40%	47%	25%
B1	67.5%	36 (68%	87.5%
B2	22.5%)	15 (28%)	0
B3	4 (10%)	2 (4%)	1 (12.5%)
Perianal Disease	1 (2.5%)	4 (7.5%)	-
Medical and Surgical Management			
Thiopurine)	50%	66%	7.5%
anti-TNF-alpha	42.5%	36%	25%
Dual immunosuppression (thiopurine + anti-TNF alpha)	27.5%	28%	25%
Surgical Intervention	27.5%	17%	25%
Mean DEXA Score			
<u>T Score Hip</u>	-1.04	-1.29	-1.74
<u>T Score Spine</u>	-0.72	-0.86	-0.88
<u>Z Score Hip</u>	-0.35	-0.68	-0.58
<u>Z score Spine</u>	-0.40	-0.46	-0.02

Age of onset(A) A1: <17 years, A2: 17-40 years, A3: >40 years; Disease location (L) L1: ileal disease, L2: colonic disease, L3: ileocolonic disease, Behaviour (B) : B1: non-stricturing, non-penetrating, B2: stricturing disease, B3: penetrating disease.

Studies on osteoclasts, osteocytes and osteoblasts have shown that impaired autophagy results in impaired bone mass while the autophagy inducer Rapamycin increases bone formation.⁵⁻¹³ These studies suggest that impaired autophagy, including impaired autophagy secondary to genetic variations, results in impaired bone formation. Our findings demonstrate that gene polymorphisms in the autophagy ATG16L1 gene may be linked to lower bone mineral density scores. Larger prospective studies are however required before this link may be put into clinical practice. While this study was not powered enough to show a statistically significant association between bone density results and impaired autophagy, the trend in decreasing T scores in patients with the rs2241880 polymorphism should encourage functional studies on osteocyte, osteoblast and osteoclast activity in patients with this polymorphism.

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