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Clinical Research

Nonsense Mutations in *BAG3* are Associated With Early-Onset Dilated Cardiomyopathy in French Canadians

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ABSTRACT

Background: Dilated cardiomyopathy (DCM) is a major cause of heart failure that may require heart transplantation. Approximately one third of DCM cases are familial. Next-generation DNA sequencing of large panels of candidate genes (ie, targeted sequencing) or of the whole exome can rapidly and economically identify pathogenic mutations in familial DCM.

Methods: We recruited 64 individuals from 26 DCM families followed at the Montreal Heart Institute Cardiovascular Genetic Center and sequenced the whole exome of 44 patients and 2 controls. Both affected and unaffected family members underwent genotyping for segregation analysis.

Results: We found 2 truncating mutations in *BAG3* in 4 DCM families (15%) and confirmed segregation with disease status by linkage (log of the odds [LOD] score = 3.8). *BAG3* nonsense mutations conferred a

RÉSUMÉ

Introduction : La cardiomyopathie dilatée (CMD) est une cause majeure d'insuffisance cardiaque qui peut nécessiter une transplantation cardiaque. Environ un tiers des cas de CMD sont d'origine familiale. La nouvelle génération de séquençage d'ADN de larges collections de gènes candidats (c.-à-d séquençage ciblé) ou de l'exome entier peut rapidement et économiquement identifier des mutations pathogènes dans la forme familiale de la CMD.

Méthodes : Nous avons recruté 64 personnes de 26 familles atteintes de CMD suivies au Centre de génétique cardiovasculaire de l'Institut de Cardiologie de Montréal et avons séquencé l'exome entier de 44 patients et 2 témoins. Les membres de ces familles, affectés ou non, ont subi un génotypage pour analyse de ségrégation.

Résultats : Nous avons trouvé 2 mutations tronquantes dans le gène BAG3 chez 4 familles avec CMD (15 %) et avons confirmé la

Dilated cardiomyopathy (DCM) is a major cause of heart failure that represents the main reason for heart transplantation.[1] One third of DCM cases are familial,[2-4] and the recognition of the familial nature of the disease is important for screening family members. The genetics of DCM is complex, with more than 40 genes involved,[5,6] As recommended in clinical practice guidelines, genetic testing is now routinely performed in familial DCM for the purpose of screening family members.[7,8] Unfortunately, because of its large genetic heterogeneity, the yield of genetic testing targeting a small number of genes is modest (15%-30%) compared with that of hypertrophic or arrhythmogenic cardiomyopathies (30%-70%).[7-9]

Advances in sequencing technologies have made it possible to perform whole-exome sequencing (WES), in which the protein coding regions of the whole genome are targeted at a reasonable cost. WES is an unbiased and efficient method to uncover potential pathogenic mutations in disease without previous assumptions about candidate genes or pathways and has proved to be successful at identifying causal mutations in several genetic disorders.[10-12] We applied WES on clinically well-characterized individuals with familial DCM to identify pathogenic mutations in those families. We present our initial focused search for pathogenic mutations (missense, nonsense, frameshift, and splice site variants) in the 41 known DCM candidate genes.

Methods

Participants

The project was approved by the Ethics Committee at the Montreal Heart Institute (MHI) and conforms to the

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worse prognosis as evidenced by a younger age of clinical onset (37 vs 48 years for carriers and noncarriers respectively; $P = 0.037$). We also found truncating mutations in *TTN* in 5 families (19%). Finally, we identified potential pathogenic mutations for 9 DCM families in 6 candidate genes (*DSP*, *LMNA*, *MYH7*, *MYPN*, *RBM20*, and *TNNT2*). We still need to confirm several of these mutations by segregation analysis.

Conclusions: Screening an extended panel of 41 candidate genes allowed us to identify probable pathogenic mutations in 69% of families with DCM in our cohort of mostly French-Canadian patients. We confirmed the prevalence of *TTN* nonsense mutations in DCM. Furthermore, to our knowledge, we are the first to present an association between nonsense mutations in *BAG3* and early-onset DCM.

ségrégation avec la maladie par liaison (degré de liaison génétique [LOD score] = 3,8). Des mutations non-sens de BAG3 entraînent un mauvais pronostique, mis en évidence par l'apparition de signes cliniques à un âge plus jeune (37 vs 48 ans pour les porteurs et les non-porteurs respectivement, P = 0,037). Nous avons également identifié des mutations tronquantes du gène TTN chez 5 familles (19 %). Enfin, nous avons mis en évidence des mutations potentiellement pathogènes pour 9 familles avec CMD dans 6 gènes candidats (DSP, LMNA, MYH7, MYPN, RBM20, et TNNT2). Nous avons encore besoin de confirmer plusieurs de ces mutations par une analyse de ségrégation.

Conclusions : Le criblage d'une gamme étendue de 41 gènes candidats nous a permis d'identifier de probables mutations pathogènes dans 69 % des familles atteintes de CMD parmi notre cohorte de patients principalement canadiens-français. Nous avons confirmé la prévalence des mutations non-sens de TTN dans la CMD. De plus, à notre connaissance, nous sommes les premiers à présenter une association entre les mutations non-sens de BAG3 et l'apparition précoce de CMD.

principles outlined in the Declaration of Helsinki. Individuals were recruited from the MHI Cardiovascular Genetic Center and signed informed consent forms. Inclusion criteria for probands were left ventricular ejection fraction (LVEF) $< 45\%$, LV dilatation end-diastolic diameter $> 117\%$ of predicted value,[13] and a first-degree relative with DCM or a familial history of premature sudden cardiac death. Inclusion and exclusion criteria are detailed in Supplemental Table S1. Family members were recruited for segregation analysis; each was classified as *affected*, *unaffected,* or *borderline* based on published criteria.[13] With the exception of 2 individuals who were tested 5 years before enrollment, all participants underwent echocardiography within the previous 3 years. Before the current study, clinical targeted genetic testing was performed in 20 of the 26 probands in the MHI molecular laboratory using Sanger sequencing. The DCM panel includes *SCN5A*, *LMNA*, *TNNT2*, *TNNI3*, *MYBPC3,* and *MYH7,* in agreement with a published Canadian Cardiovascular Society position statement on genetic testing.[8] Staff in the research laboratory was blinded to clinical testing results. To compare the sensitivity of WES and clinical Sanger sequencing, families with and families without identified mutations in the clinical laboratory were included in this study.

Whole-exome DNA sequencing

We sequenced the exome of 44 participants using the Illumina HiSeq2000 instrument and a paired-ends 2×101 base pairs protocol (Illumina, San Diego, CA). We used Illumina's TruSeqExome Enrichment Kit that targets 62 megabases, including exons from 20,794 genes. Details of the WES protocol are described in the Supplemental Note.

Sanger sequencing

We confirmed mutations identified by WES using Sanger capillary sequencing. For *TTN*, we validated only novel nonsense mutations. The primer sequences are presented in Supplemental Table S2. We also genotyped by capillary sequencing *BAG3* p.Arg309stop in the DNA of 192 unrelated French Canadians from Gaspesia (Supplemental Note).[14]

Linkage analysis

Seventeen additional affected and unaffected members from 2 families (1 and 6) with the *BAG3* p.Arg309stop nonsense mutations were recruited and genotyped by Sanger sequencing to test if the mutation segregates with disease. Within families 1, 6, and 12, we carried out linkage analysis in Merlin (Project-Wizards, Melle, Germany) using an autosomal dominant model, a recombination fraction $\theta = 0$, and a disease prevalence of 0.0004 (ref.).[15,16] Because the *BAG3* p.Arg309stop mutation is not present in public databases, we chose a disease allele frequency of 0.01%; lower allele frequencies had an insignificant effect on the calculated LOD score.

Genome-wide DNA genotyping

To look for relatedness among individuals from families 1, 6, and 12 who carry *BAG3* p.Arg309stop, we performed genome-wide DNA genotyping using the Illumina OmniExpress BeadChip array and calculated pairwise identity-by-descent (IBD) metrics. Details are presented in the Supplementary Note.

Statistical analysis

We examined whether *BAG3* or *TTN* mutations are associated with an earlier age of onset or adverse outcomes defined as cardiovascular mortality, heart transplantation, or ventricular assist device (VAD) implantation. To avoid recruitment biases, we excluded patients identified during routine screening for this analysis. Kaplan-Meier curves were generated using the *survfit* function in R (The R Project for Statistical Computing).[17] To test the association between age of onset and *BAG3* or *TTN* carrier status, we used the QFAM-total procedure implemented in PLINK that uses permutations to take into account family structure.[18]

Results

Study population

We recruited and sequenced the whole exome of 44 individuals from 26 DCM families: 42 DCM patients and 2

Table 1. Clinical characteristics for the 42 dilated cardiomyopathy (DCM) participants who were whole-exome sequenced at the Montreal Heart Institute

Characteristics	Statistics
Male sex, n (%)	21 (50)
Current age (y)*	52 ± 14
Age of onset (y)*	44 ± 12
French-Canadian descent, n (%)	34 (81)
LVEF (%)*	22 ± 12
LVEF < 35%, n (%)	34 (81)
LVEDD (mm)*	65 ± 10
History of NYHA class III-IV heart failure, n (%)	28 (67)
History of VAD implantation, n (%)	5 (12)
History of heart transplantation, n (%)	15 (36)
History of ICD implantation, n (%)	28 (67)
History of ventricular arrhythmia, n (%)	7 (17)
Coronary angiography (number performed/% abnormal)	32/0
SAECG (number performed/% abnormal)	11/91
Cardiac MRI (number performed/% abnormal)	18/100

ICD, implantable cardioverter-defibrillator; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; MRI, magnetic resonance imaging; NYHA, New York Heart Association; SAECG, signal averaged electrocardiogram; VAD, ventricular assist device.

*Mean ± standard deviation.

unaffected family members that we used as controls. The clinical characteristics of the 42 patients are described in Table 1. All probands had normal coronary angiograms, except 1 (family 7) who did not undergo angiography because of the absence of risk factors and autopsy-proven DCM in a deceased family member.

Whole-exome sequencing and variant prioritization

The summary of the sequencing results is presented in Supplemental Table S3. We achieved a mean coverage of 62X, corresponding to 83% of the targeted bases sequenced at ≥ 20X. We identified 192,464 DNA sequence variants, including 38,248 not catalogued in public databases (dbSNP build 139 and 1000 Genomes Project release 14).[19,20] To identify potential pathogenic DCM mutations, we considered only nonsynonymous coding (missense, nonsense, and frameshift) or splice site variants, with a minor allele frequency ≤ 0.001 in the National Heart Lung and Blood Institute's Exome Sequencing Project (ESP) data.[21] Of these, we initially prioritized 58 variants that lie in any of the 41 previously reported DCM candidate genes (Supplemental Tables S4 and S5).[5,6] In families with more than 1 recruited affected member, only mutations that segregated in at least another affected individual were further considered.

Truncating variants in *BAG3*

A truncating mutation in *BAG3* was identified in 3 apparently unrelated DCM families (Table 2). Family 1 is a large French-Canadian family with many affected individuals, including a member experiencing sudden cardiac death and 4 transplant recipients (Fig. 1 and Supplemental Table S6). The proband was diagnosed with postpartum cardiomyopathy at the age of 30 years and underwent urgent heart transplantation. The subsequent diagnosis of clinical DCM in first-degree relatives prompted the diagnosis of familial DCM. Before our study, the proband underwent genetic testing with negative results at MHI and also at the Laboratory of Molecular Medicine (Harvard), where she was tested for 10 genes in 2009.

We sequenced the exome of 8 affected individuals in family 1. We identified a nonsense mutation in *BAG3* (p.Arg309stop) (Table 2) that segregated in all sequenced individuals. We also identified the same mutation in families 6 and 12 (Fig. 1, Supplemental Note, and Supplemental Table S6). In the proband of family 9, we also found another novel nonsense *BAG3* mutation (p.Ser249stop).

To confirm the pathogenicity of the *BAG3* mutations, we enrolled additional affected and unaffected family members from families 1 and 6. We could not recruit additional members from families 9 and 12. The recruited individuals underwent cardiac imaging if it had not been performed within the previous 3 years, and we confirmed by capillary sequencing their *BAG3* p.Arg309stop carrier status. Genotype and phenotype information appear in Figure 1 and Supplemental Table S6.

In summary, all genotype-negative individuals are unaffected. In family 1, 3 individuals carry the mutation but are not clearly affected: individual 1.10 (24 years old) is unaffected but is still young, individual 1.9 (45 years old) had a normal echocardiogram 5 years before enrollment but was not available for clinical re-evaluation, and individual 1.16 (67 years old) has a mildly depressed LVEF (50%) but no LV dilatation and thus does not meet criteria for DCM. Interestingly, this last individual has been taking an angiotensin-receptor blocker for many years for hypertension, which could have halted disease progression.[22] In family 6, *BAG3* p.Arg309stop was fully penetrant (Fig. 1). Individual 6.11 has borderline DCM with an LV end-diastolic diameter > 112% predicted but normal LVEF at the age of 37 years. In summary, p.Arg309stop mutation segregated with disease status with high penetrance (95% if we consider individuals ≥ 40 years old). We carried out linkage analysis with all individuals from families 1, 6, and 12 (N = 30) who are ≥ 40 years old and who underwent echocardiography in the past 3 years. We calculated a LOD score of 3.8 for *BAG3* p.Arg309stop, indicating that the probability that this mutation segregates with disease status by chance in these families is approximately 1 in 4000. Note that including individuals 1.10 (young age) and 1.9 (not clinically tested in the past 4 years) in the analysis yields a LOD score of 3.2.

To evaluate the clinical importance of the *BAG3* p.Arg309stop mutation in our DCM patient population, we compared carriers and noncarriers regarding age of clinical onset and severe adverse events: heart transplantation, implantation of a VAD, or cardiovascular death. Our analyses did not include patients recruited for screening to avoid bias, and we used permutations to account for family structure. Interestingly, carrying the *BAG3* p.Arg309stop mutation was significantly associated with a younger age of clinical onset (37 vs 48 years; $P = 0.037$) (Fig. 2). The *BAG3* p.Arg309stop mutation does not modify the risk of severe adverse events in DCM patients ($P = 0.74$) (Fig. 2).

This *BAG3* p.Arg309stop mutation is absent from public databases but was previously reported in a European DCM pedigree.[19-21,23] Interestingly, families 1, 6, and 12 are all originally from the Gaspésie region in Québec. A genetic analysis based on IBD revealed a third-degree relatedness between individuals of these 3 families. We also carried out

Table 2. Mutations identified in candidate dilated cardiomyopathy genes in this whole-exome DNA sequencing experiment

Gene	Position (chr:pos)	Variant ID	Mutation	Annotation	Amino acid change	PolyPhen prediction	Minor allele frequency ESP/1000G	MHI Families	Segregation confirmed?	Identified by clinical testing
LMNA	1:156,084,983	ss836897369	c.274C>T	Missense	p.Leu92Phe	Probably damaging	-/-	26	No	Yes
LMNA	1:156,100,500	ss836897320	c.449C>T	Missense	p.Thr150Ile	Possibly damaging	-/-	14	No	Yes
LMNA	1:156,106,048	rs61094188	c.1201C>T	Missense	p.Arg401Cys	Probably damaging	-/-	3	Yes	No
LMNA	1:156,106,981	ss836897354	c.1566CG>C	Frameshift	p.Asn524Thr fs*23	–	-/-	21	Yes	Yes
LMNA	1:156,108,510	rs142000963	c.1840C>T	Missense	p.Arg614Cys	Probably damaging	0.001/-	19	No	Yes
TNNT2	1:201,333,470	ss836897393	c.415C>T	Missense	p.Arg139Cys	Probably damaging	-/-	4	No	Yes
TTN	2:179,428,202	ss836897424	c.55462G>T	Nonsense	p.Gly18488stop	–	-/-	17	No	No*
TTN	2:179,429,822	-	c.53842C>T	Nonsense	p.Arg17948stop	–	-/-	8	Yes	No*
TTN	2:179,440,999	ss836897463	c.42665G>A	Nonsense	p.Trp14222stop	–	-/-	11	Yes	No*
TTN	2:179,470,369	ss836897473	c.26458G>T	Nonsense	p.Glu8820stop	–	-/-	5	No	No*
TTN	2:179,505,980	ss836897490	c.13428T>A	Nonsense	p.Lys4476stop	–	-/-	2	Yes	No*
DSP	6:7,571,710	ss836897516	c.1796T>G	Missense	p.Met599Arg	Probably damaging	-/-	10	No	No*
MYPN	10:69,908,204	ss836897657	c.343C>T	Missense	p.Arg115Cys	Possibly damaging	-/-	20	No	No*
RBM20	10:112,572,062	ss836897756	c.1907G>A	Missense	p.Arg636His	Probably damaging	-/-	19	No	No*
BAG3	10:121,432,095	ss836897768	c.746C>A	Nonsense	p.Ser249stop	–	-/-	9	No	No*
BAG3	10:121,435,991	ss836897771	c.925C>T	Nonsense	p.Arg309stop	–	-/-	1	Yes	No*
								6	Yes	No*
								12	Yes	No*
MYH7	14:23,893,327	ss836897779	c.2711G>A	Missense	p.Arg904His	Probably damaging	-/-	16	Yes	Yes

Note: Genomic coordinates are on build UCSC hg19. Segregation was confirmed in 9 families.
* Gene is not on the dilated cardiomyopathy panel for clinical testing at the Montreal Heart Institute.

haplotype analyses to determine if *BAG3* p.Arg309stop is a founder mutation in Québec. Details are described in the Supplemental Note.

Mutations in other DCM candidate genes

We found 5 novel nonsense mutations in *TTN* in 5 families (Table 2, Supplemental Table S6, and Supplemental Note). For families 2, 8, and 11 (Supplemental Fig. S1), we confirmed segregation of the *TTN* nonsense mutation in 1 additional affected or borderline individual, but for families 5 and 17, we could not recruit other members. We did not find DCM probands with frameshift *TTN* mutations. *TTN* nonsense mutations were not associated with earlier age of onset or adverse clinical outcomes (Supplemental Fig. S2).

Before our study, 20 probands underwent clinical testing for mutations in *SCN5A*, *LMNA*, *TNNT2*, *TNNI3*, *MYBPC3,* and *MYH7* at the MHI. Likely pathogenic mutations for 6 probands were identified: in *LMNA* (families 14, 19, 21, and 26), *MYH7* (family 16) and *TNNT2* (family 4) (Table 2 and Supplemental Table S6). These mutations are described in the Supplemental Note. Our WES approach captured all 6 variants identified by clinical testing. We also found a missense mutation in *LMNA* (p.Arg401Cys) in the proband of family 3.

Besides *BAG3*, *TTN,* and the 6 genes routinely tested at the MHI, we examined 33 additional DCM genes. We identified missense mutations in *MYPN*, *DSP,* and *RBM20* (Table 2, Supplemental Table S6, and Supplemental Note). These mutations are absent from ESP and 1000 Genomes Project databases,[20,21] although the same *RBM20* mutation (p.Arg636His) was previously identified in DCM patients.[24,25] The proband from family 19 carries both *LMNA* and *RBM20* missense mutations. We still need to confirm by segregation the pathogenicity of these mutations.

Discussion

Our whole-exome DNA sequencing experiment in 26 DCM families identified rare and potentially pathogenic mutations in the following DCM candidate genes: *DSP*, *LMNA*, *MYH7*, *MYPN*, *RBM20,* and *TNNT2* in 9 families. The remaining 9 families for whom we identified a potential pathogenic mutation carry truncating alleles in *TTN* or *BAG3*. Our study reinforces the role of *BAG3* in DCM and adds to the clinical knowledge gleaned so far about BAG3. Our multiplex pedigrees allowed us to demonstrate that *BAG3* carries highly penetrant DCM mutations that are associated with a worse prognosis characterized by earlier age of onset.

Role of *BAG3* in DCM

BAG3 encodes the Bcl-2–associated athanogene 3 protein, which is a cochaperone of heat shock proteins that localize to the Z disk and was previously linked to DCM.[23,26-28] Knocking-down *bag3* translation in a zebrafish model induced a heart failure phenotype.[27] Villard et al.[23] reported the same nonsense mutation in *BAG3* (p.Arg309stop) in 2 related DCM patients of European origin. To our knowledge, no one else has reported this mutation, and it remains absent from public databases.[19-21] Given the fact that families 1, 6,

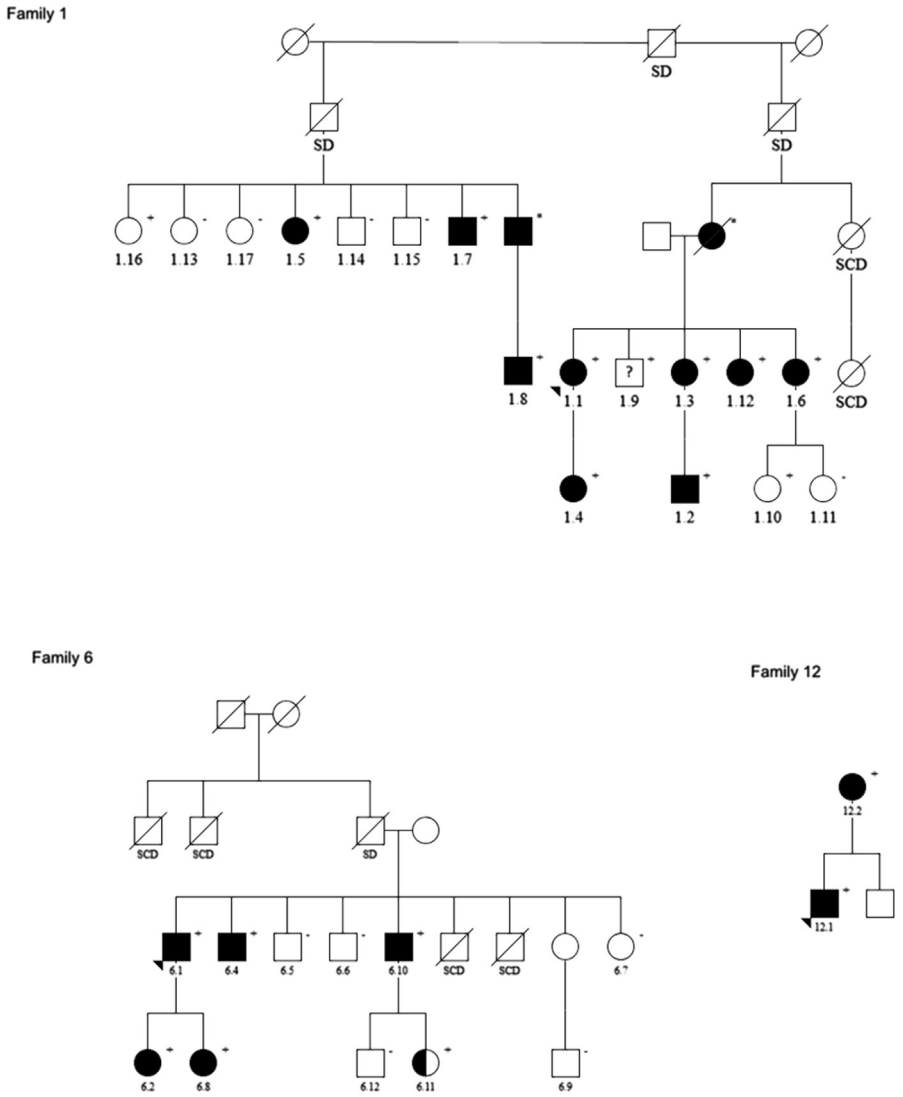

Figure 1. Pedigree of dilated cardiomyopathy families 1, 6, and 12. Probands are indicated by an arrowhead. Shaded and half-shaded symbols signify affected and borderline individuals, respectively. +, a carrier of the *BAG3* mutation p.Arg309stop; -, participant is negative for the mutation; ?, unknown affection status; SCD, sudden cardiac death; SD, sudden death. An asterisk (*) refers to an obligate carrier.

and 12 are originally from the Gaspésie region in Québec, we tested for the widespread presence of the *BAG3* p.Arg309stop allele in this region. We did not identify any carriers among 192 healthy Gaspesians. Additional genetic analyses in 3953 French Canadians did not identify potential *BAG3* p.Arg309stop carriers. The best model to explain this result is that the *BAG3* p.Arg309stop mutation arose on a *BAG3* haplotype that is common in the French-Canadian population (haplotype frequency is 9%). Although we cannot formally rule out that the European and French-Canadian DCM patients that carry the *BAG3* p.Arg309stop mutation share a common ancestor,[23] the simplest explanation is that *BAG3* p.Arg309stop is a rare familial DCM mutation that has occurred twice independently. Recently, Campbell et al.[29] combined WES and haplotype analysis to determine that a novel missense variant in *TNNT2* observed in 2 DCM families likely resulted from independent mutational events. In our case, we note that the *BAG3* p.Arg309stop mutation occurs because of a C>T nucleotide change within a CpG site. It has been suggested that DNA methylation at CpG sites can create mutation hotspots.[30]

Truncating mutations in *TTN*

TTN encodes a 33,000−amino acid protein that is important for sarcomere assembly and contractile forces in striated muscle. Several studies implicated *TTN* in DCM,[31-34] and a recent report suggested that truncating mutations in *TTN* are an important genetic cause of DCM,[35] a result corroborated by our study. We demonstrate that 19% (5 of 26) of familial DCM cases carry a truncating *TTN* mutation. In agreement with Herman et al.,[35] *TTN* nonsense mutations were not associated with earlier age of onset or more severe outcomes in our study (Supplemental Fig. S2). It will be important to validate segregation of the identified nonsense *TTN* mutations in families 5 and 17, because recent data

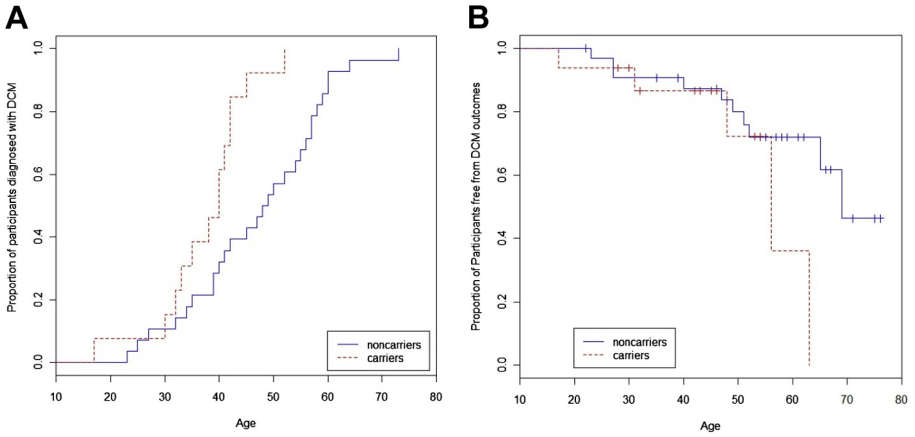

Figure 2. Kaplan-Meier curves of age of onset and severe adverse events of dilated cardiomyopathy (DCM) in carriers and noncarriers of *BAG3* nonsense mutations. (**A**) Age of onset in DCM cases (N = 41) including both probands and family members who presented to the hospital with DCM symptoms. Individuals diagnosed during screening are excluded. (**B**) Freedom of severe adverse events designated by heart transplantation, implantation of a ventricular assist device, or cardiovascular death (N = 47). Censored participants are denoted with a hatch mark.

suggest that not all truncating *TTN* alleles are fully penetrant or even pathogenic.[34,36,37]

Families or probands only?

WES generates an almost exhaustive catalogue of coding mutations found in a given patient. It is therefore a very attractive approach to identify the cause of rare mendelian diseases and works particularly well with diseases in which one or few genes are mutated. In its simplest form, one sequences a series of unrelated probands and finds the one gene in which they all carry a private mutation. In the case of DCM, however, the probands-only strategy is difficult because > 40 genes are implicated (and the list continues to grow). This approach worked well for *TTN*, but the prevalence of truncating mutations in this gene in DCM patients is very high (20%-25%).[35] For all other known DCM genes, the prevalence of mutations is small (< 5%), and it is difficult to build a convincing statistical argument by simply testing unrelated patients. Even more challenging would be the validation of a pathogenic mutation found in a single affected individual without affected family members. Functional studies in cellular or animal models could provide hints, yet the extrapolation of phenotypes observed in cells or mice to humans is not straightforward. For these reasons, we advocate that careful segregation analyses should remain the gold standard criterion to evaluate the candidacy of new DCM genes. The recruitment of family members is also essential to achieve the main goals of our DCM screening program: (1) preventing fatal cardiovascular events and (2) genetic counseling.

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Disclosures

The authors have no conflicts of interest to disclose.

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Supplementary Material

To access the supplementary material accompanying this article, visit the online version of the *Canadian Journal of Cardiology* at www.onlinecjc.ca and at http://dx.doi.org/10.1016/j.cjca.2014.09.030.