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ABOUT COVER

Editorial board member of *World Journal of Gastrointestinal Pharmacology and Therapeutics*, Dr. Laimas Virginijus Jonaitis is a Professor in the Medical Academy of Lithuanian University of Health Sciences. He also serves as Head of the sub-unit of General Gastroenterology in the Department of Gastroenterology of the Hospital of Lithuanian University of Health Sciences. Throughout his career, Dr. Jonaitis has led several international research projects in the Institute of Digestive Research of Lithuanian University of Health Sciences. His ongoing areas of scientific interest include Helicobacter pylori-related gastroduodenal pathology, gastroesophageal reflux disease, inflammatory bowel diseases, functional gastrointestinal diseases, and endoscopy. He has published more than 100 peer-reviewed articles and numerous abstracts. He is a reviewer for several international journals and a scientific reviewer for the European Crohn's and Colitis Organization. (L-Editor: Filipodia)

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ORIGINAL ARTICLE

Retrospective Study

Role of telomere shortening in anticipation of inflammatory bowel disease

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Author contributions: Truta B designed the study, performed analysis and wrote the manuscript; Wohler E performed genetic analysis, interpreted the results, reviewed the manuscript; Sobreira N performed genetic analysis; Datta L contributed to data collection; Brant S contributed to study design, data interpretation and editing the manuscript.

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Abstract

BACKGROUND

The existence of genetic anticipation has been long disputed in inflammatory bowel disease (IBD) in the absence of the explanatory mechanism.

AIM

To determine whether it was predictive of genetic anticipation, we evaluated telomere length in IBD. We hypothesized that multiplex IBD families exhibit a genetic defect impacting telomere maintenance mechanisms.

METHODS

We studied three IBD families with multiple affected members in three successive generations. We determined telomere length (TL) in lymphocytes and granulocytes from peripheral blood of the affected members using flow cytometry and fluorescence in-situ hybridization (flow FISH). We also performed whole exome sequencing in the blood of all available family members and used PhenoDB to identify potential candidate gene variants with recessive or dominant modes of inheritance.

RESULTS

Out of twenty-four patients of European descent selected to participate in the study, eleven patients, eight parent-child pairs affected by IBD, were included in the genetic anticipation analysis. Median difference in age at diagnosis between two successive generations was 16.5 years, with earlier age at onset in the younger generations. In most of the affected members, the disease harbored similar

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gastrointestinal and extraintestinal involvement but was more aggressive among the younger generations. TL was not associated with earlier age at onset or more severe disease in members of successive generations affected by IBD. NOD2 gene mutations were present in the Crohn's disease patients of one family. However, no gene variants were identified as potential candidates for inheritance.

CONCLUSION
Telomere shortening appears unlikely to be involved in mechanisms of possible genetic anticipation in IBD. Further studies using a larger sample size are required to confirm or refute our findings.

Key words: Inflammatory bowel disease; Telomere; Anticipation; Genetic; Inheritance

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Core tip: This is a retrospective study to evaluate the role of telomere shortening in genetic anticipation of inflammatory bowel disease (IBD). Genetic anticipation is a long disputed concept in IBD and lacks an explanatory mechanism. We analyzed generational changes in telomere length of eight parent-child pairs of three generation IBD families with anticipation and performed whole exome sequencing to identify genetic variants for autosomal inheritance. Neither telomere shortening or autosomal inheritance was associated with anticipation in our three generation IBD families, suggesting other potential mechanisms underlie this phenomenon.

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INTRODUCTION

Genetic anticipation is a phenomenon in which an inherited disorder manifests at a younger age and often more severely in offspring than in the affected parent[1]. This phenomenon has been reported in over twenty monogenetic degenerative neurological disorders[2]. In some of these disorders, such as Huntington's disease, familial Parkinson's disease, and fragile X syndrome, the molecular basis of anticipation has been linked to the expansion of unstable genomic triplet repeats, in step with the progression of the disease down through generations[3-7].

For inflammatory bowel disease (IBD) the involvement of genetic anticipation has been considered since the 1990's[8,9] because of observed earlier ages at diagnosis and more severe disease among offspring of patients with IBD, mainly Crohn's disease (CD). However, no mechanisms accounting for genetic anticipation have been identified, and many scientists have doubted genetic anticipation in IBD, attributing the observed differences in age at diagnosis and disease severity to biased observation[10,11].

The list of conditions exhibiting anticipation continues to grow. This list includes not only monogenic disease but also polygenic diseases such as bipolar disorder[12], schizophrenia[13], rheumatoid arthritis[14], Behcet's disease[15], and chronic leukemia[16], in which the molecular mechanism is yet to be discovered.

One new cause of genetic anticipation is telomeropathies, which encompasses a wide variety of rare diseases caused by genetic defects in telomerase maintenance mechanisms[17,18]. Telomeres are nucleoproteins that protect chromosomal ends by counteracting the erosion caused by the problems inherent in terminal replication of the chromosomes. In telomeropathies, mutations in the telomerase reverse transcriptase, the specialized polymerase that synthesizes new telomere repeats, leads to progressive telomere shortening, causing earlier onset of disease in successive generations[18]. Families with telomere disorders, such as dyskeratosis congenita, Li-Fraumeni syndrome and Von-Hippel-Lindau disease display anticipation in generational changes, both in age at onset and in appearance of specific

symptoms[17-20]. Although genetic causes have been discovered for a set of identified telomere disorders, a number of related syndromes may likewise be due to unrecognized telomeropathies[21].

Interestingly, short telomeres were found to be associated with genetic anticipations in hereditary breast cancer (in women carrying a mutation in *BRCA1* or *BRCA2* genes, and a subset of *BRCAX* families)[22] and thyroid cancer[23]. There is increasing evidence suggesting that short telomeres and subsequent genomic instability contribute to malignant transformation[24,25]. Telomere shortening has been associated with dysplasia and neoplasia in the early onset of ulcerative colitis (UC)[26-28].

These findings generate thought-provoking questions about the possibility that telomere shortening may be the cause of genetic anticipation in other familial disorders associated with increased risk of malignancies such as IBD (which has an increased risk of cancer) - and a subset of these familial patients might result from unrecognized telomeropathies, particularly if there is also an accompanying identifiable genetic mutation related to telomere biology.

In our study, the primary objective was to evaluate generational changes in telomere length in parent-child pairs of familial IBD, to determine the role of telomere shortening, and its potential implication as a mechanism of anticipation in familial IBD. The secondary objective focused on investigating potential candidate gene variants for autosomal inheritance in these multi-generation IBD families.

MATERIALS AND METHODS

Study families

Study design: We selected three multi-generation IBD families included in the Johns Hopkins IBD Family Study. Each selected family had three or more first-degree relatives affected by IBD: Either CD, UC or IBD-unclassified (IBD-U). Affected and non-affected individuals completed a multiple-choice questionnaire. This questionnaire interrogated patient demographics, family history of IBD, household specifics to childhood such as water source, number of people in the house, birth order, passive and active smoking; questions also inquired about disease onset, disease location, extraintestinal manifestations and complications, as well as medical and surgical therapy for IBD. We selected those families where all three affected generations had access to similar therapy to avoid the confounding factor of different classes of therapy on severity of the disease. Therefore, most of the patients were treated with mesalamine, thiopurine and steroids. None of the patients were treated with methotrexate. The patients were treated before biologics were commonly used as part of the IBD therapy.

Blood samples also obtained from participants were used to isolate lymphocytes for storage, serum for serological analysis of antibodies and other proteins relevant to IBD and DNA for genotyping or sequencing.

The Johns Hopkins IBD Family Study, conducted with the overall goal of identifying IBD susceptibility genes, was initially approved in 1996 and later registered with clinical trials in 2010. The review of electronic and paper records and the genetic analysis for the current study was performed in 2019.

IBD diagnosis: To confirm the diagnosis of IBD, define the severity of the disease, and determine current and past therapies, we reviewed electronic and paper medical records including consult notes, imaging tests (computer tomography, upper gastrointestinal, small bowel and barium enemas studies), endoscopic (upper endoscopy, colonoscopy and capsule endoscopy) and pathology reports of all available family members. We used the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) IBD Genetic Consortium phenotype operating manual (version May 10, 2006) and forms to classify of disease as either CD, UC or IBD-U (https://repository.niddk.nih.gov/media/studies/ibd/ibd_phenotyping-manual.pdf).

Genetic analysis

Telomere length measurement: DNA was purified from whole blood using Qiagen Puregene kit. To measure average length of telomere repeats in cells, we used fluorescent *in situ* hybridization with labeled peptide nucleic acid probes specific for telomere repeats in combination with fluorescence measurements by flow cytometry (flow FISH) as previously described[29,30]. This method was developed to measure single-cell Telomere length (TL) using a fluorescently labeled probe that hybridizes to telomere DNA and has the unique advantage over other methods for measuring

telomere length by providing multi-parameter information on the length of telomere repeats in thousands of individual cells. The accuracy and reproducibility of the measurements are augmented by the automation of most pipetting (aspiration and dispensing) steps and by including an internal standard (control cells) with a known TL in every tube. Individuals were diagnosed with short telomeres if TL was shorter than the 10th percentile of the age-matched control population.

Whole exome sequencing: Performed on eight individuals from the three families as part of the Baylor-Hopkins Center for Mendelian Genomics. Whole exome sequencing (WES) was performed using Agilent SureSelect HumanAllExonV5Clinicalon the Illumina HiSeq2000 platform. Libraries were sequenced on the HiSeq2500 platform with onboard clustering using 125 bp-paired end runs and sequencing chemistry kits HiSeq PE Cluster Kit v4 and HiSeq SBS Kit v4. Fastq files were aligned with BWA mem[31] version 0.7.8 to the 1000 genomes phase 2 (GRCh37) human genome reference[32,33]. Duplicate molecules were flagged with Picard version 1.109(1716). Local realignment around indels and base call quality score recalibration were performed using the Genome Analysis Toolkit 26 version v3.1-1- g07a4bf8 or v3.3-0-g37228af. Variant filtering was done using the Variant Quality Score Recalibration (VQSR) method[34]. Variants that passed VQSR filtering were annotated using Annovar (version 2013_09_11) against a variety of data sources. Ultimately, PhenoDB[35] was used to analyze the data for coding, frameshifting, nonsense, or missense, rare (minor allele frequency > 1% in ExAC, gnomAD, 1000 Genomes, or Exome Variant Server) variants in genes that were shared between the three affected families based on recessive or dominant modes of inheritance.

Statistical analysis

Results are expressed as mean ± SD for n number of samples. Analysis of difference between groups was conducted using an analysis of variance, *t* test, or Fisher exact test, as appropriate. A two-sided *P* value < 0.05 was used for statistical significance. All analysis was done using GraphPad Prism software (GraphPad 5.0).

Approval of the study was obtained from the Institutional Review Board of Johns Hopkins University.

RESULTS

Clinical characteristics of patient population

There were twenty-four individuals selected to participate in the study. Only eleven out of the twenty-four were eligible (successive generations affected by IBD) for genetic anticipation analysis: Five patients with UC, four patients with CD and two patients with IBD-U. These represent eight parent-child pairs from the three families studied (Table 1).

IBD diagnosis confirmation: Through electronic and paper medical records review and the IBD phenotype was defined per NIDDK IBD Genetic Consortium phenotype operating manual (version May 10, 2006) to classify disease as either CD, UC or IBD-U.

Children's age at diagnosis: The median age of parents at diagnosis was 16.5 years older than that of the children affected by IBD and the median age at blood draw was 39.6 ± 23 years for the entire group. The mean age of parents at diagnosis was 40 ± 17 and the mean age of children was 21 ± 13, $P < 0.0014$. The UC families were of mixed descent Italian/Irish/Japanese and Poland/Russian/Puerto Rican while the CD family was of European descent (Polish/Russian). The later two families were also identified as being of Ashkenazi Jewish descent.

Disease extent and severity in parent-child pairs: Table 1 shows the extent of disease involvement in the CD, UC, and mixed families. Similar extent of the disease was noted in either parents or their children. In the family with CD, the disease extended into the large and small intestine in all three generations of affected individuals. All four affected members had a penetrating form (entero-enteral fistula, anal fistulas) of the disease. Arthropathy was the most common extraintestinal manifestation of IBD. In UC patients, the disease involved mainly the rectum and sigmoid colon (left side disease) and showed increasing severity as the disease was passed to the subsequent generation. All the affected members of the second family underwent total colectomy for disease refractory to medical therapy with the youngest member requiring surgery at only 6 years of age.

Table 1 Patient included in genetic anticipation analysis- characteristics and disease severity

ID	Member	IBD	Tobacco	AOD	NOD2	Extent	Behavior	Medications	Surgery
1-01	Mother	CD	None	21	+	Ileum, colon	B2	Steroids 5-ASA	Yes
1-02	Proband/F	CD	Former	9	+	Ileum, jejunum, colon	B3p	Steroids 5-ASA	No
1-05	Sister	CD	None	14	+	Ileum, colon	B2	Steroids 5-ASA thiopurine	No
1-07	Grandmother	CD	Passive	42	+	Ileum, colon	B2	5-ASA	Yes
2-01	Father	IBD-U	Active	21	_	Left colon	NA	5-ASA, thiopurine	Yes
2-02	Proband/F	UC	Active	4	_	Left colon	NA	Steroids 5-ASA, thiopurine	Yes
2-03	Grandfather	UC	Former	63	_	Left colon	NA	Steroids 5-ASA	Yes
3-01	Mother	UC	None	40	_	Left colon	NA	Steroids 5-ASA	Yes
3-03	Proband/M	UC	None	19	_	Left colon	NA	Steroids	No
3-04	Uncle	UC	Active	41	_	Left colon	NA	Steroids 5-ASA	No
3-05	Grandfather	IBD-U	Former	56	_	Pancolitis	NA	Steroids	No

There was no available TL data for the member 3-05. B2: Stricturing disease; and B3: Penetrating disease, as per Montreal Classification; Steroids: Refers to prednisone; Surgery: Refers to total colectomy for ulcerative colitis and segmental bowel resection for Crohn's disease. F: Female; M: Male; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Indeterminate colitis; 5-ASA: 5–aminosalicilates; AOD: Age at diagnosis; TL: Telomere length; NOD2: NOD2 gene; NA: Not applicable.

Results of genetic analysis

TL in successive generations: In order to evaluate the hypothesis that telomere shortening may be associated with the earlier onset of the disease, we investigated the mean relative TL of blood lymphocytes and granulocytes (Figure 1). Individuals were diagnosed with short telomeres if their telomere length was shorter than the 10th percentile of the age matched control population[17]. No associations were found between TL in lymphocytes and granulocytes and anticipation in the age at onset observed in successive generations.

WES for genetic inheritance: Finally, we explored the possibility of a gene variant with potential for autosomal inheritance in these Mendelian families. We used WES and PhenoDB to identify a genetic variant common to all three IBD families. NOD2 gene mutations were present in the CD affected patients of one family. WES did not detect any rare frameshift, nonsense or missense coding variants in genes shared by these three families for either dominant or recessive modes of inheritance. NOD2 gene mutations were present in the Crohn's affected patients of one family.

DISCUSSION

Awareness of the changing of disease pattern in IBD families with multiply affected generations not only has implications for genetic counseling and surveillance of at-risk members but also for implementation of correct treatment strategies. Genetic anticipation in IBD, although observed in most studies, has been questioned by many scientists due to lack of evidence for a molecular mechanism to explain this phenomenon. It has been suggested that anticipation may result from observation bias[10,11]. Recently, a Bayesian method (that corrects for random effects, isolating the confounding effect of changes in secular trends, screening and medical practices, and adjusts for changes in age-specific incidence across birth cohorts), confirmed anticipation observed among successive generations of Lynch Syndrome families, a syndrome that likewise had controversy regarding genetic anticipation[36]. Nevertheless, the molecular mechanism of genetic anticipation in Lynch Syndrome has not been identified. It is therefore possible, that genetic anticipation in IBD may be demonstrated using an optimal study design and statistical methods and/or evidence of a molecular mechanism[37].

In several monogenetic diseases, including Huntington disease[3], myotonic

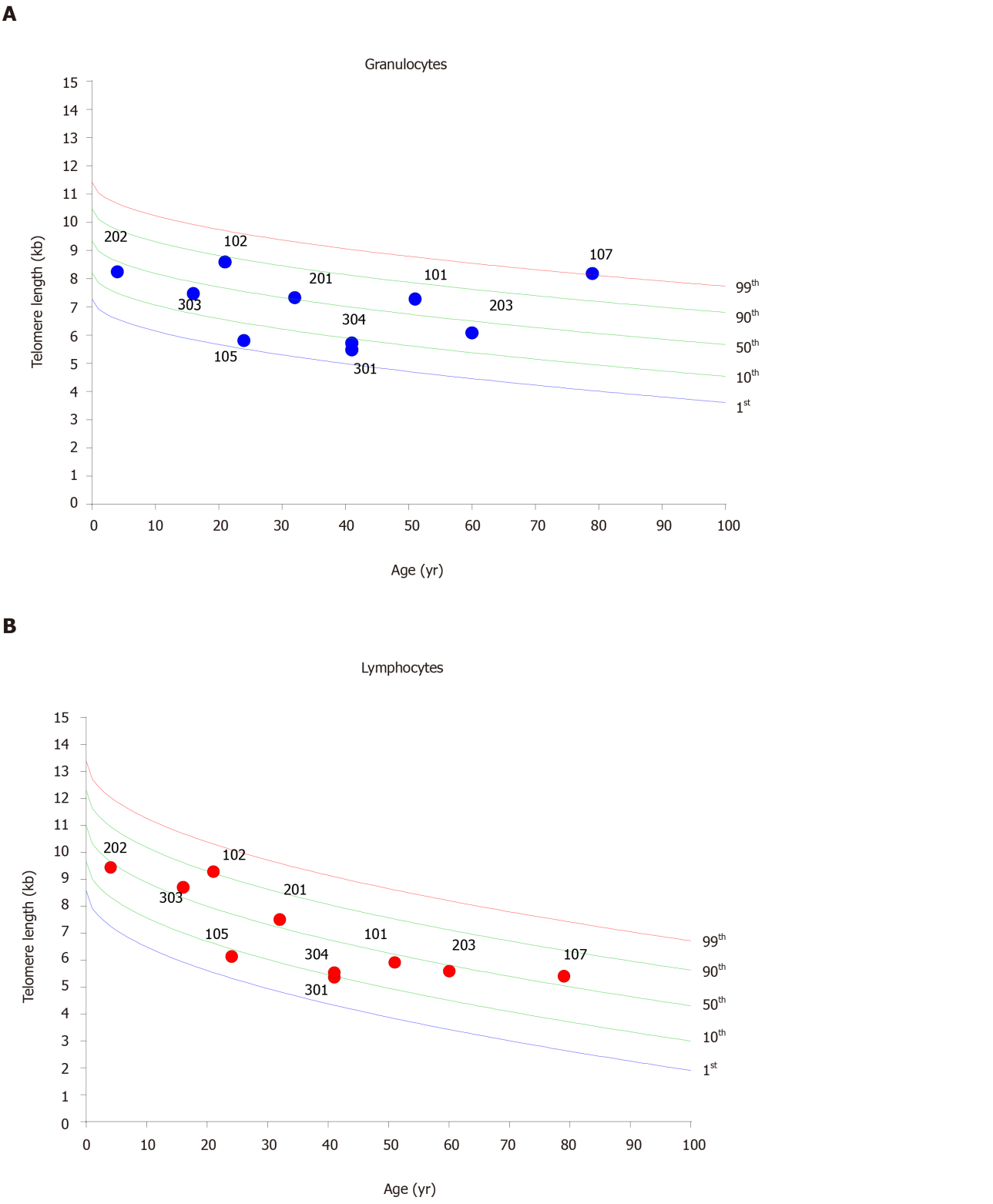


Figure 1 Telomeres length in the granulocytes and lymphocytes of affected members from the three inflammatory bowel disease families. Telomere length measured by flow cytometry with fluorescence in situ hybridization in affected individuals of Family 1, Family 2, and Family 3 plotted against nomogram from Johns Hopkins controls are within normal age adjusted range.

dystrophy[38], and Friedreich ataxia[39], the biological basis for genetic anticipation resides in expansion of unstable genomic triple repeats. In polygenic diseases, the complexity of multifactorial inheritance makes the identification of such molecular mechanism very challenging. In IBD, genomic imprinting of age at diagnosis has been proposed but when tested in 137 affected parent-child pairs from 96 families, it was not observed[40].

The patterns of genetic anticipation discovered for some telomeropathies generated

the question of whether a similar defect in telomere maintenance mechanisms may have a role in IBD. The TL has been studied in IBD in association with colon cancer and severity of inflammation in UC but not in the context of genetic anticipation[26-28,40]. In particular, we have questioned if these familial forms of IBD with observed anticipation might be due to unrecognized telomerophaties.

In our study, we examined three IBD families with clinical features of genetic anticipation. In all three families, the disease onset was reported at an early age (median 16.5) and became more severe in successive generations. In order to encompass three successive generations, we reviewed the disease history of families who received care at our center as early as 1996. Confusion related to the potential impact of newer therapy on the severity of the disease was avoided since all the recruited affected members had to receive treatment before the introduction of the biologics. Since population genetics has temporal stability, the recruitment methodology did not influence the results of the study which remain valid today despite the age of our families.

We have shown that when controlling for age, no association exists between TL and age at disease onset among members of successive generations affected by IBD. Genetic anticipation is unlikely to be the result of telomere shortening and other mechanism should be explored. We also investigated the potential for these IBD families with Mendelian pattern of inheritance to represent a monogenic form of IBD similar to Very Early Onset IBD[41]. In particular, we wanted to learn if there is an accompanying identifiable genetic mutation related to telomere biology[32] in these families and if its effects were overlooked due to our study sample size. Moreover, in some monogenic diseases, genetic anticipation has been linked to the expansion of unstable genomic triplet repeats[3], a mechanism that we would have explored in IBD had it been monogenetic. However, besides the *NOD2* mutations identified among the affected members one of the CD families, WES did not detect any rare frameshift, nonsense or missense coding variants in genes shared by these three families for either dominant or recessive modes of inheritance. In adults, *NOD2* is typically the most highly penetrant established gene associated with CD but the population attributable risk of *NOD2* variance to CD is only 26%[43]. No mutation in genes involved in the telomere maintenance mechanism were detected.

Strengths and limitations

The study represents the first evaluation of TL defects in genetic anticipation of IBD. Even though no association was found between TL and age at disease onset among members of successive generations, this pilot study is very valuable as an attempt to find the molecular mechanism of genetic anticipation in IBD. While we have shown that genetic anticipation is unlikely to be the result of telomere shortening, our results do point the way to explore other mechanisms. Nonetheless, the sample size may be too small to rule out whether telomeropathies may play a role in other families with similar strong evidence for anticipation. Further studies to confirm our findings should address a larger population of familial IBD that likewise have strong evidence for anticipation among successive generations.

ARTICLE HIGHLIGHTS

Research background

The existence of genetic anticipation, a phenomenon in which an inherited disorder manifests at a younger age and often more severely in offspring than in the affected parent, has been long disputed in inflammatory bowel disease (IBD). In the absence of the explanatory mechanism, it has been suggested that anticipation may result from observation bias. In monogenetic degenerative neurological disorders the mechanism for genetic anticipation was identified as unstable genomic triple repeats. In polygenic diseases, such as IBD, the complexity of multifactorial inheritance makes the identification of genetic mechanism very challenging. I Most recently, mutations in the telomerase reverse transcriptase, have been shown to lead to progressive telomere shortening, causing earlier onset of disease in successive generations with telemeropahities. Telomere length (TL) has also been found to be associated with severity of inflammation and colon cancer in IBD.

Research motivation

The role of TL in genetic anticipation of patients with IBD has not been studied.

Research objectives

In our study, the primary objective was to evaluate generational changes in TL in parent-child pairs of familial IBD, to determine the role of telomere shortening, and its potential implication as a mechanism of anticipation in familial IBD. The secondary objective focused on investigating potential candidate gene variants for autosomal inheritance in these multi-generation IBD families.

Research methods

We studied three IBD families with multiple affected members in three successive generations. We determined TL in lymphocytes and granulocytes from peripheral blood of the affected members using flow cytometry and fluorescence in-situ hybridization (flow FISH). We also performed whole exome sequencing (WES) in the blood of all available family members and used PhenoDB to identify potential candidate gene variants for recessive or dominant modes of inheritance.

Research results

Out of twenty-four patients of European descent selected to participate in the study, eleven patients, eight parent-child pairs affected by IBD, were included in the genetic anticipation analysis. Median difference in age at diagnosis between two successive generations was 16.5 years, with earlier age at onset in the younger generations. Five patients were affected by ulcerative colitis, four patients by Crohn's disease and two patients by IBD-Unclassified. In most of the affected members, the disease harbored similar gastrointestinal and extraintestinal involvement but was more aggressive among the younger generations. TL was not associated with earlier age at onset or with more severe disease in members of successive generations affected by IBD. NOD2 gene mutations were present in the Crohn's affected patients of one family. However, no gene variants were identified as potential candidates for Mendelian inheritance.

Research conclusions

The study represents the first evaluation of TL defects in genetic anticipation of IBD. Even though no association was found between TL and age at disease onset among members of successive generations, this pilot study is very valuable as an attempt to find the molecular mechanism of genetic anticipation in IBD.

Research perspectives

While we have shown that genetic anticipation is unlikely to be the result of telomere shortening, our results do point the way to exploration of other mechanisms. Further studies to confirm our findings should address a larger population of familial IBD that likewise have strong evidence for anticipation among successive generations.

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