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The Fragile X CGG Repeat Shows a Marked Level of Instability in Hereditary Non-Polyposis Colorectal Cancer Patients

Key Words
Fragile X syndrome
Unstable repeat
HNPCC
Mismatch repair genes
Microsatellite slippage

Abstract
The allelic variation of the FMR1 CGG repeat was investigated by small-pool PCR in nonneoplastic peripheral blood leukocytes from HNPCC patients and matched controls for similar CGG repeat lengths. The allelic variation for repeat lengths appears to be roughly twice as frequent in HNPCC patients as in controls, especially when patients are mutated in hMLH1. There are more expansions in HNPCC patients (42%) than in controls (20%) but this difference is statistically borderline. The mean length of expansions relative to the genuine size did not differ in HNPCC patients or controls (respectively 17% and 20% of the constitutional allelic length). The reported data suggest that instability within nonneoplastic cells of a subset of HNPCC patients might be one mechanism for transition from normal to the premutation range of the FMR1 CGG repeat.

Introduction

Fragile X syndrome is the most frequent cause of inherited mental retardation, affecting 1/4,000 males and 1/8,000 females [1]. In almost all patients the disorder is based on the expansion of a CGG repeat in the 5′ UTR of the FMR1 gene. This expanded CGG with more than 200 copies, termed full mutation, is associated with hypermethylation of CpG islands resulting in the repression of the transcription of the FMR1 gene and in absence of the FMR1 protein. In families segregating for the fragile X syndrome, unaffected carriers bear a premutation in the 54–200 repeat range. A premutation is unstable and can expand meiotically to larger premutations in both sexes, or to a full mutation when transmitted by females [see ref. 2 for a review].

Instability in both meiosis and mitosis critically depends on the length of pure CGG tracts within the 3′ end of the array [3, 4]. The instability threshold is similar to the other triplet-repeat disorders and lies around 34 pure repeats [4–6]. In normal individuals, the CGG arrays range from 6 to 52–54 copies, most of them being 30, and are stable in transmission [6–8] either because rare pure CGG tracts are largely below the instability threshold or because, in most cases, the arrays are interrupted by regularly interspersed AGG, every 9 or 10 CGG repeat units [3–5, 9, 10]. Indeed, in vitro studies [11] have established that AGG interspersions within a CGG tract prevent the

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formation of stable hairpin structures implicated in replication slippage. 68% of normal alleles have 2 interspersed AGG [12]; by contrast, most premutations have no AGG or have but one (respectively 63% and 37%) and show a 3′ pure CGG tract up to the instability threshold [10]. A loss of one AGG within the 3′ end of the repeat would predispose the resulting pure CGG tract to be unstable only if it approaches the 34 pure repeat threshold. However, in most of normal arrays present in studied populations [5, 10, 12], the loss of an interspersed AGG is not sufficient to raise the 3′ pure CGG tract up to this instability threshold, even in the gray zone. So it must be hypothetized that microsatellite slippage at the 3′ end had already expanded it before or could expand if after the AGG loss.

Familial studies have clearly established that cancer predisposition in most individuals with hereditary nonpolyposis colorectal cancer (HNPCC) is attributable to defects in any one mismatch repair (MMR) genes, human homologs to the MutS or MutL genes of *Escherichia coli.* To date, five MMR genes have been identified, hMSH2, hPMS1, hPMS2, hMLH1 and GTBP [13–16]. A phenotypic consequence of MMR deficiency within neoplastic cells is known as replication errors [RER+] phenotype resulting in microsatellite instability [17, 18]. Furthermore, recent studies of extratumoral tissues of HNPCC patients have evidenced microsatellite instability in nonneoplastic cells due to dominant mutations of hMLH1 or hPMS2 [19]. So it could be hypothesized that in nonneoplastic cells of a subset of HNPCC patients even a moderate mutator phenotype [RER+/–] could display instability within the CGG array of FMR1.

To clarify this issue, we have investigated the variation of CGG arrays within nonneoplastic cells of 7 HNPCC patients with an [RER+] tumor. The reported data are consistent with the hypothesis that HNPCC background might play a role in the dynamics of the FMR1 CGG repeat.

Material and Methods

Patient Sample

Seven unrelated patients were investigated. All of them exhibit a HNPCC using the ‘Amsterdam’ criteria: all were from families (1) with 3 patients, or more, affected with HNPCC; (2) whose patients are first-degree relatives in two generations; (3) with 1 patient, or more, who is less than 50 years old.

Three of them (H5, H6, H8) have been proved, after the reported study, to bear an identified mutation in the sequence of hMLH1. Denaturing gradient gel electrophoresis (DGGE) failed to evidence any mutation (splicing or coding sequence) in the hMLH1 gene of other patients and is carried out in other genes. All 7 patients display a high microsatellite instability in tumors, i.e. a [RER+] tumoral phenotype.

DNA Studied

DNA from nontransformed nonneoplastic peripheral blood leukocytes was amplified by small-pool PCR (SP-PCR) for the FMR1 CGG repeat.

Small-Pool PCR

SP-PCR was performed according to published procedures [20, 21], with a modification of the PCR protocol adapted from others [7, 22]. Genomic DNA was diluted to 1.2 ng/µl corresponding to the 184 diploid genome equivalent per microliter. A 0.5-µl aliquot of diluted DNA (92 genomes equivalent) was denatured with 0.5 µl of 0.8 *M* NaOH, 1 m*M* EDTA for 5 min at room temperature, placed in ice, and then neutralized with 0.5 µl of 0.5 *M* NH_4Ac at pH 5.4. Samples were amplified by PCR in 10 m*M* Tris-HCl at pH 8.3, 50 m*M* KCl, using 2 m*M* $MgCl_2$, 500 µ*M* each dATP, dGTP, dCTP and deaza-dGTP, 10% dimethylsufoxide and 1 unit of Taq polymerase (Eurobio, France). The reaction mixture was heated to 95 °C for 10 min, followed by 5 cycles of DNA denaturation (2 min 30 s at 95 °C)/ annealing (1 min at 65 °C/extension (2 min 30 s at 72 °C) and 25 cycles of DNA denaturation (1 min 30 s at 95 °C)/annealing (1 min at 55 °C)/extension (2 min 30 s at 72 °C). The sense primer used was 5′AGCCCCGCACTTCCACCACCAGCTCCTCCA and the antisense primer was 5′GCTCAGCTCCGTTTCGGTTTCACTTCCGGT. PCR products were migrated onto a 4% polyacrylamide sequencing gel in a ‘GATC 1500 direct blotting electrophoresis system’ (B. Braun Sciencetec) and hybridized with a (GCC)7 oligonucleotide probe end-labeled with terminal transferase (Boehringer) and digoxigenin-ddUTP. Hybridization was performed at 62 °C in 5 × SSC, 0.1% laurylsarcosine, 0.02% SDS and 1% blocking reagent (Boehringer). Membranes were washed for 20 min at 62 °C in 2 × SSC, 0.1% SDS and detected by chemiluminescence. This routine has been proved to detect large alleles up to 100 repeats. The constitutional sizes of alleles within each patient or control were estimated by amplification of 200 ng of DNA followed by comigration with a sequencing reaction of bacteriophage M13.

Results and Discussion

Seven unrelated patients who exhibit a HNPCC and display microsatellite instability in tumors, i.e. a [RER+] tumoral phenotype, were investigated for slippage at the CGG repeat of the FMR1 gene. DNA from nonneoplastic peripheral blood leukocytes was amplified by SP-PCR. In order to account for the correlation between repeat size and in vitro slippage, every HNPCC patient was matched with a control bearing CGG of similar length, for SP-PCR, electrophoresis and Southern blot hybridizations (fig. 1). The observed allelic variations in nonneoplastic cells of patients and controls are reported in table 1.

In controls, as expected, the greater the length of the array, the higher the frequency of allelic variations. Con-

trols with small CGG arrays (C5, C4, C8, C6) exhibited rare allelic variation, at a mean frequency of 1.5×10^{-4} per chromosome X. Those with a premutation allele (C1, C2) exhibited more allelic variation, at a mean frequency of 8×10^{-4}. However, four expansions up to the larger CGG arrays (in females) were observed in controls with 29, 31 and 42 repeats and none within premutated controls, but roughly 4-fold less premutated genomes were studied. The allelic variants lying between the two allelic sizes (in females) are more likely contractions of the higher repeat than expansions of the smaller, because, as shown by previous studies [4, 9], the smaller the repeat length, the greater its genetic stability, and because the contractions were shown to be more frequent than expansions in both somatic and germinal tissues [21, 23, 24].

By contrast, the allelic variation of CGG repeats in nonneoplastic cells of HNPCC patients is quite different. Allelic variation for the repeat length appears to be roughly twice as frequent in HNPCC patients as in their matched controls. Overall, the mean frequency of allelic variation in HNPCC patients is significantly twice that in

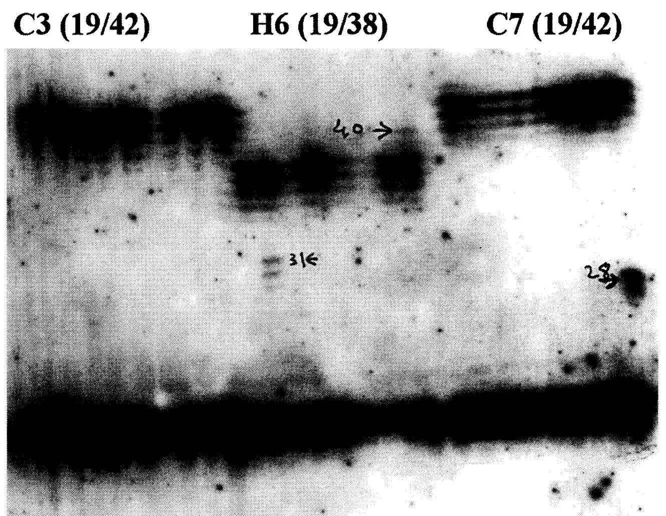

Fig. 1. Allelic variation of the FMR1 CGG repeat for the HNPCC patients H6, and for controls C3 and C7. H6 displays two variations (40 and 31) from the genuine alleles 38 or 19, and C7 displays only one variant allele (28). DNA from patients and controls is amplified in the same routine, then electrophoresed on the same gel.

Table 1. Frequencies, numbers and various kinds of allelic variation at the FMR1 CGG array investigated by SP-PCR within the somatic DNA of controls (C) matched with HNPCC patients (H)

Individuals: CGG sizes:	C5 29/19	C4 29/22	C8 31	C6 32/29	C3 42/19	C7 42/19	C1 56/30	C2 59/29	H5[1] 29/23	H1 31	H7 31/22	H4 32/28	H8[1] 36/19	H6[1] 38/19	H3 51/30
Total number of X chromosomes investigated	25,760	27,416	23,000	8,280	29,624	20,056	9,200	5,520	23,552	23,920	16,560	7,912	23,000	22,080	13,432
Total number of observed allelic variations	0	3	3	0	9	5	4	8	5	5	4	0	12	9	3
Frequency per X chromosome, $\times 10^{-4}$	<0.4	1.1	1.3	<1.2	3	2.5	4.3	14.5	2.1	2.1	2.4	<1.3	5.2	4.1	2.2
Number of expansions up to larger allele size	0	1	1	0	2	0	0	0	5	1	1	0	5	4	0
Size of expansion		+4	+8		+2 +15				+2[2] +8 +12 +15	+24	+2		+2 +3 +4 +5 +8	+2[2] +3 +11	
Number of variations between allele sizes	0	0	–	0	7	5	3	7	0	–	0	0	6	5	0
Number of contractions down to smaller allele size	0	2	2	0	0	0	1	1	0	4	3	0	1	0	3

1 Patients with proven mutations in the sequence of hMLH1 after SP-PCR investigations.
2 Sizes of expansions observed twice in independent SP-PCR.

Table 2. Allelic variation of the FMR1 CGG repeat within non-neoplastic cells of HNPCC patients versus controls

			Observed numbers	Expected numbers	χ^2 value	p value
A	Patients	variant alleles	38	28.59		
		genuine alleles	130,418	130,485.51	6.115	<0.015
	Controls	variant alleles	20	29.41		
		genuine alleles	134,116	134,106.59		
B	Patients	expansions	16	13.11		
		contractions	22	124.89	2.82	0.1
	Controls	expansions	4	6.89		
		contractions	16	13.11		
C	hMLH1 patients	expansions	14	10.28		
		contractions	12	15.72	5.63	<0.02
	Controls (C4, C3, C7)	expansions	3	6.72		
		contractions	14	10.28		

Line A: all variations within HNPCC versus controls; Line B: expansions versus contractions, within HNPCC versus controls; Line C: expansions versus contractions, within HNPCC mutated in hMLH1, versus their controls.

controls (respectively $2.9 \times 10^{-4}$and 1.5×10^{-4}, at $p < 0.015$; table 2, line A).

There are more expansions up to the larger CGG allele in HNPCC (16 out of 38 variations: 42%) than in controls (4 out of 20 variations: 20%). But due to the small number of observed allelic variants, the overall excess of expansions fails to be significant ($p = 0.1$; table 2, line B). However there is a significant excess of expansions in patients (H5, H8, H6) bearing a mutation in hMLH1 ($p < 0.02$; table 2, line C). It must be emphasized that the excess of expansions within HNPCC versus controls (lines B and C) has been tested under the conservative hypothesis that all the variant alleles lying between the two allelic sizes (in C3, C7, H8, H6) were considered as contractions. The fact that most expansions are confined in patients clearly mutated in hMLH1 is in agreement with data recently reported on other microsatellites [19]. The mean length of expansions relative to the genuine size did not differ in HNPCC patients or controls (respectively 17 and 20% of the constitutional allelic length).

By contrast with controls, the frequency of allelic variation seems less correlated to the length of CGG. This observation is consistent with the hypothesis that some HNPCC background could display instability within the CGG, even within a short tract or interrupted array. Indeed, a recent study [25] established (in yeast) that a poly GT tract without a variant repeat was 3- to 4-fold less stable, within an MMR deficient background, than an interspersed tract. Due to significant logistical difficulties, it was not possible to test germinal instability in HNPCC patients, but a recent study [24] established that microsatellite instability at the FMR1 locus is well correlated in both somatic and germinal cells.

So these results support the hypothesis that an MMR deficiency could affect the FMR1 CGG repeat in a subset of individuals and might be responsible for transition from normal to a premutation range of the array. Such promoted expansions might be partially responsible for the specific 3 and 2% of chromosomes with a longer 3′ CGG pure tract within the FMR1 triplet array structure of type 1 and type 2 [9], especially when considering the high incidence of HNPCC (1/200). But in the absence of confirming sequence data, it might be that partial deficiency in repair processes might be also responsible for both the increase in size and the loss of an intervening AGG triplet.

The additional effect of the loss of an interspersed AGG and microsatellite slippage, evenly promoted by MMR deficiency, would generate reservoirs of protomutations from which premutations would originate. Because they have already passed through a multistep process of transition from the normal range, these protomutations need only one mutational event, either another loss of AGG or microsatellite slippage, in order to become a premutation. The so-called 'founder effect' observed on fragile X chromosomes [26, 27] still exists in reservoirs of protomutations because it results from the previous multistep process of transition. At each generation, a set of

protomutations is expanded to the premutation level with a frequency high enough to account for the fragile X incidence. The question as to whether a moderate instability would occur for other triplet diseases is being studied, especially for the CAG repeat responsible for SCA1.

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