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Stage-specificity of spontaneous mutation at a tandem repeat DNA locus in the mouse germline

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Abstract

Mouse expanded simple tandem repeat (ESTR) loci are the most unstable loci in the mouse genome. Despite the fact that over the last decade these loci have been extensively used for studying germline mutation induction in mice, to date little is known about the mechanisms underlying spontaneous and induced ESTR mutation. Here we used flow cytometry and single-molecule PCR to compare the frequency of ESTR mutation in four flow-sorted fractions of the mouse male germ cells – spermatogonia, spermatocytes I, round and elongated spermatids. The frequency and the spectrum of ESTR mutation did not significantly differ between different stages of mouse spermatogenesis. Considering these data and the results of other publications, we propose that spontaneous ESTR mutation is mostly attributed to replication slippage in spermatogonia and these loci may be regarded as a class of expanded microsatellites.

1. Introduction

The results of some recent publication show that mouse ESTR loci provide a sensitive technique for studying germline mutation induction following exposure to ionising radiation and chemical mutagens [1, 2]. ESTR loci consist of long homogenous arrays of relatively short repeats (4-9 bp) and show a very high spontaneous mutation rate of length changes both in germline and somatic cells [3-5]. In this respect they clearly differ from another group of highly unstable tandem repeat DNA loci – the GC-rich human minisatellites, where the bulk of spontaneous mutations occur in the germline with very rare events taking place in the somatic cells [6]. Given this, ESTRs more closely resemble some microsatellite loci showing equally high instability in the germline and somatic tissues. In many publications it has been suggested, though not experimentally proven, that microsatellite mutation is most probably attributed to replication slippage [7]. However, numerous studies have also identified a unique class of the expanded disease-causing human microsatellites i.e., trinucleotide instability, which is clearly attributed to non-replication mechanisms [8]. It therefore remains unclear whether the very high spontaneous instability at ESTR loci is replication-driven or may be attributed to some other mechanisms. Here, to elucidate the still unknown mechanisms of ESTR spontaneous mutation, we have compared ESTR mutation frequencies in flow-sorted fractions of the male germ cells.

2. Materials and methods

2.1. Flow cytometry

CBA/J male mice (Iffa Credo, Charles River, France) were used in this study. Cells were isolated from 3 month-old male mice using a two-step enzymatic digestion as previously described [9]. Magnetic-activated cell sorting (MACS) of α_6 -integrin positive cells was performed as we previously described [9] using rat anti- α_6 -integrin antibodies (GoH3, BD Biosciences) and mouse anti-rat Kappa antibody coupled to magnetic microbeads (Miltenyi Biotec, Paris, France). Testicular cell suspension, MACS α_6 -integrin-positive and negative cellular fractions were collected and suspended at 10^6 cells/ml for DNA staining with Hoechst 33342 (5 μ g/ml, 1 hour at 32° C), and stained with propidium iodide (2 μ g/ml) to exclude dead cells. Various fractions of germ cells were sorted with a purity of at least 97% on a dual-laser FACStarPlus flow cytometer (Becton Dickinson, Le Pont de Claix, France). Spermatogonia (SG) were sorted on MACS α_6 -integrin-positive cells fraction according to their ability to efflux the Hoechst 33342 (the ‘Side Population’ phenotype), while meiotic

spermatocytes I (SC) and round spermatids (RS) were sorted from the α_6 -integrin-negative cells according to their DNA content [9]. Elongated spermatids (ES) were isolated according to forward scatter and their haploid DNA content on the testicular single-cell suspensions [10, 11].

2.2 *ESTR mutation typing*

DNA was extracted using the QIAmp DNA micro kit from Qiagen according to the manufacturer instructions. The frequency of ESTR mutation was evaluated using a single-molecule PCR (SM-PCR) approach [12, 13]. DNA was amplified on an MJ DNA engine PTC 220 in 10 μ l reactions using 0.6 μ M flanking primers, 1 U enzyme mix for 30 sec, and 68°C for 3 min for 30 cycles, ending with 10-min incubation at 68°C. PCR products were resolved on a 40 cm long agarose gel and detected by Southern blot hybridisation [14]. To increase the robustness of the estimates of individual ESTR mutation frequencies, on average 150 amplifiable molecules were analysed for each tissue for each male mouse. The frequencies of ESTR mutation, 95% CI's and standard errors were estimated using modified approach proposed by Chakraborty [15].

3. Results and discussion

Using flow cytometry sorting of testicular cell suspensions from CBA/J male mice, four fractions of germ cells were isolated according to α_6 -integrin labelling and Hoechst 33342 DNA staining (Figure 1A). The Hoechst 33342 fluorescence of DNA in these cells depends on ploidy, accessibility of chromatin and Hoechst efflux out of the cells due to ATP-binding cassette transporter activity. As previously reported [9, 10], the 'Side Population' phenotype of the male germ cells is based on efflux of Hoechst 33342 out of the cells determined by the expression of ABC transporter BCRP1. Given that there is no saturation of the Hoechst dye in spermatogonia, DNA in these cells is poorly stained and their ploidy cannot therefore be established [9, 10]. As a result, in this protocol the fraction of spermatogonia found in the 'Side Population' do not co-localise with the 4N DNA content plot. In contrast, as spermatocytes and spermatids do not exhibit an efflux of Hoechst, the concentration of Hoechst inside those cells is saturated [9, 10] and they can effectively be separated by the technique used in our study.

Using SM-PCR, the frequency of ESTR mutation at the *Msh6-hm* locus was evaluated in sorted spermatogonia, meiotic spermatocytes I as well as post-meiotic round and elongated spermatids. This approach involves diluting bulk genomic DNA and amplifying multiple samples of DNA, each containing approximately one amplifiable ESTR molecule (Figure 1B). Table 1 presents a summary of ESTR mutation data. The frequency of ESTR mutation did not significantly differ across the different stages of mouse spermatogenesis and was close to that in sperm of BALB/c mice [12, 16]. Further analysis of ESTR mutation frequencies in individual males confirmed the lack of measurable inter-stage differences (Figure 1C). We next defined the germline length changes for the *Msh6-hm* mutations found in DNA samples extracted from the four groups of germ cells. Again, the incidence of gains and losses of repeats did not significantly differ across all stages of spermatogenesis (Table 1). We therefore conclude that both the frequency of ESTR mutation and its spectrum remain similar in spermatogonia, meiotic spermatocytes I as well as post-meiotic round and elongated spermatids.

The homogeneity of ESTR mutation frequency and its spectrum across all stages of mouse spermatogenesis strongly argues that spontaneous mutations at these loci mostly occur in replication-proficient mitotic spermatogonia. Indeed if, similar to the human minisatellites,

mutation at these loci were mainly attributed to meiotic gene-conversion like events [17], then the frequency of ESTR mutation in meiotic spermatocytes and post-meiotic spermatids should substantially exceed that in pre-meiotic spermatogonia. The data showing the lack of measurable effects of meiotic events on the frequency of ESTR mutation in the male germline is consistent with the results of our previous study which failed to detect any correlation between meiotic crossing over and ESTR mutation induction [18]. Using a similar approach for cell sorting and a small-pool PCR technique, the frequency of microsatellite mutation was studied in transgenic mice carrying expanded disease-causing human microsatellite [19]. The results of this study showed that microsatellite expansion almost exclusively occurs in haploid spermatids and thus may be attributed to the repair of DNA gaps within repeats inappropriately folded into secondary structures. Despite the ability of the *Ms6-hm* repeats to form hairpins and intrastrand structures [20], we did not detect any measurable increases in the frequency of ESTR mutation in haploid round and elongated spermatids. This result suggests that, in contrast to the expanded disease-causing human microsatellites, the presence of secondary structures in ESTR arrays does not substantially destabilise them in non-replicating cells. Considering these data and the results of other publication, it therefore appears that spontaneous ESTR mutation is mostly attributed to replication slippage occurring in mitotic spermatogonia and, given the similarities in the proposed mutational mechanisms, these loci may be regarded as a class of expanded microsatellites.

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Figure legend

Figure 1. Separation of mouse germ cells and ESTR mutation frequencies in sorted cells. (A) Hoechst 33342 and propidium iodide (PI) fluorescence analysis by flow cytometry of testicular cell suspension. The spermatogonia (SG) contained in the 'Side Population', meiotic spermatocytes I (SC), round spermatids (RS) and elongated spermatids (ES) are indicated. (B) Mutation detection at the *Ms6-hm* locus by SM-PCR in DNA sample containing two progenitor alleles. Mutant is indicated with arrowhead. (C) ESTR mutation frequencies in sorted male germ cells. The 95% confidence intervals (CI) of are shown.

Table 1
Summary of mutation data

Stage of spermatogenesis	No mutations ^a	Frequency \pm s.e. ^b	t^c	Prob ^c	Type of mutants (%)	
					Gains	Losses
Spermatogonia	58 (447)	0.1298 ± 0.0185	-	-	38 (65.5)	20 (34.5)
Spermatocytes I	53 (425)	0.1247 ± 0.0186	0.19	0.8474	45 (84.9)	8 (15.1)
Round spermatids	59 (514)	0.1148 ± 0.0162	0.61	0.5431	43 (72.9)	16 (27.1)
Elongated spermatids	47 (410)	0.1146 ± 0.0180	0.59	0.5580	34 (72.3)	13 (27.7)
χ^2 , df=3 ^d					5.72	$P=0.1262$

^a Number of amplifiable molecules is given in brackets.

^b \pm standard error.

^c Student's test and probability for difference from mutation frequency in spermatogonia.

^d Chi-square test for homogeneity of the type of mutants between all stages.

Figure 1

