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SHORT REPORT

Direct estimation of the recombination frequency between the *RB1* gene and two closely linked microsatellites using sperm typing

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In this study, single sperm typing has been used for high-resolution recombination analysis between the retinoblastoma gene and two closely linked extragenic microsatellites (D13S284 and D13S1307). The analysis of 1198 single sperm from three donors allowed the determination of recombination fractions between RB1.20 and D13S284 and RB1.20 and D13S1307 of 0.022 and 0.033, respectively. These results show that *RB1* gene and the two microsatellites are closely linked, which validates their potential use in indirect genetic diagnosis of retinoblastoma.

Keywords: sperm typing; recombination frequency; *RB1* gene; microsatellites

Introduction

Retinoblastoma is a rare intraocular tumour that arises in early childhood, initiated by the loss of function of both alleles of the retinoblastoma gene (*RB1*).[1] In families affected with hereditary retinoblastoma, when the causative mutation has not been identified, it is possible to follow the segregation of the mutant allele by studying polymorphisms located within the *RB1* gene.[2] However, 5–10% of families show a lack of heterozygosity for all of the markers described. An alternative is to study extragenic markers as close as possible to the *RB1* gene, which minimizes the chance of a recombination event occurring.

In humans, the resolution of the genetic distance between closely linked markers is low due to the fact that these distances are estimated from very rare events in a limited sample size. To overcome this problem, the genetic study of isolated single sperm constitutes an original alternative to indirect procedures based on linkage analysis.[3]

Several extragenic microsatellites located on chromosome 13 around the *RB1* gene were described in the most recent Généthon genetic map.[4] Among the polymorphisms for which the donors in our study were

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heterozygous, D13S284 and D13S1307 presented a high polymorphism information content (0.87 and 0.68, respectively) and were easily amplified and analysed. The recombination frequencies between these loci and the *RB1* gene have never been fully established, because of the lack of recombinant chromosomes in the offspring of the CEPH (Centre d'Etudes du Polymorphisme Humain) pedigrees studied.

The aim of the present study was to estimate by sperm typing technique the male recombination frequency between a short tandem repeat of the retinoblastoma gene (*RB1.20*) and each of the microsatellite markers D13S284 and D13S1307, in order to validate their use in indirect genetic diagnosis of predisposition to retinoblastoma in affected families. The ability to type microsatellites might have a major implication for future preimplantation genetic disease diagnosis, due to the high level of allelic heterogeneity of most disease-causing mutations.

Materials and Methods

Single Sperm Isolation and Preparation

Sperm samples were collected from two donors heterozygous for both RB1.20 and D13S284 microsatellites (donors 1 and 2) and one donor heterozygous for RB1.20 and D13S1307 (donor 3).

Single sperm were sorted by fluorescence-activated cell sorting, lysed and neutralized as previously described.[5]

PCR Conditions

In the first-round PCR, the two polymorphisms (RB1.20/D13S284 and RB1.20/D13S1307) were co-amplified for 35 cycles, in the same microtiter well using a specific pair of outside primers, each pair flanking one of the microsatellites (Table 1).

The two microsatellites were then amplified in separate second-round reactions using two microliters of first-round product, for 40 cycles. The forward primers were labelled with a fluorescent dye (6-FAM) to visualize the PCR products in an Automated DNA-sequencer model ABI 377 (Applied Biosystems, Foster City, CA, USA).

Second-round PCR products were loaded onto denaturing 4% polyacrylamide gels and analysed with the Genescan 2.1 software (Applied Biosystems).

Statistical Analysis

Data were analysed using two programs especially designed for statistical analysis of sperm typing data: TWOLOC[5] and SPERM.[6] In addition to the estimation of recombination fractions (θs) and the standard errors (SEs), the programs calculate amplification efficiencies (α) and contamination rates (β) for each allele, and the probability (γ) of n sperm present in a tube (n = 0, 1, 2).

Results and Discussion

We typed 868 single sperm (416 from donor 1 and 452 from donor 2) for both RB1.20 and D13S284 microsatellites and 330 single sperm for RB1.20 and D13S1307 (donor 3). No amplification was obtained for the negative controls (wells without cells), which is consistent with absence of contamination. The distribution of observed RB1.20, D13S284 and D13S1307 alleles for the 1198 amplified spermatozoa is given in Table 2. The maximum likelihood estimates and their asymptotic standard errors are given in Table 3.

The estimated recombination fractions for the RB1.20–D13S284 interval using TWOLOC program were not significantly different between donors 1 and 2 (0.0256 ± 0.0100 and 0.0194 ± 0.0083, respectively). Combined data from the two donors (868 sperm cells) using the SPERM program gave an estimated recombination rate of 0.0222 with a standard error of 0.0064. For donor 3, the recombination fraction between RB1.20 and D13S1307 was 0.0326 with a standard error of 0.0124. However, more individuals have to be studied further to determine if an interindividual

Table 1 Primers and conditions for the two rounds of amplification

Locus	*Primers*	*Sequence (5′ to 3′)*	*PCR 1*		*PCR 2*	
			Concentration (μM)	*Annealing t°C*	*Concentration (μM)*	*Annealing t°C*
RB1.20	AF2	ACT CAT GAG AGA CAG GCA TTT G	0.5	56	–	–
	AR2	GTA CAC GCC TGT ATC CTA GCT	0.5	56	–	–
	BF2	CTT CAC CTT CTC TCC TCC CTA C	–	–	0.2	64
	BR	GGG TAA CAG AGT GAG ACT CTA TC	–	–	0.2	64
D13S284	284 Fext	GAG TGT CCT CTG TTG CAG AAC	0.8	56	–	–
	284 R	AAA AGG CTA ACA TCG AAG GGA G	0.8	56	0.4	56
	284 F	CAG GTG GAA ACA GAA TTC ATT CA	–	–	0.4	56
D13S1307	1307 Fext	CTG CCA AAA TGG GAG TTA GCA	0.6	56	–	–
	1307 R	CTC CTT CAA ACA GAC TCT GAC	0.6	56	0.2	60
	1307 F	CAA GGT ATG GGA TCT CAA AGA A	–	–	0.2	60

Table 2 Typing data from 1198 sperm cells for alleles RB1.20 (locus A), D13S284 (locus B for donors 1 and 2) and D13S1307 (locus B for donor 3)

	Number of sperm		
Observed sperm type	*Donor 1 phase (ab/AB)*	*Donor 2 phase (ab/AB)*	*Donor 3 phase (aB/Ab)*
(– – – –)	43	35	31
(a – – –)	28	23	28
(– A – –)	33	22	17
(aA – –)	0	1	0
(– – b –)	21	21	16
(– – – B)	29	26	11
(– – bB)	0	0	1
(a – b –)	129	141	7
(a – – B)	2	3	130
(aAb –)	1	2	0
(aA–B)	0	0	1
(a–bB)	1	2	2
(–AbB)	0	0	0
(–A–B)	124	166	1
(–Ab–)	5	4	85
(aAbB)	0	6	0
Total	416	452	330
Recombination frequency	2.56%	1.94%	3.26%
95% confidence interval	0.6–4.5%	0.3–3.6%	0.8–5.7%

Dashes indicate that the allele was not detected.

variability of the recombination rate exists between these markers, as has been already reported for other specific genomic intervals.[7] Moreover, several studies have discussed the possible effect of different parameters on the recombination rate such as sex, age and environmental factors. Some chromosomes show an increasing map length with increasing age, whilst others show the opposite, but it is still not known if recombination phenomena are restricted to specific intervals or are genomewide. Sex-specific differences in recombination are well characterized in many organisms. The human genetic map shows on average 50% more recombination events for females than for males[4] but some specific DNA intervals display more recombination events in males.

These sperm typing data indicate that both D13S284 and D13S1307 polymorphisms are tightly linked to the retinoblastoma gene with estimated θs which do not differ significantly which is consistent with the CEPH data. Thus, as a first approximation, the two markers may be equally used in linkage analysis. However, we only measured the male recombination fraction; different results may be obtained in females, which cannot be estimated from the CEPH data as no recombination occurred in the families analysed.

Table 3 Maximum likelihood estimates of the parameters θ, α, β and γ

L	*Parameter estimate*	
	Donor 1	
L_1 = –1487.782	α_A =0.8629 (0.0281)	α_a =0.8160 (0.0322)
	α_B =0.8286 (0.0300)	α_b =0.7929 (0.0332)
	β_A =0.0	β_a =0.0067 (0.0063)
	β_B =0.0	β_b =0.0060 (0.0061)
	θ_{AB} = 0.0256 (0.0100)	
	γ_0 =0.0763 (0.0166)	γ_1 =0.9237 (0.0166)
	γ_2 =0.0	
	Donor 2	
L_2 = –1569.571	α_A =0.8737 (0.0253)	α_a =0.8653 (0.0264)
	α_B =0.8599 (0.0261)	α_b =0.8861 (0.0250)
	β_A =0.0	β_a =0.0144 (0.0104)
	β_B =0.0	β_b =0.0071 (0.0087)
	θ_{AB} = 0.0194 (0.0083)	
	γ_0 =0.0643 (0.0135)	γ_1 =0.9015 (0.0190)
	γ_2 =0.0342 (0.0145)	
	Donor 3	
L_3 = –1155.345	α_A =0.9265 (0.0245)	α_a =0.8333 (0.0345)
	α_B =0.8295 (0.0350)	α_b =0.8273 (0.0330)
	β_A =0.0	β_a =0.0055 (0.0062)
	β_B =0.0210 (0.0127)	β_b =0.0
	θ_{Ab} = 0.0326 (0.0124)	
	γ_0 =0.0769 (0.0176)	γ_1 =0.9231 (0.0176)
	γ_2 =0.0	
	Donors 1 + 2	
L_4 = –1537.68	α_A =0.8696 (0.0192)	α_a =0.8448 (0.0198)
	α_B =0.8460 (0.0200)	α_b =0.8450 (0.0199)
	β_A =0.0	β_a =0.0099 (0.0065)
	β_B =0.0	β_b =0.0061 (0.0054)
	θ_{Ab} = 0.0222 (0.0064)	
	γ_0 =0.0711 (0.0106)	γ_1 =0.9160 (0.0115)
	γ_2 =0.0	

Values in parentheses are standard errors. *L*, maximum log likelihood.

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