

Ethnic variations of a retinoblastoma susceptibility gene (*RBI*) polymorphism in eight Asian populations

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Abstract

An A → G single nucleotide polymorphism (SNP) at nucleotide 153,104 in the retinoblastoma susceptibility locus (*RBI*) at 13q14 was previously reported to be present only in Asians. In this study, we determined the distribution of this SNP in normal Southeast Asian populations (Chinese, Malay, Javanese, Thai, Filipino), in South Asian populations (Bangladeshi, Pakistani Pushtun and Indian) and in Chinese retinoblastoma cases and control subjects. The *RBI* SNP was present in all populations at an overall frequency of ≤ 0.18. Heterozygosity was higher in the Southeast Asian groups (0.14–0.34) than in the South Asian groups (Bangladeshi and Indian) (0.04–0.06). Significant differences in allele frequencies were found between the two population groups. Interestingly, our Pakistani population comprised of ethnic Pushtuns from northwest Pakistan was significantly different from the neighbouring Bangladeshi and Indian populations. No significant difference was found between Chinese case patients and control subjects. This *RBI* SNP appears to be an ethnic variant prevalent in Southeast Asian populations and may be useful for studying *RBI* inheritance by pedigree analysis.

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Introduction

Retinoblastoma is the most common childhood intraocular malignant neoplasm that arises in the retina (Knudson 1971). It occurs in hereditary and nonhereditary forms, the hereditary forms being transmitted as an autosomal dominant trait (Hogg *et al.* 1992). The gene associated with the disease is a tumour suppressor gene called the retinoblastoma susceptibility gene, *RBI*, on chromosome 13q14 (Toguchida *et al.* 1993). Although polymorphisms in *RBI* have already been reported (Cavenee *et al.* 1984; Dryja *et al.* 1984; Yandell and Dryja 1989; Bookstein *et al.* 1990; Lohmann *et al.* 1996; Lohmann 1999; Alonso *et al.*

2001), limited and fragmentary information is available on their incidence among Asians. Given that the presence or absence of alleles and their frequencies among different ethnic groups are markedly varied in as much as 40% of all known human SNPs (Iwasaki *et al.* 2001), a better understanding of the distribution of *RBI* polymorphisms among Asian populations will facilitate the design of specifically tailored strategies for studying *RBI* inheritance by pedigree segregation analysis in different Asian ethnicities.

The diallelic polymorphism investigated here was previously detected as a novel variant (an A → G change at nucleotide 153,104 in intron 18 of the *RBI* gene) and affects a *Tsp509I* restriction site (Schubert and Hansen 1996). It was found to occur only in Asians and not in North American populations. To our knowledge, no other population and case studies involving this marker have

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since been reported. The present work is an extensive study of the incidence of this *RBI* SNP in Asian populations to validate this marker and determine genotype frequencies for pedigree segregation analysis for retinoblastoma among Asians. Allele frequency distributions of the SNP were studied in two major ethnic groupings, Southeast Asian (Chinese, Malay, Javanese, Thai, Filipino) and South Asian (Bangladeshi, Pakistani Pushtun, Indian) populations, and in Chinese retinoblastoma patients for a case-control association study. The heterozygosity values and allele frequencies will aid in the prediction of retinoblastoma susceptibility in affected families among Asians and may be used for population-genetic studies in the region.

Materials and methods

Study subjects: The following populations were studied: Chinese (Han Chinese from southern China), Malay from the Malayan peninsula, Javanese from central Java, Thais, Filipinos, Bangladeshi, Pakistani Pushtun (Pushuns from northwest Pakistan) and Indians (Dravidians from southern India). Blood samples were obtained with informed consent from fifty normal unrelated subjects in each population, as well as from 47 unrelated Chinese (Han Chinese from southern China) retinoblastoma patients for the case-control association study. The retinoblastoma patients were simplex cases with no known family history. Of the 47 patient cases, 25 were unilateral sporadic while 22 were germline (20 bilateral and 2 unilateral) cases. Patients were designated as germline if there was bilateral disease or if a mutation in the *RBI* gene was previously identified to be present in their constitutional DNA. The *RBI* SNP profiles of the patient cases were compared to the normal Chinese population sample described earlier.

Genotyping of the *RBI* SNP by RFLP: Genomic DNA was prepared using standard high-salt precipitation method (Miller *et al.* 1988). Primers used to amplify exon 19 of *RBI* and its flanking intronic sequences (GenBank Accession No. L11910) were forward 5'-AGGCAGT-AATCCCCAGGAAAAGCCA-3' and reverse 5'-CACAGAGATATTAAGTGAAGTGGCC-3' (Hogg *et al.* 1992). Amplification was performed in a total volume of 50 μ l, containing 200 ng genomic DNA, 1.5 mM MgCl₂, 2.5 U *Taq* DNA polymerase (AP Biotech, England), 200 nM dNTPs, 200 nM each of forward and reverse primer, and 3 μ l DMSO. The amplification conditions were: 94°C for 7 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min, ending with a single 3-min extension step at 72°C. The SNP affects a *Tsp509I* site within the 485-bp amplified fragment, allowing the use of *Tsp509I* enzyme for rapid genotyping of the *RBI* SNP in the populations.

Eight μ l of the PCR product was digested with *Tsp509I* (New England Biolabs, USA) in a 20- μ l mixture at 65°C for 90 min, following manufacturer's instructions. Restriction fragments were resolved by electrophoresis on 3% NuSieve™ (FMC BioProducts, USA) agarose gel. To confirm heterozygous samples for 153104A/G, direct sequencing of randomly selected samples was performed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, USA) using BigDye™ Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

Statistical analysis: Allele and genotype frequencies, expected heterozygosity by Levene's correction, and Hardy-Weinberg conformity by Guo and Thompson's exact test were determined using GENEPOP (v. 3.1d) software (Raymond and Rousset 1995). Significance levels of comparisons were determined by χ^2 statistics.

Results and discussion

The primers used amplified a 485-bp fragment spanning exon 19 of *RBI* and its flanking intronic sequences. The SNP studied here is an A \rightarrow G transition at nucleotide 153,104 in intron 18, affecting one of three *Tsp509I* restriction sites within the amplified fragment. Thus the variant allele, designated here as *G*, has three fragments, the uninterrupted 237-bp (cut into 157-bp and 80-bp fragments in the wild-type allele *A*), 210-bp and 38-bp fragments. Heterozygotes are characterized by a composite profile of five bands: 237, 210, 157, 80 and 38 bp (figure 1a) and the presence of 153104A/G in the DNA sequence chromatogram (figure 1b).

Three genotypes, designated as *G/G*, *A/G*, and *A/A*, were detected in varying frequencies across the Asian populations (table 1). The frequency of allele *G*, which indicates presence of the *RBI* SNP, was ≤ 0.18 across the populations. This value is slightly lower than what was previously observed in a mixed Asian population (Schubert and Hansen 1996), although the sample size of the latter, i.e. 25 individuals, was relatively limited. All groups conformed to Hardy-Weinberg expectations, with the exception of the Chinese and Malay populations, which exhibited homozygote excess. In this study, randomness of the sampling of unrelated subjects from each population was ascertained by interview prior to collection and surname (family name) check, thus discounting or at least reducing the possibility of sampling errors that may account for the deviations. Another causative factor may be limitations in the sample size, but, as demonstrated by the other populations in this study, the sample size of 50 individuals is sufficient to attain Hardy-Weinberg proportions for the genotype distributions of the *RBI* SNP. The deviations are also unlikely to be due to genotyping errors since the methods used in this study give discrete

and unambiguous results considering the ease of detection of the *RB1* SNP. Notwithstanding the interplay of indeterminate population-genetic factors that may be responsible for the deviations, the allele and genotype data can still be used as the departures in the Chinese ($P \leq 0.0361$) and Malay ($P \leq 0.0212$) were only slightly significant. Heterozygosity values were clearly divided into two sets corresponding to the two population groups in this study. South Asian populations, i.e. Indian and Bangladeshi, gave lower heterozygosities, 0.04 to 0.06, respectively, compared to the Southeast Asian populations where the values ranged from 0.14 to 0.34 (table 1). This observation underlines the importance of determining

the frequencies of polymorphisms in various ethnic groups to identify markers that are informative for use in a particular population.

The allele frequency distributions between the two population groups were significantly different based on χ^2 statistics (table 2), indicating the genetic heterogeneity of the Asian populations with respect to the incidence of the *RB1* SNP. A case of genetic isolation by distance was observed in that the geographically related Southeast Asian populations had similar allele frequencies and distributions, distinct from those of the South Asian Bangladeshi and Indian groups. This observation supports previous findings on the differentiation of Chinese and Malay populations from Asian Indians based on other DNA polymorphisms (Tan *et al.* 1996; Heng *et al.* 1999). Our Pakistani population sample composed of ethnic Pushtuns from northwest Pakistan, however, did not share the same allele frequency profile as the other South Asian groups in this study. The results obtained from the Pushtun population could not be extrapolated to the entire Pakistani population, as comparisons with other non-Pushtun Pakistani groups would be necessary. In any case, it would be interesting to explore the possibility of genetic uniqueness of the Pushtun ethnic minority group using additional polymorphic markers, non-Pushtun Pakistani samples and more South Asian populations. Presently, there are no other reported population-based data available for the *RB1* SNP with which the allele frequencies observed in this study can be compared.

There was no significant difference in the genotypic proportions of the *RB1* SNP between case patients and control subjects in our Chinese population. Also, comparison between allele and genotype frequencies between germline and sporadic unilateral cases showed no significant difference. This suggests that this *RB1* SNP may not influence the germline or sporadic origin of retinoblastoma (table 1). As retinoblastoma is a rare disease it is difficult to carry out large case-control studies. However, on the basis of the present results from the comparison of genotypic frequencies, this *RB1* SNP is not likely to be directly involved in the pathogenesis of retinoblastoma. This is in agreement with previous observations of its high incidence in unaffected Asian individuals (Schubert and Hansen 1996). It is likely that the *RB1* SNP has attained evolutionary stability in the Asian populations and any variation seen may have been derived from early migrations and random drift events, under limited or no selective pressure. Therefore it is also a potentially useful marker for population studies unrelated to retinoblastoma, such as investigations on the phylogenetic relationships between populations, as well as association studies for gene mapping. The validation of its precise location at nucleotide 153,104 of the *RB1* locus in 13q14 and allele frequency data among these populations contribute to a finer definition of the chromosome 13 genetic map of SNPs. Although

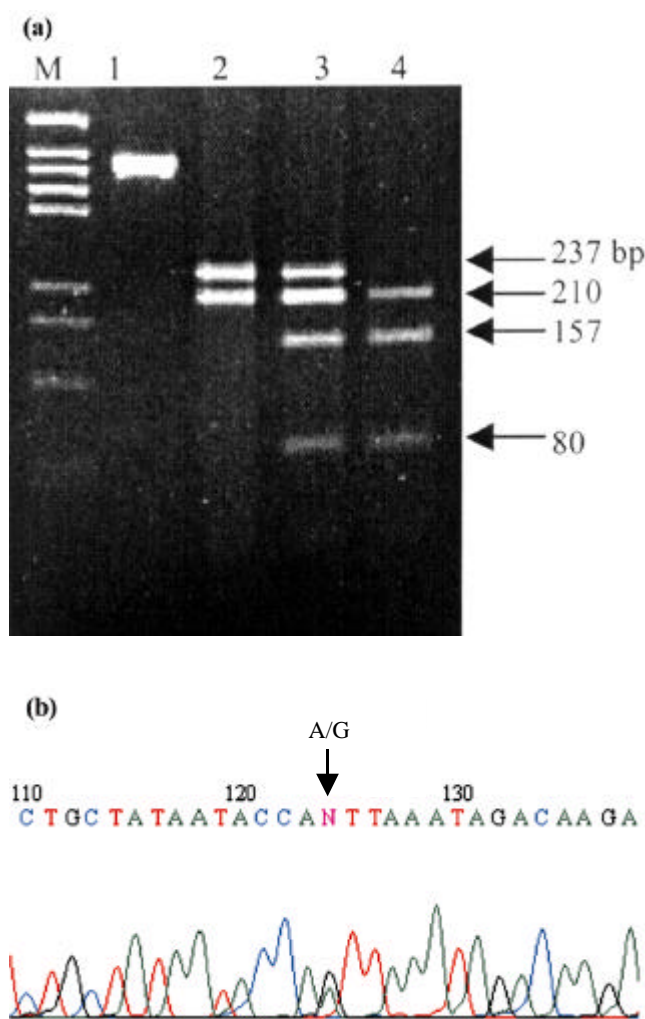


Figure 1. *Tsp509I* polymorphism in the *RB1* locus. (a) Observed genotypes of the *Tsp509I* polymorphism. The restriction fragments were resolved on 3% NuSieve™ agarose gel and visualized by ethidium bromide staining. M, pGEM size ladder (Promega, USA); lane 1, undigested 485-bp PCR product; lane 2, *G/G* genotype; lane 3, *A/G* genotype; lane 4, *A/A* genotype. The 38-bp fragment of the three genotypes is not visible. (b) Sequence chromatogram of the 153104A/G heterozygous state in intron 18 of *RB1*.

Table 1. Allele and genotype frequencies of the *RBI* 153104A→G SNP and Hardy–Weinberg conformity in Asian populations.

Population	N	Genotype frequency ^a								HW conformity ^b P value
		Allele frequency		G/G		G/A		A/A		
		G	A	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
Chinese ^c	50	0.14	0.86	0.06	0.02	0.16	0.24	0.78	0.74	0.0361*
Malay	50	0.13	0.87	0.06	0.16	0.14	0.23	0.80	0.76	0.0212*
Javanese	50	0.10	0.90	0.02	0.01	0.16	0.18	0.82	0.81	0.3883
Thai	50	0.12	0.88	0.02	0.01	0.20	0.21	0.78	0.77	0.5266
Filipino	50	0.17	0.83	0	0.03	0.34	0.28	0.66	0.69	0.3172
Bangladeshi	50	0.03	0.97	0	0	0.06	0.06	0.94	0.94	1.0
Pakistani (Pushtuns)	50	0.11	0.89	0	0.01	0.22	0.20	0.78	0.79	1.0
Indian	50	0.02	0.98	0	0	0.04	0.04	0.96	0.96	1.0
Chinese retinoblastoma patients (unrelated)										
	47	0.15	0.85	0.02	0.02	0.28	0.25	0.70	0.72	0.9995
Germline cases (20 bilateral + 2 unilateral)										
	22	0.14	0.86	0	0.06	0.27	0.24	0.73	0.82	0.9716
Unilateral sporadic cases										
	25	0.22	0.78	0.08	0.13	0.28	0.34	0.64	0.53	0.9861

Cases vs control $\chi^2 = 0.0004$; d.f. = 2; $P = 0.9998$, not significant.
 Germline vs unilateral sporadic $\chi^2 = 0.2168$; d.f. = 1; $P = 0.8829$, not significant.

^aObs., observed; Exp., expected; computed using Levene's correction.

^bDetermined by Guo and Thompson's exact test using GENEPOP v. 3.1c software.

^cControl population for the case–control study.

*Significant at 5% level.

Table 2. Pairwise test for differences in allele frequencies in Asian populations by χ^2 statistics.

Population	Malay N = 50	Javanese N = 50	Thai N = 50	Filipino N = 50	Bangladeshi N = 50	Pakistani N = 50	Indian N = 50
Chinese							
χ^2	0.0428	0.7575	0.1768	0.3435	7.7788	0.4114	9.7826
P	0.8361	0.3841	0.6741	0.5578	0.0053*	0.5213	0.0018*
Malay							
χ^2		0.4421	0.0457	0.6274	6.7934	0.1893	8.7207
P		0.5061	0.8307	0.4283	0.0091*	0.6635	0.0031*
Javanese							
χ^2			0.2042	2.0980	4.0312	0.0532	5.6737
P			0.6514	0.1475	0.0447*	0.8176	0.0172*
Thai							
χ^2				1.0082	5.8378	0.0491	7.6804
P				0.3153	0.0157*	0.8246	0.0056*
Filipino							
χ^2					10.8888	1.4950	13.0851
P					0.001*	0.2214	0.0003*
Bangladeshi							
χ^2						4.9155	0.2051
P						0.0266*	0.6506
Pakistani (Pushtuns)							
χ^2							6.6639
P							0.0098*

*Significant at 5% level.

there are now approximately 60,000 SNPs mapped to chromosome 13, on the basis of the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP>), only about 200 SNPs and variants are known in *RB1*, most of them having been studied in Caucasian populations (Cavenee *et al.* 1984; Dryja *et al.* 1984; Yandell and Dryja 1989; Bookstein *et al.* 1990; Lohmann *et al.* 1996; Lohmann 1999; Alonso *et al.* 2001).

The differences in the distribution of the *RB1* SNP allele frequencies in the Asian populations indicate that the polymorphism is an ethnic variant, found more frequently among Southeast Asians than among South Asians. While the acid test of the genetic diversity of these populations is only upon inclusion of more markers and ethnicities in the analysis, the results already support previous studies showing that the incidences of many SNPs among different ethnic groups vary considerably (Cao and Hegele 2001; Iwasaki *et al.* 2001). The baseline data presented here may contribute to the definition of a strategy for carrier detection and prenatal diagnosis of retinoblastoma by haplotype segregation analysis for Asian populations, as well as to population genetics.

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References

- Alonso J., Garcia-Miguel P., Abelairas J., Mendiola M., Sarret E., Vendrell M. T., Navajas A. and Pastana A. 2001 Spectrum of germline *RB1* gene mutations in Spanish retinoblastoma patients: Phenotypic and molecular epidemiological implications. *Hum. Mutat.* **17**, 412–422.
- Bookstein R., Lai C., To H. and Lee W. H. 1990 PCR-based detection of a polymorphic *Bam*HI site in intron 1 of the human retinoblastoma (*RB*) gene. *Nucl. Acids Res.* **18**, 666.
- Cao H. and Hegele R. 2001 Single nucleotide polymorphisms of the resistin (*RSTN*) gene. *J. Hum. Genet.* **46**, 553–555.
- Cavenee W., Leach R., Mohandas T., Pearson P. and White R. 1984 Isolation and regional localization of DNA segments reveal polymorphic loci from human chromosome 13. *Am. J. Hum. Genet.* **36**, 10–24.
- Dryja T. P., Cavenee W., White R., Rapaport J. M., Petersen R., Albert D. M. and Bruns G. A. 1984 Homozygosity of chromosome 13 in retinoblastoma. *N. Engl. J. Med.* **310**, 550–553.
- Heng C. K., Saha N. and Low P. S. 1999 Evolution of the apolipoprotein B gene and coronary artery disease: a study in low and high risk Asians. *Ann. Hum. Genet.* **63**, 45–62.
- Hogg A., Onadim Z., Baird P. N. and Cowell J. K. 1992 Detection of heterozygous mutations in the *RB1* gene in retinoblastoma patients using single strand conformation polymorphism (SSCP) analysis and PCR sequencing. *Oncogene* **7**, 1444–1451.
- Iwasaki H., Shinohara Y., Ezura Y., Ishida R., Kodaira M., Kajita M., Nakajima T., Shiba T. and Emi M. 2001 Thirteen single-nucleotide polymorphisms in the human osteopontin gene identified by sequencing of the entire gene in Japanese individuals. *J. Hum. Genet.* **46**, 544–546.
- Knudson A. G. 1971 Mutation and cancer: Statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA* **68**, 820–823.
- Lohmann D. R. 1999 *RB1* gene mutations in retinoblastoma. *Hum. Mutat.* **14**, 283–288.
- Lohmann D. R., Brandt B., Hopping W., Passarge E. and Horsthemke B. 1996 The spectrum of *RB1* germ-line mutations in hereditary retinoblastoma. *Am. J. Hum. Genet.* **58**, 940–949.
- Miller S. A., Dykes D. and Polesky H. F. 1988 A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl. Acids Res.* **16**, 1215.
- Raymond M. and Rousset F. 1995 Genepop (version 1.2): A population genetic software for exact tests and ecumenicism. *J. Hered.* **86**, 248–249.
- Schubert E. L. and Hansen M. F. 1996 A previously unknown polymorphism located within the *RB1* locus only present in Asian individuals. *Hum. Hered.* **46**, 118–120.
- Tan J. A., Tay J. S., Aziz N. B. and Saha N. 1996 T cell receptor beta chain RFLP in Chinese, Indians and Malays from Singapore. *Hum. Hered.* **46**, 236–238.
- Toguchida J., McGee T. L., Paterson J. C., Eagle J. R., Tucker S., Yandell D. W. and Dryja T. P. 1993 Complete genomic sequence of the human retinoblastoma susceptibility gene. *Genomics* **17**, 535–543.
- Yandell D. W. and Dryja T. P. 1989 Detection of DNA sequence polymorphisms by enzymatic amplification and direct genomic sequencing. *Am. J. Hum. Genet.* **45**, 547–555.

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