

Prediction of retinoblastoma and osteosarcoma: linkage analysis of families by using polymorphic markers around RB1 locus

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Summary

Purpose: Linkage analysis at the retinoblastoma gene (RB1) locus is required for identification of individuals at risk of developing retinoblastoma and osteosarcoma. Identification of disease causing mutations is necessary for accurate risk prediction. However, the usefulness of direct mutation analysis is impeded by the size and complexity of the RB1 gene. The authors report an alternative polymerase chain reaction (PCR)-based method for genotyping the RB1 locus using polymorphic microsatellite markers for the prediction of risk of developing the disease.

Materials and methods: For this purpose, we have used 2 intragenic microsatellite markers of the RB1 gene,

D13S153 and RB1.20 VNTR, and 2 flanking markers D13S218 and D13S176. The segregations of the 4 polymorphic markers within and flanking the RB1 gene were analyzed in 3 families with osteosarcoma and 2 families with retinoblastoma.

Results and conclusion: Our results showed that linkage analysis of families by using the intragenic and flanking markers could be applied to detect carriers and for prenatal diagnosis in families with retinoblastoma and osteosarcoma. Moreover, this PCR-based genotyping is simpler and faster than other conventional methodologies.

Key words: linkage analysis, microsatellite marker, osteosarcoma, RB1 locus, retinoblastoma

Introduction

Retinoblastoma, the most common intraocular cancer of the childhood, occurs in both hereditary and sporadic forms. The former is characterized by early age of onset and multiple tumor foci compared to the nonhereditary type, which occurs later with a single, unilateral tumor. Susceptibility to hereditary retinoblas-

toma is transmissible to offspring as an autosomal dominant trait with 90% penetrance [1]. There is also an increased risk of other specific extraocular primary neoplasms among the patients cured of retinoblastoma (collectively called second primary tumors). Most of the second primary cancers are osteosarcomas, soft tissue sarcomas, or melanomas [2]. These tumors are usually manifested in adolescence or adulthood. A majority of osteosarcoma tumors from patients with or without a history of retinoblastoma also showed inactivation of the RB1 gene [3].

The retinoblastoma susceptibility gene (RB1) is located in chromosomal 13q14.2 locus and consists of 27 coding exons spanning 179035 basepairs of the genome. Mainly nonsense mutations of the RB1 gene are found, resulting in truncated gene product [4-6], though missense mutation and also mutation of the promoter have been reported [7-9]. The 'two-hit' hypothesis proposed by Knudson for the development of retinoblastoma suggests inactivation of both alleles

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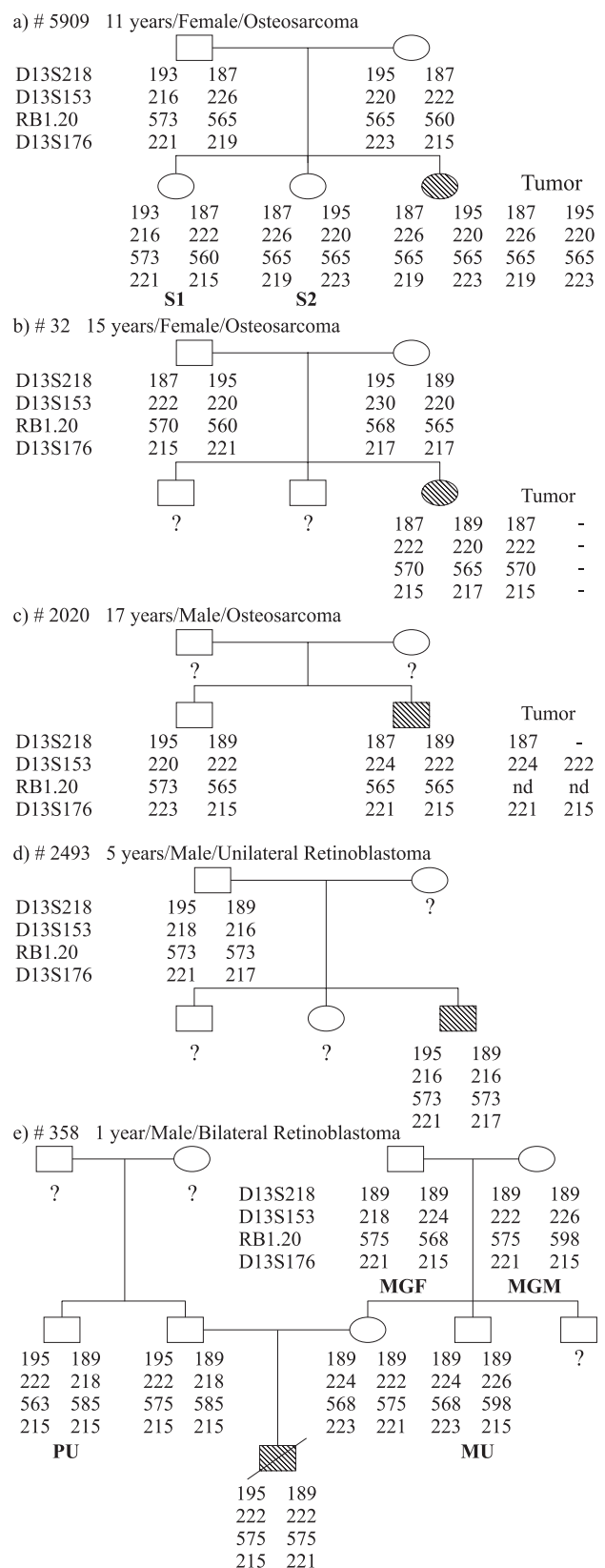
[10]. The hereditary form of the disease is caused by the germline mutations in one allele, and therefore, the mutant gene is present in all somatic cells. Because the second tumorigenic event, the somatic inactivating mutation of RB1 in retinoblasts, occurs spontaneously at a relatively frequent rate (1:1,000) [11], the prevalent phenotype in this group of patients is mainly bilateral, multifocal retinoblastomas with an early onset (mean age at diagnosis, 1 year) and a lifetime predisposition to other RB1-dependent tumors, such as osteosarcoma. In the nonhereditary (sporadic) form of the disease, both inactivating events occur during somatic development of a single retinal cell, resulting in the relatively late onset of a single tumor in one eye (i.e., unilateral disease with a median age of 2 years at diagnosis) [11]. However, in no patient the presence of a germline mutation can be excluded clinically. Hence, the relatives of these patients are also at an increased risk for the disease. Molecular testing is required for prediction of the risk among the family members. An increased risk of the relatives can be excluded by segregation analysis. Mutational analysis or loss of heterozygosity (LOH) analysis may be used to screen the family members. Though identification of the disease causing mutation is necessary for accurate risk prediction, its usefulness as a screening method to approach the familial form is hampered by the fact that mutations of the RB1 gene are scattered among 27 exons and the promoter region of the RB1 gene, and no single hotspot has been found [12]. Alternately, LOH analysis using intragenic polymorphic markers could be applied to detect carriers and for prenatal diagnosis in families as this approach is simpler and less labor-intensive.

In the current study we describe a simple predictive test that screens for susceptibility to retinoblastoma and osteosarcoma. For this purpose, we have used two intragenic and two flanking highly polymorphic microsatellite markers around the RB1 locus.

Materials and methods

Subjects

A total of 5 patients and their families were studied. Three patients (# 5909, 32, 2020) were affected with osteosarcoma (age range 11-17 years). The patient (# 2493) aged 5 years presented with unilateral retinoblastoma. The patient (# 358) presenting with bilateral retinoblastoma developed the tumor in the left eye at the age of 1.5 years and the second tumor in the right eye at the age of 2 years (Figure 1a-e). All these



The numbers beneath a symbol represent the haplotype comprising of alleles in base pairs.

For abbreviations see text.

Figure 1. Linkage analysis of families using microsatellite markers D13S218, D13S153, RB1.20 and D13S176.

patients did not have any other affected siblings or a family history of retinoblastoma or osteosarcoma.

Tumor samples and matching normal peripheral lymphocytes (PBL) were available from the osteosarcoma patients who underwent surgery at the hospital section of Chittaranjan National Cancer Institute, Kolkata. Blood samples were obtained from the retinoblastoma patients and available family members of all the osteosarcoma and retinoblastoma patients for genetic testing. The tumor tissues and the blood samples were frozen immediately and stored at -80°C until further use. Informed consent from both patients with their family members and hospital authorities were obtained for sample collection.

Microdissection and DNA isolation

The contaminant normal cells in the osteosarcoma tumor specimens were removed by microdissection procedure. More than 50 serial sections (10–20 μm) were taken on glass slides using a cryostat (Leica CM 1800, Germany). The representative 5 μm sections from different regions of the specimens were stained with hematoxyline and eosin for diagnosis, as well as for marking of tumor-rich regions. These marked regions were meticulously dissected by microdissection procedure. Samples containing $> 60\%$ tumor cells were taken for further analysis [13].

High molecular weight DNA from the microdissected osteosarcoma tumors and the PBL from the osteosarcoma, retinoblastoma patients and their family members was extracted by proteinase-K digestion followed by phenol-chloroform extraction [14].

Linkage analysis

Two intragenic markers of the RB1 gene, microsatellite D13S153 located in intron 2 and RB1.20 VNTR intron sequence 54 bp from 3' end of exon 20 were used [15,16]. Two flanking microsatellite markers

D13S218 and D13S176 were also used. Primer sequences of these markers were obtained from Genome Database (<http://www.gdb.org>).

The isolated DNA were amplified by PCR in a 20 μl reaction mixture containing 1X PCR buffer [67 mM Tris-HCl (pH 8.7), 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.01% Tween-20], 25–50 ng genomic DNA, 1–1.5 mM MgCl_2 , 4 pmol of each primer (except RB1.20 VNTR), 0.2 mM of each dNTPs (Gibco-BRL, USA), 0.5–1 unit of Taq polymerase (Gibco-BRL, USA). One of the paired primers in the reaction mixture was end-labeled with $[\gamma^{32}\text{P}]$ ATP (specific activity 3000 ci/mmol, Perkin Elmer Life Sciences Inc., USA) using T4-polynucleotide kinase (Gibco-BRL, USA). In case of RB1.20 VNTR, the PCR was performed in 20 μl reaction volume containing 1X PCR buffer [50 mM Tris-HCL (pH 8.3), 10 mM KCL, 5 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 0.2 mM of each dNTP, 5 pmol of each primer, 0.1 μl of $[\alpha^{32}\text{P}]$ dCTP (specific activity 3000ci/mmol, Perkin Elmer Life Sciences Inc., U.S.A) and 2 units of FastStart Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The labeled PCR products were electrophoresed on 7% denaturing polyacrylamide gel containing 8M urea and autoradiographed [13]. The PCR conditions are given in Table 1.

LOH in osteosarcoma tumors was evaluated according to the method described by Chunder et al. [13].

Results and Discussion

We studied 5 pedigrees including 3 osteosarcoma and 2 retinoblastoma cases using 2 flanking and 2 intragenic microsatellite markers selected on the basis of their reported heterozygosity and close linkage to the RB1 gene [15,16].

In case #5909 (osteosarcoma) illustrated in Figure 1a, the patient along with one of her sisters (S2 aged 14 years) carries the same allelic profile that segregated with the disease. However, S2 is free of

Table 1. Characteristics of microsatellite markers

Marker	Position	Size range (bp)	Heterozygosity	No. of reported alleles	Annealing temperature	MgCl_2 (mM)	No. of observed alleles
D13S218	Flanking	187-195	0.66	5	55° C for 30 cycles	1.5	4
D13S153	intragenic	212-236	0.82	10	55° C for 30 cycles	1.5	7
RB1.20	intragenic	550-600	0.94	n.a	61° C for 5 cycles 57° C for 30 cycles	2	10
D13S176	Flanking	211-227	0.79	7	60° C for 30 cycles	1	6

n.a: not available

disease to date. The tumor of the patient showed no LOH of the markers in any of the haplotype inherited from the parents. From the linkage analysis, it could be observed that any one of the parents may be a carrier of a mutant haplotype that is also present in both the sisters. The patient has acquired a second hit according to Knudson's "two-hit" hypothesis that has led to the development of the disease. The second hit did not occur in S2 and hence she showed no disease manifestation but is at the risk of developing other forms of cancer later in her lifetime. As a carrier of a mutant allele, the offspring of S2 may also be at risk. The eldest sister (S1 aged 19 years) did not carry the alleles that segregated with the disease, indicating that she is not at the risk of developing the disease. Supporting this prognosis, she is free of the disease to date.

In case #32 (osteosarcoma) in Figure 1b, the maternal haplotype was found to be deleted in the tumor as all the markers showed LOH. This indicates that the haplotype inherited from the father may be mutated leading to the inactivation of the RB1 gene. The 2 elder brothers (aged 21 and 25 years respectively) of the patient are disease-free to date, but no blood samples could be collected from them and hence their risk for the disease could not be predicted. If they carry the father's haplotype that is present in the patient, they may be at risk of disease development.

In case #2020 (osteosarcoma) in Figure 1c, both the patient and his unaffected brother (aged 26 years) carry a common haplotype that showed LOH at only flanking D13S218 marker in the tumor of the patient. The tumor showed retention of the intragenic D13S153 and flanking D13S176 markers. However, allelic status of RB1.20 marker could not be determined (nd) due to paucity of the tumor DNA. It seemed that both RB1 alleles in the tumor may be mutated. However, the haplotype (187 - 224 - 565 - 221) of the patient may have germline mutation as the disease-free elder brother has the other haplotype.

In case #2493 (unilateral retinoblastoma) in Figure 1d, it is observed that the RB1 locus was homozygous though the flanking markers were heterozygous. No blood samples were available from the patient's mother, elder brother and sister, hence their risk could not be predicted. But if the patient's siblings carry the same haplotype they are at risk.

In case #358 (bilateral retinoblastoma) in Figure 1e, the RB1 locus was found to be homozygous while the flanking markers are heterozygous as also seen in the previous case (Figure 1d). But the early age of onset (1.5 years) and bilateral disease indicate that both parents may have a cancer-predisposing

germline mutation of RB1 gene that is transmitted to the patient. The patient has inherited one of its haplotype from his mother that could be traced to his maternal grandmother (MGM). His maternal uncle (MU) is not at risk as he has inherited the other haplotype of his mother (MGM). The paternal haplotype of the child is absent in his paternal uncle (PU) and he is disease-free. Both parents are apparently unaffected. In the presence of a cancer-predisposing mutation, the risk to each sib (if born) of the patient is 50% of inheriting the cancer-predisposing mutation from the parents [17,18].

Thus the linkage analysis by the microsatellite markers can be an alternative for screening of susceptibility of the disease among the family members. Genotyping is essential for the identification of carriers and genetic counseling. The microsatellite analysis is useful for the detection of large deletions, and as pre-screening in search of mutations that help determine the risk in offspring. Moreover, this methodology provides a rapid and reliable alternative to other methodologies.

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