Identification of Four Novel RB1 Germline Mutations in Korean Retinoblastoma Patients

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To elucidate RB1 germline mutations in Korean retinoblastoma patients, DNA samples from 14 children with bilateral (including three familial cases) and 19 children with unilateral retinoblastoma were analyzed. We found germline mutations in three out of 14 bilateral cases and one out of 19 unilateral cases. There were no germline mutations in the three familial cases. PCR-SSCP from each exon showed bandshifts in four patients which, upon sequencing, were shown to be K616E in exon 19 (c.1846A>G), an AA insertion in exon 7 (c.684-685insAA), R500G in exon 16 (c.1498A>G), and an A insertion in exon 23 (c.2391-2392insA), respectively. © 2001 Wiley-Liss, Inc.

KEY WORDS: Retinoblastoma; RB1; Korea; bilateral RB; blindness

INTRODUCTION

Retinoblastoma (MIM# 180200) is a malignant tumor, often found in children, which can damage sight and eventually make it impossible to retain the eyeball. When it spreads to other parts of the body, it can cause death. The disease occurs in one out of 15,000-20,000 individuals. Although the average diagnosis age is 1.5 to 2-years old, it can be diagnosed earlier [Tamboli et al., 1990].

While Korea’s annual incidence of retinoblastoma is around 40 out of 600,000, similar in proportion to that of the West [Yu, 1995], it is the most frequently found intraocular tumor in Korea because the incidence of malignant melanoma is much lower in Korea compared to the West [Roh et al., 1987]. It is hereditary in about 40% of all patients, in which case the disease is bilateral and multifocal. In only 10-15% of these cases was there any

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indication of a family history of the disease, and the remaining subjects are considered to have developed the disease due to new germline mutations [Hogg et al., 1992]. In Korea, the occurrence of retinoblastoma following a family history of the disease is considered to be similar to that of the West [Yu et al., 1996]. The RB1 gene is located on chromosome 13q.14.2, is about 180kb in size, and consists of 27 exons. It has been determined that a germline mutation of the RB1 gene is related to hereditary retinoblastoma, and the somatic mutation of the gene has also been known to occur in sporadic retinoblastoma and other kinds of tumors as well [Yandell et al., 1989]. To elucidate this germline mutation of the RB1 gene in Korean retinoblastoma patients, we collected DNA samples from the patients’ blood and analyzed this with the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) method. Subsequently, the germline mutations of the RB1 gene were confirmed by bidirectional DNA sequencing.

MATERIALS AND METHODS

Patient samples

Blood samples were collected from 33 Korean retinoblastoma patients at Seoul National University Hospital (designated as SNU-RB1-SNU-RB33). Clinical manifestations in the retinoblastoma patients are shown in Table 1. Three out of 33 patients had a family history of the disease. Fourteen patients were bilaterally affected in early childhood and nineteen patients were unilateral cases. Mutational screening for the RB1 gene was performed with DNA samples from all 33 patients. Informed consent was obtained from the parents of all participants before testing.

DNA extraction

Peripheral blood lymphocytes from the retinoblastoma patients were isolated using Ficoll-Paque, following the manufacturer's instructions (Pharmacia Biotech, Uppsala, Sweden). Total genomic DNA was extracted using TRI reagent, following the manufacturer's instructions (Molecular Research Center, Cincinnati, OH, USA).

PCR amplification and Single-strand conformation polymorphism (SSCP) analysis

PCR primer pairs and conditions were used as described by Liu et al. [Liu et al., 1995]. Non-radioactive PCR reactions were carried out in a volume of 25 µl containing 100 ng of genomic DNA, 2.5 pmoles of each primer, four dNTP at 250 µM each, 0.5 units of Taq polymerase and the reaction buffer provided by the supplier (Boehringer- Mannheim, Germany). Reactions were performed in a programmable thermal cycler (Perkin Elmer Cetus 9600; Roche Molecular Systems, Inc., New Jersey, USA).

To detect genetic alterations of the RB1 gene, we investigated all 27 exons in the samples from 33 Korean retinoblastoma patients by PCR-SSCP. For SSCP, the genomic DNA was amplified, in a final volume of 10 µl, for each exon of the RB1 gene through the same PCR procedure as described above, with the addition of [α-32P]-dCTP (Amersham, Arlington Heights, IL, USA). Exons 2, 8, 15-16 and 17 were divided into two overlapping products to obtain a higher sensitivity in detecting mutations by PCR-SSCP analysis as described by Liu et al [1995].

Radio-labeled PCR reaction products were mixed with 95% formamide dye, denatured at°C 94°C for 5 min. and chilled on ice. 3.5 µl of each mixture was loaded on a non-denaturing SSCP gel, 6% polyacrylamide (19:1) with 10% glycerol in 1x TBE buffer, and separated for 12 to 16 hours at 4°C at a constant level of 300V. Following, electrophoresis, the gel was transferred to 3MM Whatman paper, dried on a gel dryer, and subjected to autoradiography.

Cloning and Sequencing

The samples where the SSCP showed abnormal bands were directly sequenced (SNU-RB11) or subjected to cloning for DNA sequencing analysis. Fresh PCR products were ligated into PCR-TOPO vectors (Invitrogen, Carlsbad, CA, USA) and subcloned using the TA cloning system (Invitrogen, Carlsbad, CA, USA). A minimum of ten individual colonies were taken and cultured overnight in Luria-Bertani (LB) medium containing 50 µg /ml
ampicillin. Plasmid DNA was isolated and used for DNA sequencing analysis. Bi-directional sequencing was performed using the Taq dideoxy terminator cycle sequencing kit and ABI 377 DNA sequencers (Perkin-Elmer, Foster City, CA, USA). Sequences of the target DNA were determined using the original PCR primers.

**LOH studies**

To evaluate LOH in SNU-RB24 and SNU-RB29, we microdissected normal and cancer area under microscopic visualization. The primer sequences used for chromosome 13q14 were obtained from the Genome Database. (www.gdb.org, primer name: RB-660 and RB-672).

**RESULTS**

As a result of PCR-SSCP analysis, abnormal band patterns were found in the four patients, identified as SNU-RB3, SNU-RB11, SNU-RB24 and SNU-RB29. To confirm this aberrant band pattern, we analyzed samples from these four patients by bi-directional sequencing. Patient SNU-RB3 exhibited a missense mutation of the RB1 gene in exon 19 (K616E, SNU-RB3 was reported in the Journal of Korean Cancer Association, Ku et al., 1997). Patient SNU-RB24 showed a missense mutation of the RB1 gene in exon 16 (R500G, AGCAgtaagt→AGCGgtaagt). To rule out the possibility of a polymorphism in cases SNU-RB3 and SNU-RB24, we respectively investigated samples from 50 unrelated, normal individuals as a control. None of these samples showed these variants. Patient SNU-RB11 and patient SNU-RB29 respectively harbored a frameshift of the RB1 gene in exon 7 (c.684-685insAA), and in exon 23 (c.2391-2392insA). For the LOH studies, two (SNU-RB24 and SNU-RB 29) out of four patients with RB1 germline mutations were available. SNU-RB29 showed LOH at chromosome 13q14. But, SNU-RB24 did not show LOH (Table 2).

**DISCUSSION**

According to reports in Korea, there are 40 new retinoblastoma patients a year, which is similar to the level of Western countries, where it occurs in one out of every 15,000-20,000 individuals [Yu, 1995]. It is thought that the
chances of having a family history of retinoblastoma existing among Korean patients are about similar to those of foreign ones, while only a little has been discovered about the gene mutation. Therefore, it may be significant to identify the gene mutation in Korean patients and compare that with its Western counterpart. In this study, the occurrence rate of the mutation in Korean retinoblastoma patients was three out of 11 patients, or 27.3%, who were affected bilaterally and showed no family history of the disease. However, among patients with unilateral retinoblastoma and no family history of the disease, one in 19 patients, or 5.3%, exhibited RB1 germline mutation. There were no germline mutations in the three familial cases. Frequencies of the mutations in familial cases ranged from about 36% to 100%. It was reported that 16 out of 25 (64%) familial cases exhibited no germline mutations by Blanquet V. et al. (1995, Hum Mol Genet 4:383-388). As there were so low numbers (3 cases) of familial cases in our studies, mutation rates of familial cases would be changed with the increasing number of familial cases. Through studies, we detected missense mutations in exon 16 and 19, and frameshift in exons 7 and 23, all of which were observed in four patients. So far, nonsense mutation and frameshift have been the most frequently reported mutations, accounting for about 78% of the total [Harbour, 1998]. In our study, we also observed frameshift in two patients. Among the four patients in which mutations were observed, three exhibited retinoblastoma in both eyes. Patient SNU-RB11 was diagnosed with retinoblastoma one month after birth, but the clinical result of this patient's treatment remains unknown, as the parents of the patient decided to terminate treatment after the disease developed into a fatal case of systemic metastasis. Meanwhile, patient SNU-RB3 was diagnosed with bilateral retinoblastoma two months after birth and was treated with a combination of enucleation in the left eye and external x-ray radiation therapy. Fifteen years later, tumors were found in the right orbit and nasal cavity and were identified as osteosarcoma after biopsy. The patient ultimately died of sepsis during the course of chemotherapy treatment. Patient SNU-RB29 was diagnosed with retinoblastoma in both eyes at the age of 7 months and treatment with chemotherapy followed after left-eye enucleation, laser and cryotherapy. The tumor appeared to be in remission by the time the patient was two years old and at the time of this writing, the patient is undergoing follow-up examinations. Patient SNU-RB24 was diagnosed with retinoblastoma in the right eye at the age of two-and-a-half, and underwent enucleation in the right eye. There were no relapses by the time the patient was eight years old and the patient is also undergoing follow-up examinations. SNU-RB24 showed A to G variation at –1 position from the splicing donor site (AGCgtaagt→AGCGgtaagt). We could not rule out the possibility that this A to G variation result in a splicing variant. Blanquet et al. [Blanquet et al., 1995] reported tumors in areas other than the eyes in retinoblastoma patients who were found to have mutations in exon 19. In this study, we also found missense mutation in exon 19 in SNU-RB3 patient and observed osteosarcoma in areas other than the eyes after 15 years. However, Lohmann et al. [1996] maintained that the secondary tumors in other areas were not correlated with the location of the RB1 gene germline mutation, but were rather related with the patient’s age and treatment methods. In this study, we were unable to identify a correlation between the location of the germline mutation and clinical manifestations of retinoblastoma. The CGA codon experienced variation at the TGA codon as C→T transitions were frequently observed in many genes, including RB1 [Yandell et al., 1989]. There are 14 CGA codons within the RB1 gene and C→T transitions were observed at most of the 14 CGA codons [Lohmann et al., 1996]. However, we did not detect C→T transitions in our samples.

Cowell and Cragg suggested the possibility of constitutional mutation of genes even in unilateral cases when the tumor is in its early stage, which they maintained is very important in genetic screening [Cowell and Cragg, 1996; Lohmann et al., 1997]. In this study, three out of four patients who showed mutations were diagnosed with the disease within six months after birth and were bilateral cases. The one unilateral case was diagnosed at the age of two-and-a-half. Out of 19 unilateral patients, six patients who were diagnosed before they were a year-old did not show any mutation at all. In hereditary tumors such as retinoblastoma, it is vital to conduct hereditary consulting and screening among relatives as well as patients. When no RB1 mutation is found in the affected child, regular eye examinations for the relatives are still warranted. And it is expected that in the future, examination for mutations will become more established, both to eliminate fears among those who do not need to undergo full examination, and to obtain better treatment results for those who actually have the disease by making way for earlier, more detailed ophthalmic examination of those who are most at risk. Finding the reason for the rarity of RB1 mutations in Korean retinoblastoma patients requires further studies.
REFERENCES


