Genomic Amplification in Retinoblastoma Narrowed to 0.6 Megabase on Chromosome 6p Containing a Kinesin-like Gene, RBKIN

Danian Chen, Sanja Pajovic, Allison Duckett, Vivette D. Brown, Jeremy A. Squire, and Brenda L. Gallie

Division of Cancer Informatics [D. C., S. P., A. D., V. D. B., B. L. G.] and Cellular and Molecular Biology [D. C., S. P., A. D., V. D. B., B. L. G., J. A. S.], Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, Toronto, M5G 2M9 Canada; Departments of Ophthalmology [B. L. G.], Laboratory Medicine and Pathobiology [J. A. S.], and Medical Biophysics [B. L. G., J. A. S.], University of Toronto, Toronto, M5G 2M9 Canada; and Department of Ophthalmology, West China University of Medical Sciences, Chengdu 610041, People’s Republic of China [D. C.]

Abstract

All retinoblastomas (RBs) show genomic changes in addition to loss of both RB1 alleles. Quantitative-multiplex PCR analysis identified in 41 of 70 (59%) RBs a 0.6-Mb minimal region of chromosome 6p22 gain from which we cloned the human kinesin gene, RBKIN/KIF13A, by rapid amplification of retinal cDNA. RBKIN is expressed ubiquitously in adult tissues, at low levels in fetal tissues, and at high levels in RB. Antisense RBKIN oligonucleotide reduced the growth rate of RB cell lines. RBKIN and/or another closely linked gene are candidate oncogenes contributing to malignant progression of RB.

Introduction

All RBs contain mutations of both RB1 gene alleles with additional genetic events. In RB1−/− mice, apoptosis is the usual consequence of loss of RB1 (1–3). Human RBs all contain other genomic alterations (4), some of which may be critical in the process of tumor initiation, whereas others may contribute to tumor progression and resistance to therapy. Almost unique to RB is a specific marker, i(6p), which is identified in 60% of RB tumor karyotypes (5). The result of the i(6p) marker is four copies of genes on chromosome 6p, or low level amplification (6). Extra copies of 6p in RB tumor have been reported to correlate with undifferentiated histology and features of poor prognosis (7).

We and others have sublocalized the altered genomic regions in RB by CGH (8–10). In our analysis of 50 RBs, the minimal regions most frequently gained were 1q31 (52%), 6p22 (44%), 2p24–25 (30%), and 13q32–34 (12%). The minimal region most frequently lost was 16q22 (14%). The overall total number of gains or losses evident on CGH was significantly greater in tumors with either or both 6p or 1q gain than in tumors with neither 6p nor 1q gain, suggesting that these markers contribute to malignant progression in the tumors. Further definition of the region of genomic gain at 6p22 is beyond the limits of resolution of CGH.

We now report a minimal 0.6-Mb region of 6p22 gain in RB, detected by QM-PCR, from which we cloned a novel human kinesin-like gene (RBKIN) that is a candidate oncogene in the development of RB.

Materials and Methods

DNA Samples. DNA was extracted directly from 69 RB tumors and 3 RB cell lines, WERI-RB1, Y79, and RB409. The tumors were from patients (35 males and 34 females) with hereditary (17 bilaterally affected) and nonhereditary (52 unilaterally affected) RBs. Both RB1 mutant alleles were detected in the 52 unilateral tumors and were not detected in the blood of the patients, strongly indicating that they had nonhereditary RB. There remains the possibility that some of the patients had germ-line mosaicism for one of the mutant RB1 alleles identified in the tumor. Previously, 46 of these tumor DNA samples had been analyzed by CGH, and the karyotypes of tumors RB302, RB383, RB386, and RB445 were published previously (4).

QM-PCR. We selected nine STSs from NCBI within human 6p21.3–6p23, each of which had a PCR product of >200 bp. For an internal control, the STS P4HA1 (WI-7221) in human 10q21 was selected because our previous work with CGH indicated that chromosome 10 was not involved in gains or losses in RB and, therefore, could be expected to consist of two copies in the RB samples. All forward primers for the STS markers were labeled by fluorescent dye Cy5.0 (Integrated DNA Technologies, Inc.).

In each reaction, we amplified three or four STS markers on 6p and the chromosome 10q internal control marker. Reactions were performed in 20 μl of total reaction volume containing 100 ng of template DNA, 2–4 pmol of each primer, 2× Taq polymerase buffer, 4 mM deoxynucleotide triphosphates, and 1 unit of Taq polymerase (BioLab). The template was amplified for only 18–19 cycles to maintain linearity of generation of the PCR products. The PCR conditions included an initial denaturing step at 94°C for 10 min and 18–19 cycles of 94°C for 30 s, 60°C for 23 s, and 72°C for 23 s using a PTC 100 thermal cycler (MJ Research, Watertown, MA). The PCR products were resolved on a 6% denaturing polyacrylamide gel using the VIGI Micro-Geno Clipper sequencer. The areas under all STS marker peaks were measured using VIGI Gene Objects software. Those values were used to calculate the ratio of the area under the curve of each 6p STS marker to the area under the curve of the 10q control STS, which was set to two copies to determine the copy number of each 6p STS marker. Two external controls were included in each run. For four separate runs, with non-tumor DNA set to two copies, the calculated copy number of all 6p STS loci was 1.94 ± 0.37 (mean ± SD) for RB445, which has neither i(6p) (Ref. 4) nor 6p gain (10) and 4.28 ± 1.25 for RB302 and 3.52 ± 0.41 for RB383, which both have i(6p) (Ref. 4) and gain of 6p by CGH (10). Copy number for an STS >3.0 was considered amplification.

Computational Analysis of DNA Sequences. The exon predictions of PAC AL023807 were carried out using Grailexp and Genescan 5 and MZEF. Homologies of nucleotide and amino acids sequences were analyzed through the BLAST server at NCBI using BLASTN, BLASTP, and BLASTX.

cDNA Cloning. cDNA cloning was performed by internal amplification of cDNAs and 5′- and 3′-RACE according to the manufacturer’s protocol using primers summarized in Table 1. The primers F40, F108, R253, and R330 were designed according to the partial predicted cDNA sequence. Other primers

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2 To whom requests for reprints should be addressed, at Division of Cancer Informatics, Room 8-415, Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, 610 University Avenue, Toronto, M5G 2M9 Canada. Phone: (416) 946-2324; Fax: (416) 946-4619; E-mail: gallie@attglobal.net.

3 The abbreviations used are: RB, retinoblastoma; CGH, comparative genomic hybridization; QM-PCR, quantitative multiplex PCR; RT-PCR, reverse transcription-PCR; STS, sequence-tagged site; RACE, rapid amplification of cDNA ends; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NCBI, National Center for Biotechnology Information; i(6p), isochromosome 6p; M6PR, mannose-6-phosphate receptor.


5 Internet address: http://compbio.ornl.gov.

were designed according to the sequences obtained. RACE and internal fragments were amplified by Advantage 2 PCR Enzyme System (Clontech) from retinal cDNA pools (Marathon-Ready cDNA; Clontech), under the condition of 94°C for 30 s, 68°C for 2 min 30 s for 30 cycles on an automated thermal cycler and then subcloned into Adv T/A vector (Clontech) for sequencing on a Beckman Coulter CEQ2000XL DNA analyzer.

Expression Studies. Total RNAs were isolated from RB1406, RB1541, and RB1513 and human normal retina using RNA TRIzol reagent (Life Technologies, Inc.) following the manufacturer’s instructions. Human cDNA of RB and adult normal retina were synthesized using 4 μg of total RNA and random primer with transcript II reverse transcriptase (Life Technologies, Inc.). RT-PCR expression analyses of RBKIN were performed using the Human Multiple Tissue cDNA (MTC) panels (Clontech; I and Fetal) containing cDNA from 16 human tissues and human RB and adult normal retina cDNA with primers F9 and R1367 (Table 1). As a control, PCR was performed with the G3PDH control amplifier set using the same amount of cDNA template as RBKIN.

Intron/Exon Mapping. The RBKIN cDNA sequence was compared with genomic sequence of contig NT-007180 containing PAC AL023807 and AL138724, obtained from NCBI, to determine the intron/exon organization in 27 tumors (Fig. 2B). Examination of the data from all markers (Fig. 2B) shows that 18 of 70 RBs that showed gains for nine STS markers, scored by calculated copy number >3.0 by QM-PCR, showed gain for adjacent markers (STS-X46229, 244 kb centromeric, 5 tumors; WI-19208, 366 kb telomeric, 1 tumor; and both in 2 tumors), possibly involving regulatory sequences of a gene near SHGC-103950.

For the 20 tumors that showed no 6p gain, the SHGC-103950 copy number values (2.3 ± 0.4, mean ± SD) were similar to the other markers (2.1 ± 0.4; Fig. 2B). A few copy number values were below the number that we consider to indicate one copy, which may be attributable to the experimental error of QM-PCR. Interestingly, for the “i(6p)” tumors, the SHGC-103950 copy number value (7.1 ± 2.5) was significantly higher (P = 0.0003) than the copy number value (4.6 ± 1.1) for all of the other markers in the broad region of gain (Fig. 2B). This suggests that in addition to increased copy number across the broad region of an i(6p) chromosome, further genomic rearrangements around SHGC-103950 may be present.

Cloning of a Kinesin-Family Gene cDNA (RBKIN) from a Retina cDNA Library. The RBKIN gene encompasses a region of contig NT_007180, within one PAC, AL023807, from which the sequence is available from NCBI. Exon prediction using Genescan, Grailexp, and MZEF identified 20, 34, and 6 potential exons by each program, respectively. We ran BLASTN using those exon sequences against the dbEST and found three unigene clusters located on this contig: hs.146286, hs.188750, and hs.189915. At this time, no human genes had been cloned in this region. Unigene hs.146286 has 19 cDNA sequences in the dbEST. By analysis of those sequences, we predicted 494 bp of the partial cDNA sequence of a gene located at 6p22 within the region of gain in RB and deduced 164 amino acids of a protein that has homology to the motor domain of the kinesin superfamily of motor proteins. We named this gene RBKIN, described recently as KIF13A (11).

Using the predicted sequence, we designed PCR primers F108 and F9.
R253 for 5′- and 3′-RACE and amplified fragments from a human retina cDNA fragment of 1377 bp, the 3′ 550 bp of which extended out of PAC AL023807. BLASTN search of this 550-bp sequence indicated that it was contained within another chromosome 6p PAC, AL138724, and a 4200-bp cDNA clone AK025303. Using primers designed from the 1377-bp fragment and the clone AK025303, we ran internal amplification and 3′-RACE and obtained the full-length cDNA for RBKIN. The full-length RBKIN cDNA was cloned into a mammalian expression vector pcDNA3 (Invitrogen).

Human RBKIN has 39 exons and spans about 223 kb of genomic DNA. The intron/exon boundaries all conform to the GT/AG rule with the exception of intron 33–34. The gene is contained in two overlapping PACs AL023807 and AL138724, both elements of a recently submitted contig NT_007180.

Expression Pattern of RBKIN. We have used two methods to examine the pattern of expression of RBKIN in various tissues, Northern blotting (not shown) and RT-PCR. RBKIN is ubiquitously expressed in adult tissues (Fig. 3A) but is expressed at very low levels in most fetal tissues (except skeletal muscle and lung; Fig. 3B). The two transcripts are evident in adult kidney and pancreas and fetal skeletal muscle. RB tumors express higher levels of RBKIN than normal adult retina (Fig. 3C).

RBKIN Antisense Oligonucleotides Inhibit Proliferation of RB Cell Lines. The 15-mer oligonucleotides to RBKIN were designed at the ATG start codon for translation: antisense, 5′-GGTATCCGACATGT-3′; and sense, 5′-AACATGTCGGATACC-3′ (nucleotides 17–31 of RBKIN1). The antisense but not the sense oligonucleotide caused a reduction in the amount of RBKIN mRNA after 14 h of...
treatment of fibroblast D551 (Fig. 3D), indicating that the antisense oligonucleotide was effective.

Two RB cell lines, Y79 and RB409, were treated with the antisense or sense oligonucleotides every 24 h, and viable cell counts were determined over 4 days. Both cell lines showed a decrease in growth rate after 48 h when treated with antisense but not sense oligonucleotides (Fig. 4). The copy number of SHGC-103950 was determined to be 1.8 copies in Y79 and 5.3 copies in RB409. (Neither tumor was studied with the other markers.)

Discussion

The most specific common genomic change after loss of both RB1 alleles in RB is the i(6p), found in 25–60% of RB tumors by karyotype analysis (4). This unusual chromosomal rearrangement results in four copies of genes on chromosome 6p (6), which may result in positive selection in the development of RB by inducing low level amplification of an oncogene located on chromosome 6p. We previously narrowed the common region of gain in RB to 6p22 using CGH (10). In the present study, we used a strategy to further narrow the common region of gain using evaluation of genome copy number by QM-PCR for STSs that span 6p22. We deliberately did not investigate in detail the many candidate genes in the 20 Mb of 6p22 but chose to narrow our studies to genes within the smallest possible region of gain.

By QM-PCR for 6p22 STS primer sets on DNA from 70 RB tumors, we narrowed the 6p gain to a 0.6-Mb region that lies 19.5 Mb from 6pter. This region is just telomic of the minimal region of 6p22 gain detected in six urinary bladder carcinoma cell lines, between D6S285 (20.4 Mb from 6pter) and D6S1921 (26.9 Mb from 6pter; Ref. 13).

Exon prediction using public database information suggested that there are at least three unigene clusters in this 0.6 Mb. Using the RACE methodology, we cloned from one of those unigene clusters the RBKIN gene, also known as KIF13A. The region of 6p that contains KIF13A has been identified recently to show linkage to schizophrenia (11). The two other predicted genes in the region of gain remain uncloned and are also candidates to be proto-oncogenes in RB.

The RBKIN protein has 90% amino acid identity with its homologue, mouse kinesin-like protein Kif13a (12). Kif13a lacks exons 26 and 38 of RBKIN. Kif13a is a plus end-directed, microtubule-dependent motor protein. The cargo vesicles of Kif13a were shown to contain AP-1 and the M6PR. Overexpression of Kif13a resulted in mislocalization of AP-1 and M6PR. Functional blockade of Kif13a reduced cell surface expression of the M6PR. Because M6PR is often mutated in several forms of cancer, it is suggested that it may function as a tumor suppressor (14). It is possible the amplification and high expression of RBKIN in a RB1−/− retinal cell may change the function of M6PR and result in rescue from the usual consequences of loss of RB1 (apoptosis and endoreduplication without cell division), permitting uncontrolled cell growth and tumor formation.

Y79 has no i(6p) but contains the (4;5)(p15;p22.1) with the breakpoint localized on 6p21.3 at least 13 Mb away from RBKIN (15). Perhaps expression of RBKIN is up-regulated in Y79 by translocation rather than the genomic gain detected in the other RB tumors. The antisense RBKIN oligonucleotide decreased growth rate in both cell lines.

Jamain et al. (11) and our results indicate that RBKIN/KIF13A is ubiquitously expressed in adult human tissues but is expressed at very low levels in most human fetal tissues. In mouse embryos, Kif13A is expressed essentially in the central nervous system and strongly in optic vesicles (11). We show that RB tumors highly express RBKIN mRNA. An antisense oligonucleotide targeting the RBKIN gene reduces the growth rate of RB cell lines, suggesting that RBKIN may have a role in cell division in RB, supporting but not confirming its candidacy to be the proto-oncogene accounting for the 6p gain in RB.

Kinesin-related proteins are microtubule motor proteins that can bind to and move on microtubules (16) and have roles in vesicle/organelle transport, spindle function, and chromosome motility in mitosis and meiosis. These proteins could account for many of the movements of the spindle and chromosomes during cell division. Whether or not RBKIN is related to movements of the spindle and chromosomes is unknown. The role of RBKIN in RB will only be clear after much further study, including demonstration that it can function as an oncogene in model systems.

The other two unigene clusters in 6p22 must also be tested. Before cloning these genes, we will first demonstrate using RT-PCR for the predicted cDNAs if there is expression in RB. If there is, we will follow a similar strategy to the cloning and study of these genes as we have for RBKIN.

We have used QM-PCR with STS primers to advantage to rapidly narrow one of the common regions of genomic gain in RB. This strategy allowed us to test a large number of samples with a few STS and then generate more STS primers in the hotspot identified and to test many samples rapidly. This approach emphasizes the testing of many samples, in comparison with microarray approaches that provide high-density screening of genomic or expressed sequences, but which are limited by efficiency and cost in how many samples can be tested. We have also used QM-PCR to delineate a minimal region of 16q genomic loss in RB3 and identify cadherin-11 and cadherin-13 as candidate tumor suppressor genes, submitted for publication.

to be candidate tumor suppressor genes in the progression of \textit{RB1}/\textit{H11002} cells to RB.

Our data on RB indicate the power of QM-PCR to quickly refine regions of gain and loss in cancer cells. With the advantage of the Human Genome Project, the limit to identify important oncogenes and tumor suppressor genes becomes the number of banked, high-quality DNA samples and the quality of clinical data about the patients who donated the samples, rather than the technology to get high-resolution determination of genomic rearrangements.

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References

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