Abstract Although it is established that the loss of function of both alleles of the RB1 gene is a prerequisite for the development of retinoblastoma, little is known about the genetic events that are required for tumor progression. We used comparative genomic hybridization (CGH) to search for DNA copy number changes in isolated unilateral retinoblastomas. From a series of 66 patients with retinoblastomas with somatic mutations in both RB1 alleles, tumor samples from 13 children with the youngest (2.0–9.8 months) and 13 with the oldest (36.2–84.1 months) age at operation were studied. Loss at 13q14, the location of RB1, was demonstrated in two tumors only. Recurring chromosome imbalances included gains at 6p (11/26), 1q (10/26), 2p (4/26), and 17q (4/26), gains of the entire chromosome 19 (3/26), and losses at 16q (9/26). A commonly gained region at 1q32 was identified. Increased dosage of GAC1, a candidate oncogene located in 1q32, was found in two of four tumors by Southern blot analysis. Comparison of the CGH findings revealed that retinoblastomas from children with an older age at operation showed significantly more frequent (13/13 cases vs 4/13 cases; P = 0.0005) and more complex genetic abnormalities (median, 5 changes/abnormal tumor vs median, 1.5 changes/abnormal tumor; P = 0.003) than retinoblastomas from children with a young age at operation. Gains at 1q, 2p, 17q, of the entire chromosome 19 and losses of 16q were restricted to the older age group. Our results suggest that the progression of retinoblastomas from older patients follows mutational pathways different from those of younger patients.

Introduction Retinoblastoma (Rb) is the most common malignant intraocular tumor in childhood, with an incidence of 1 in 13,500–25,000 live births (Newsham et al. 1998). Current knowledge indicates that mutations in both alleles of the RB1 gene, a tumor suppressor located in 13q14, are a prerequisite for the development of Rb. About one third of the children with Rb have a hereditary predisposition to this tumor caused by germline mutations in the RB1 gene (Horsthemke 1992; Lohmann et al. 1996, 1997). Children with hereditary disease usually develop multifocal tumors in both eyes and are diagnosed in the first six months of life. In two thirds of the patients, the tumor is restricted to one eye, and in most of them, the causative RB1 gene mutations are of somatic origin and non-hereditary. The incidence of unilateral isolated Rb peaks between 24 and 29 months of life. About half of these children are diagnosed before the age of 30 months and only about 6% at ages 60 months and older (Sanders et al. 1988).

Chromosome-banding studies have shown that genetic changes in addition to RB1 mutations occur during the development of Rb. One or two extra copies of an isochromosome of the short arm of chromosome 6, i(6p), partial trisomy of the long arm of chromosome 1, 1q, monosomy of chromosome 16, and abnormalities of the short arm of chromosome 1, 1p, have been described most frequently. Homogeneously staining regions (HSR) and double minutes (dmin), which are cytogenetic correlates of gene amplifications, have been observed in 9% of tumors with chromosome abnormalities (Squire et al. 1985; Potluri et al. 1986; Oliveros and Yunis 1995). However, poor in vitro growth of some tumor cells, possible selection of clones with specific alterations during cultivation, especially if grafted onto nude or SCID mice, and the presence of complex karyotype aberrations interfere with the assessment of the true frequency of aberrations by chromosome-banding analyses (Yan et al. 2000; Mairal et al. 2000).

Comparative genomic hybridization (CGH) allows the genome-scale detection of complete and partial chromo-
some gains and losses in tissues without prior in vitro cultivation. This technique generates a profile of the changes in the DNA sequence copy number in a tumor sample. Increased DNA dosage of chromosomal regions may point to the location of major oncogenes, whereas regions with decreased copy numbers may harbor important tumor suppressor genes (Kallioniemi et al. 1992; Du Manoir et al. 1993). A large degree of agreement of CGH results with those of karyotypic analysis has been demonstrated in a recent study in 24 hereditary and non-hereditary Rb cases (Mairal et al. 2000).

In children with hereditary Rb, the distance between the tumor and the macula at time of diagnosis increases with age after birth (Brinkert et al. 1998). Thus, in Rb, biological differences may exist that are related to the age at diagnosis.

To investigate the frequency and type of chromosome abnormalities in addition to RB1 gene mutations, we performed CGH analyses on tumor samples of children with unilateral isolated Rb in which somatic mutations in both RB1 alleles had been previously identified. In addition, samples from children with a young age and with an older age at operation were selected to explore age-related differences in the chromosome changes. To our surprise, we observed a marked divergence in the incidence and the extent of chromosomal imbalances between tumors from children with a young age and those with an old age at operation.

### Materials and methods

#### Patients

Tumor samples from a series of 66 patients with unilateral isolated Rb (age: 2.0–84.1 months; median: 18.1 months) carrying somatic mutations of both RB1 alleles were available for CGH analysis (Lohmann et al. 1997). All patients tested negative for the respective RB1 mutations in peripheral blood DNA. From this series, we selected samples of 26 patients representing 13 children with the youngest (age: 2.0–9.8 months; median: 6 months) and 13 children with the oldest (age: 36.2–84.1 months; median: 50 months) age at operation. In almost all patients, enucleation had been performed within 2 weeks following the initial diagnosis, because the tumor involved more than half of the retina. Therefore, tumor sizes were similar in young and older children, and the age at operation did not differ significantly from the age at presentation.

Comparative genomic hybridization

CGH was performed essentially as described (Du Manoir et al. 1993; Kallioniemi et al. 1994b). Briefly, tumor DNA was extracted by using standard protocols. In previous Rb1 mutation analyses and loss of heterozygosity (LOH) studies, no normal Rb1 allele had been found in the tumor DNA. Thus, in the samples used for CGH analyses, the presence of relevant amounts of contaminating normal tissue DNA could be ruled out. The tumor DNA was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany), and normal male reference DNA was labeled with biotin-11-dUTP (Sigma, St. Louis, Mo.) by using 1 µg of each DNA and a nick translation kit (Vysis, Downer’s Grove, Ill.). Equal amounts (400 ng each) of labeled tumor and reference DNA were mixed with 1 µg/µl COT1-DNA (BRL Life Sciences, Gaithersburg, Md.), 0.75 µg/µl sonicated herring sperm DNA, 50% formamide, and 2×SSC (1×SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0). The mixture was denatured at 73°C for 10 min, incubated for 10 min at 37°C, and hybridized for 24 h at 37°C. The slides were washed twice in 4×SSC/0.1% Triton X-100 for 5 min. After washing in 0.1×SSC at 75°C for 5 min followed by 2×SSC/0.1% Triton X-100 for 2 min at ambient temperature. Following hybridization with 3% bovine serum albumin (BSA)/4×SSC at 37°C for 15 min, the hybridized biotinylated and digoxigenin-labeled DNA were detected by incubation with 5 µg/ml Texas-Red-conjugated streptavidin (Vector Laboratories, Burlingame, Calif.) and 2 µg/ml fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxigenin antibodies (Boehringer Mannheim, Germany) in 1% BSA/4×SSC at 37°C for 30 min. The slides were washed twice in 4×SSC/0.1% Triton X-100 for 5 min, counterstained with 4′,6-diamidino-phenylindole (DAPI; Sigma) for the identification of the chromosomes, and mounted in anti-fade mounting media (Vector Laboratories). The hybridized metaphase chromosomes were analyzed by using a Zeiss epifluorescence microscope and a digital image analysis system (ISIS, MetaSystems, Altussheim, Germany). DAPI (blue), FITC (green), or Texas Red (red) fluorescence of the chromosomes was recorded separately. The images were processed further for CGH analysis by using ISIS-CGH software (MetaSystems). The normalized green/red fluorescence ratio along the chromosome axis was calculated for each chromosome on the basis of at least 10 metaphases for each sample. DNA copy number gains were recorded at three different levels of imbalances by using fixed thresholds. Level 1 corresponded to a green/red fluorescence ratio at a range of 1.2–1.3, level 2 to a range of 1.3–1.5, and level 3 to a ratio greater than 1.5. DNA copy number losses were recorded at a green/red fluorescence ratio of less than 0.8. Losses of 1p32-pter and 16p, of the entire chromosome 19 or 22 and of pericentric, heterochromatic, and telomeric regions were excluded from the analysis (Kallioniemi et al. 1994b; Du Manoir et al. 1995). Reverse-labeling CGH was performed on selected cases (Table 1, cases 25 and 26), which confirmed the alterations detected by the standard technique. CGH findings were described according to the International System for Human Cytogenetic Nomenclature (Mitelman 1995).

#### Southern blot analysis

Southern blots of tumor DNA digested with SacI were hybridized simultaneously with probes that were derived from the GAC1 (accession no. NM006338, position 1234–1993) and REN (accession no. M10128, position 145–1341) genes and that recognize genomic SacI fragments of 2.8 kb and 6.0 kb/4.2 kb, respectively. These probes were created by using PCR-primers kindly provided by B. Malfoy (Almeida et al. 1998). To control for the amount of DNA loaded, probes derived from the Prader-Willi syndrome region on chromosome 15 were used, viz., D15S63, which recognizes a 4.2-kb genomic SacI fragment (Buiting et al. 1990), or 71.19.19, which recognizes a 8.8-kb fragment (Saftoiu et al. 1996). All Southern blot hybridizations were performed in triplicate. The relative intensities of the resulting autoradiography signals were evaluated by scanning densitometry (Dual wavelength scanner CS-9000, Shimadzu).

#### Statistical analyses

Following the argumentation of Kato and colleagues (1993), age at operation was used for the analysis of age-related differences. The differences in the frequency of abnormal GCH findings between children with a young age and an old age at operation were tested by Fisher’s exact test with two-tailed Ps. The differences in the frequency of the chromosomal imbalances in abnormal tumor samples between both age groups was tested by the Mann-Whitney U-test.