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# Somatic Events Unmask Recessive Cancer Genes to Initiate Malignancy

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A heritable mutation predisposes an individual to certain childhood malignancies, such as retinoblastoma and Wilms' tumor. The chromosomal locations of the genes responsible for the predisposition are known by linkage with chromosomal deletions and enzyme markers. A study of these tumors in comparison to the normal constitutional cells of the patients, using enzyme and DNA markers near the predisposing genes, has shown that these genes are recessive to normal wild-type alleles at the cellular level. Expression of the recessive phenotype (malignancy) involves the same genetic events that were observed in Chinese hamster cell hybrids carrying recessive drug resistance genes. In both the experimental and clinical situations, the wild-type allele is most commonly eliminated by chromosome loss with duplication of the mutant chromosome. Simple chromosome loss and mitotic recombination have been documented in both systems. In the remaining 30% of cases, inactivation or microdeletion of the wild-type allele are assumed to be responsible for expression of the recessive phenotype. Osteosarcoma is a common second tumor in patients who have had retinoblastoma. Studies with markers in osteosarcoma show that these tumors also result from unmasking of the recessive phenotype by loss of the normal allele at the retinoblastoma locus, whether or not the patient had retinoblastoma. Subsequent chromosomal rearrangements and amplification of oncogenes that occur in these homozygous tumors provide progressive growth advantage. In other malignancies, in which studies have so far focused on oncogene amplification and chromosomal rearrangements, unmasking of recessive mutations may also be the critical initiating events.

**Key words: retinoblastoma, recessive, oncogene, somatic**

In most cases, comparison of tumor tissue and normal cells does not distinguish initiating events from subsequent progressive changes. By the time tumors are studied in the laboratory, many differences from normal cells are often apparent: Aneuploidy may be obvious on karyotype analysis; oncogenes may be amplified or rearranged, and expression of growth factors may be aberrant. Which events are critical in the genesis of the tumors and which are subsequent changes related to the inherent genetic

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instability of malignant cells, cannot be easily determined. On the other hand, the rare tumors with a hereditary predisposition do allow identification of the earliest genetic abnormality, the premalignant change. Since the majority of the cells predisposed to the malignancy function quite normally in these individuals, the first predisposing mutation is not sufficient for malignancy. Therefore, it was proposed that these mutations might be recessive to the normal wild-type allele [1,2]. Malignancy would only arise following elimination of the dominant wild-type allele.

## RETINOBLASTOMA

One of the best studied of the tumors with hereditary predisposition is retinoblastoma. Because surgical cure was possible, the affected individuals lived to reproduce and the inheritance patterns became evident at the beginning of the twentieth century [3,4]. Retinoblastoma occurs in three different genetic situations: 60% of cases develop only one tumor and have no inherited predisposition to the tumor; 40% of cases carry a germline mutation that predisposes them to multiple retinoblastoma tumors and other tumors (mainly osteogenic sarcoma) later in life; and a small number of cases occur in association with deletion of chromosome 13 that includes band q14.1.

Virtually all cases of retinoblastoma are diagnosed before the age of 3 years, strongly suggesting that the target cell for the malignant change must disappear from the retina early in life. When the age of diagnosis is plotted against the logarithm of the proportion of cases not yet diagnosed (100% at birth), the shape of the curve for the unilateral cases (probably nonhereditary) suggests that two or more rate limiting steps are involved in tumor initiation [2]. The shape of the curve for bilateral (hereditary) cases is a decreasing exponential, strongly suggesting that only one rate limiting event is required for the genesis of the tumors in the patients that carry the predisposing mutation. Based on such analysis of clinical data, Knudson et al [5,6] hypothesized that as few as two mutations could lead to retinoblastoma: In hereditary tumors the first mutation occurs in the germline, whereas in nonhereditary tumors the first mutation occurs in the somatic cell that forms the tumor. For both types of retinoblastoma the second mutation occurs in the somatic cell that becomes malignant. Since it is infinitely unlikely for two rare somatic mutations to occur in the same cell in an individual, the nonhereditary retinoblastoma patients have only one tumor. On the other hand, the multiple tumors observed in patients predisposed to retinoblastoma are explained by the relatively high likelihood that the second mutation occurs in more than one retinal precursor cell in an individual. Knudson also predicted that the first and second mutations could be at the same genetic locus: If the inherited predisposing mutation was recessive, tumors would form only when the remaining normal allele was lost by the second event. This second event could then be a second mutation or a "segregation" event that results in loss or inactivation of the second allele allowing expression of the recessive phenotype. Thus the genetic basis for deletion, and hereditary and nonhereditary retinoblastoma could all involve mutations in the same gene.

## EXPRESSION OF THE RECESSIVE PHENOTYPE BY SOMATIC REARRANGEMENTS

Apart from the study of tumor cells themselves we have also utilized an experimental approach to investigate mechanisms for the expression of recessive

phenotypes in somatic cells that are heterozygous for a recessive marker. In Chinese hamster cell hybrid lines that were constructed to be heterozygous for a recessive drug resistance gene [7], it had been expected that expression of the recessive (drug-resistant) phenotype might occur by loss of the normal chromosome carrying the wild-type (drug sensitive) allele. This occurred in only about 20% of drug-resistant colonies analyzed [8]. More extensive analysis using hybrids with karyotypically marked chromosomes, revealed that loss of the normal chromosome was often associated with the presence of two copies of the mutant chromosome and this accounted for the majority of drug resistant segregants [9]. Inactivation of the normal allele, probably by DNA methylation, was shown to account for the drug-resistant phenotype in one segregant, in which re-expression of the dominant wild-type gene was documented under demethylating conditions [10]. The fourth mechanism examined in these studies was mitotic recombination. Although no drug resistant lines were found to be the result of mitotic recombination, in other studies with a very similar system including proximal chromosomal markers, mitotic recombination has been observed in 2 of 20 segregants [11].

A variety of segregation mechanisms allowing expression of recessive genes have been documented in mammalian hybrid cells, using a selectable phenotype (drug resistance) and chromosomes distinguishable by markers. A number of other studies have revealed similar mechanisms for the expression of recessive drug resistance markers in near-diploid cells [12]. To test if tumor formation in the hereditary malignancies represents expression of a recessive mutation, markers surrounding the mutant genes were studied.

The chromosomal location of the mutation predisposing the retinoblastoma was suggested to be chromosome 13q14.1 by mapping with rare deletion patients [13]. The ubiquitous enzyme, esterase D (EsD), the only enzyme marker on chromosome 13, was mapped to the same chromosomal band by the observation of hemizygosity in deletion retinoblastoma patients [14]. Studies of families with heritable retinoblastoma without deletion have shown tight linkage of esterase D isoenzymes to the occurrence of retinoblastoma [15–17]. In all informative families reported so far, no meiotic recombination has been observed. Assuming a uniform likelihood of meiotic recombination for any chromosomal region, Mukai et al [17] estimated that the retinoblastoma gene and EsD gene were less than 1,000 kilobase pairs (Kb) apart. One patient with retinoblastoma and a deletion ending in band 13q14.1 has been reported with normal levels of EsD, suggesting that the genes can be separated [18]. Using the recently cloned ESD gene [19], 24 retinoblastoma tumors were found by gene dosage studies to be diploid for EsD with no evidence of DNA rearrangement or deletion. It may be that the region from the EsD gene to the retinoblastoma gene is too large to be homozygously deleted. Since EsD is ubiquitous, total absence of EsD may be lethal to cells. Lack of any EsD rearrangement in 24 retinoblastoma tumors also suggests that the two genes are separated by at least 30 kB (the estimated size of the EsD gene).

In a few tumors, derived from ESD heterozygotes, the isoenzymes of EsD were used as markers for the homologous chromosomes, in a study of segregation events in retinoblastoma tumors involving adjacent regions of chromosome 13 [20]. In 70% of informative tumors, one of the isoenzymes that was present in the constitutional cells, was missing from the tumor cells, suggesting loss or inactivation of this chromosomal region in one homologue. However, two karyotypically normal chro-

mosomes 13 were present. This was the first evidence that segregation mechanisms similar to those observed in Chinese hamster cell hybrids could be involved in retinoblastoma tumor formation.

In order to further analyze chromosomal loss or rearrangement in retinoblastoma tumors, two groups developed restriction fragment length polymorphic (RFLP) markers on chromosome 13 [21,22]. Thus, detailed analysis of chromosomal events around the retinoblastoma locus could be carried out [23,24]. It was observed that in 70% of tumors of informative individuals (heterozygous for chromosome 13 markers), the heterozygous RFLP markers on chromosome 13 were reduced to a homozygous state. Control RFLP's on other chromosomes remained heterozygous in the retinoblastoma tumors. As in the Chinese hamster cell hybrids, the most common mechanism for segregation appeared to be loss of one chromosome 13 with duplication of the homologous chromosome. Subsequent studies with cases of familial retinoblastoma showed that the lost chromosome carried the wild-type allele, while the retained or duplicated chromosome carried the retinoblastoma mutation [25]. Simple loss of the normal chromosome occurred in less than 10% of tumors. In most tumor studies it was not possible to distinguish clearly between loss and duplication of homologous chromosomes on the one hand and mitotic recombination on the other, as both events convert all markers distal to the locus to the homozygous state. In a few cases where both proximal and distal markers were available it was possible to document mitotic recombination events in 10% of tumors [23]. We found that 30% of the tumors did not reduce to homozygosity for informative markers surrounding the retinoblastoma locus. It was presumed that expression from the normal wild-type allele in these cases was eliminatead by mutation, microdeletion, or gene inactivation. Since nonheritable retinoblastoma tumors reduced to homozygosity for chromosome 13q at the same frequency as heritable tumors, the two somatic events leading to nonheritable retinoblastoma appear to be identical to the events in heritable tumors, involving the retinoblastoma locus on chromosome 13.

It has been noticed that the rare deletion retinoblastoma patients generally have fewer tumors than the usual hereditary mutation patients without deletion [26]. This suggested that in the presence of a large deletion around the retinoblastoma locus, the options for segregation of the recessive phenotype were reduced [27]. This can be understood when the mechanisms of segregation of the recessive phenotype are recognized. Loss of the normal and reduplication of the mutant chromosome, mitotic recombination, and simple chromosomal loss would all result in total absence of all the genetic material within a deletion, and would probably be lethal to the cell. Only mutation, gene inactivation, or microdeletion would result in tumor formation. These deletion patients therefore should develop only 30% as many tumors as patients with classical mutations in the retinoblastoma gene. This explains why these deletion patients are unilaterally affected more frequently than expected.

## RELATIONSHIP OF THE RECESSIVE, INITIATING MUTATION TO OTHER GENETIC CHANGES IN THE TUMORS

Although the losses and rearrangements documented on chromosome 13 using specific markers are extensive, karyotypic studies of retinoblastoma tumors show only rare cytogenetic abnormalities involving chromosome 13 [28,29]. However all the tumors examined to date do show aneuploidy. Many of the rearrangments are

random, but two rearrangements occur with high frequency: Extra copies of chromosome 1q occur in 90% of retinoblastoma tumors; and 70% of tumors carry a marker, isochromosome 6p, that is almost unique to retinoblastoma [29–32].

The oncogene N-*myc* is frequently amplified in neuroblastoma cell lines in association with progressive increase in tumor growth rate and tumor autonomy [33,34]. Similar DNA amplification of N-*myc* has been documented in some retinoblastoma tumors [35–37]. However, in a survey of 18 recently derived retinoblastoma cell lines, N-*myc* DNA amplification was found in only one tumor and the oncogene was expressed in all unamplified retinoblastoma tumors at a level comparable to fetal retina [38]. A later manifestation of the inheritance of the retinoblastoma mutation is a second malignant tumor, most commonly osteosarcoma or soft tissue sarcoma [39]. Although the sarcomas have been shown to be initiated by the retinoblastoma mutation [40], no N-*myc* expression was observed [38]. Although the normal product of the retinoblastoma locus may control or regulate genes related to cell division, there is no evidence to suggest that N-*myc* is the unique target of the retinoblastoma gene product. On the other hand, karyotypic evidence of DNA amplification and the presence of unidentified marker chromosomes has been reported in tumor cells with no amplification of N-*myc* or other known available oncogenes [29]. These unidentified rearrangements could be related to amplification for other genes encoding unknown growth factors.

## SEGREGATION OF RECESSIVE ALLELES IN OTHER TUMORS

Osteogenic sarcomas were found to reduce to homozygosity for markers on chromosome 13, both from patients who had previously had retinoblastoma [40] and unassociated with retinoblastoma [40,41]. This suggests a wider role in specific malignancies for the recessive mutation previously associated only with retinoblastoma.

The data for somatic shift to homozygosity in malignant tumors is summarized in Table I. The gene that predisposes to Wilms' tumor was localized to chromosome 11p13 by constitutional deletion, analogous to the localization of the retinoblastoma locus [42]. Study of Wilms' tumor has also shown reduction to homozygosity for markers on chromosome 11p, at a similar frequency and with similar mechanisms to retinoblastoma [43–46]. Hepatoblastoma and rhabdomyosarcoma were also studied with markers on 11p, since these tumors may occur in the rare Beckwith–Widemann Syndrome, characterized by specific congenital anomalies. Both tumor types were found to reduce to homozygosity for 11p markers [47]. In Ewing's sarcoma, on the other hand, neither chromosome 11p nor 13q reduces to homozygosity. Markers on chromosome 13 and other chromosomes do not change in the tumors thought to be induced by a recessive tumor gene on chromosome 11p.

Transitional cell carcinoma of the bladder, linked to Wilms' tumor only because it may arise in a similar embryological tissue, has also been shown to reduce to homozygosity for chromosome 11p markers [48]. However, the frequency of homozygosity demonstrated was not as high as in Wilms' tumor, and other chromosomes also became homozygous at low frequency. Random aneuploidy, developing subsequent to the initiating reduction to homozygosity, can presumably occasionally result in homozygosity of other chromosomes.

**TABLE I. Frequency of Reduction to Homozygosity in Tumors**

| Tumor type | Chromosome 13q | Chromosome 11p | Other chromosomes (chromosomes tested) | References |
|---|---|---|---|---|
| Retinoblastoma | 11/18[a] | 0/11 | 0/11 (1,3,10,12,15,17,18,20) | 23,24 |
| Osteosarcoma | | | | |
| with retinoblastoma | 2/3 | 0/3 | 0/3 (2,3,5,6,20,22) | 40 |
| without retinoblastoma | 4/10 | 0/4 | 0/4 (3,6,17,20,22) | 40,41 |
| Wilms' tumor | 0/6 | 12/22 | 0/7 (1,5,6,14,17,18,21) | 42–45 |
| Hepatoblastoma | 0/3 | 2/3 | 0/3 (6,14,20,22,) | 47 |
| Rhabdomyosarcoma | 0/3 | 2/3 | 0/3 (6,14,20,22) | 47 |
| Transitional cell carcinoma of bladder | 0/12 | 5/12 | 3/12 (1,2[b],3,12,14[b],15,17,18,20) | 48 |

[a]Tumors homozygous/informative tumors tested.
[b]Homozygosity shown for these chromosomes.

## DISCUSSION

The genes documented to lead to malignancy by segregation of the recessive tumor forming phenotype, are tissue specific and may be developmentally regulated. Patients with the retinoblastoma germline mutation develop hemopoietic malignancies at the same frequency as the normal population, although we would expect that the segregation events involving 13q occur randomly in all tissues, not only in retinal cells. Therefore, the retinoblastoma gene and its mutations must be irrelevant in hemopoietic tissues. Somatic loss of the gene product in specific tissues may be the critical factor in tumor formation.

The only difference between hereditary and nonhereditary retinoblastoma is the timing of the first mutation, which occurs in the germline in hereditary cases and in the somatic cell that becomes malignant in nonhereditary cases. The first mutation leading to retinoblastoma is a rare (estimated $10^{-6}$ events per cell division) [2] deletion, microdeletion, or point mutation in a tissue-specific gene localized at 13q14. The mutation is recessive, and as long as the normal allele is present no cellular growth abnormality is detected in the tissue. The second mutation may be a more frequent segregation event. In experimental situations, similar segregation events, usually an abberrant chromosome disjunction that results in loss of the wild-type allele and duplication of the mutant allele, occur at an estimated frequency of $10^{-3}$ per cell division [11]. Since the number of retinal cells in which the retinoblastoma gene product is relevant is unknown, the frequency of the segregation event resulting in the tumors can not be estimated.

In retinoblastoma, both the first and the second events are rate limiting for the occurrence of the tumor. However, many tertiary mechanisms are available to increase growth advantage subsequently. Oncogenes can be amplified or rearranged, presumably providing an augmentation of growth factors. A single tumor type appears to use more than one tertiary mechanism, for example isochromosome 6p, extra copies of chromosome region 1q and N-amplification all occur in retinoblastoma

tumors. Different tumor types also can use the same tertiary mechanism, for example amplification of N-*myc* occurs in both retinoblastoma and neuroblastoma.

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