HOW DO RETINOBLASTOMA TUMOURS FORM?

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SUMMARY

The causes of retinoblastoma (RB) can now be described
with considerable accuracy, although many details are
still unclear. Understanding the genetic changes leading
to RB has provided an awareness of general mechanisms
of cancer development and progression, previously only
suspected. From the basic understanding have come new
diagnostic technologies that are now ready to be applied
directly to RB patients and their families, and a rational
approach, based on this understanding, will help us to
develop new therapies that avoid the severe complica-
tions of conventional treatment.

MUTATIONS OF THE *RB1* GENE INITIATE RB TUMOURS

The *RB1* gene was identified because mutations in both
alleles result in RB.[1,2] All children with bilateral RB have a
germline mutation in *RB1* and develop tumours when the
second, normal allele is lost or mutated. The non-heritable
RB tumours arise when both alleles are mutated in one ret-
inoblast in an individual with normal *RB1* alleles; these
events are each so rare, that the tumour is always unifocal
and unilateral. However, 15% of the unilaterally affected
patients do have a germline *RB1* mutation.

Although bilaterally affected patients may inherit a
defective *RB1* gene, most have no family history of RB
and have new germline mutations, proven in some
cases.[3,4,5] In these patients, the answer to the title question
begins when poorly-defined factors cause that initial
germline mutation. Cytogenetic[6] and molecular ana-
lyses[7,8,9] have shown that these new mutations arise more
commonly on the paternal chromosome. In contrast, uni-
lateral tumours, in which both *RB1* alleles were mutated
only in the somatic retinal cell that became the tumour,

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show the initial mutation on either maternal or paternal
chromosomes, ruling out somatic imprinting as a mech-
anism in RB tumours. The paternal germline mutation of
RB1 may arise because of the opportunity for mutation in
the many cell divisions of spermatogenesis,[10] or by
increased exposure of fathers to mutagens. A case control
study has shown a significant association of new germline
RB with paternal employment in the military and metal
industries.[11,12] Consistent with accumulation of mutations
in sperm, several studies have suggested a small paternal
age effect in bilateral but not unilateral RB.[13,14,15]

Types of *RB1* mutations

The *RB1* gene was localised to chromosome 13 by the
observation of a constitutional deletion of chromosome 13
in 3% to 5% of RB patients.[16,17,18,19] These children have
many abnormalities in addition to RB, for example, card-
iac abnormalities, mental retardation, extra digits and a
characteristic facial appearance,[20] depending on the size
and extent of the chromosome 13 deletion.

However, most patients with germline mutations affect-
ing only *RB1* are of normal intelligence and are otherwise
normal in every way except for tumour formation. The
smallest deletion was specifically localised to band 13q14
(13q14.1–13q14.3).[21] The gene was designated '*RB1*' to
leave room for another, unmapped, gene '*RB2*' in which
mutations could also lead to RB tumours. It is now likely
that all RB tumours are caused by *RB1* mutations.

Chromosomal localisation of *RB1* was refined by
family studies linking a chromosome 13q14 marker (este-
rase D) to the occurrence of RB.[22,23,24] The first molecular
clone of *RB1* was obtained by identifying that a unique
13q14 DNA fragment was homozygously deleted in a few
RB tumours.[1] This genomic DNA fragment was then used
to obtain the cDNA of the *RB1* gene.[2] *RB1* spans approxi-
mately 200 kb and contains 27 exons.[25]

Many *RB1* mutations in RB tumours and other cancers
have been characterised,[3,4,5,26,27,28] but a complete survey
that might indicate 'hot spots' for mutation has not yet
been reported. The mutations have been detected by vari-
ous technologies.[3,4,5] Currently, the most efficient method

to find unknown mutations appears to be screening for variations from normal by single-stranded conformation polymorphism[29,30] of the PCR-amplified exons. The types of mutations leading to RB tumours include deletions, duplications, and point mutations (See figure 1).

As long as one normal *RB1* allele is present, cells are normal. Although it has been shown in RB tumour cells that both alleles of *RB1* are expressed[4] and contribute to the total cellular p110,[RB1] only one functional allele is required. The patient with one mutant *RB1* allele in every retinal cell has normal retinal development, but is highly predisposed to develop RB tumours.

Loss of the remaining normal chromosome 13 commonly initiates RB

The malignancy is initiated when the only remaining normal *RB1* allele is lost, leaving the developing retinal cell with no normal *RB1* gene (See figure 1). In more than 70% of RB tumours this involves chromosomal events resulting in loss of all or most of the normal chromosome 13. This process was demonstrated initially by esterase D studies,[31] elaborated by restriction fragment length polymorphism (RFLP) analysis[32,33,34] and finally was confirmed by *RB1* mutation identification.[3,4,5,26,27] In the tumours, the mutant and normal chromosomes recombine to duplicate the region around the mutant *RB1* allele, while the normal allele is lost[35] by loss of heterozygosity (LOH) (See Figure 1). In 30% of RB, the second *RB1* mutation is a separate mutation different from the first[4]. The independent, multifocal RB tumours in a single patient each arise by different second *RB1* mutations.

The cell of origin of RB, the 'retinoblast' is an embryonic cell of the retina that is uniquely susceptible to malignant transformation by *RB1* mutations. Morphologically, RB tumour cells resemble developing retinal cells prior to differentiation into multiple retinal cell types,[36,37] but the basis for the preferential impact of *RB1* mutation in developing retina is not known. When babies with germline *RB1* mutations are examined at birth, most already have RB. However, about 30% with no tumours at birth develop multiple tumours in both retinas in the first year of life. The mean number of separate RB tumours arising in patients with germline *RB1* mutation is three to four.[38,39] After three years of age new RB tumours are rarely observed.[40] Presumably, within the first few years of life, the normal, fully differentiated, retinal cells cease proliferation, terminally differentiate, and can no longer form RB tumours. These children are at about a 40,000 times risk to develop RB tumours compared to a child with two normal *RB1* alleles. The process of LOH is presumably occurring in all tissues at a similar frequency. Non-retinal cells may tolerate loss of *RB1* without proliferating out of control, or *RB1* may be lethal to the cells, so that LOH does not demonstrate any phenotype. However, once malignancy of many other tissues is initiated by other factors, loss of *RB1* may contribute a relative growth advantage and is frequently observed, particularly in tissue culture cell lines.

The product of RB1: properties, function and mutant forms

What is the protein that is so critical in retinal development? The product of the *RB1* gene is a 110 KD protein (p110[RB1]) that is transported into the nucleus in all tissues and cell types studied.[41,42,43,44] In resting normal cells (G_0 and early G_1 of the cell cycle), only hypophosphorylated p110[RB1] is present.[45,46,47] As cells begin to prepare for, and initiate DNA synthesis (late G_1 and S phase of the cell cycle) many p110[RB1] serine and threonine amino acids become phosphorylated[48,49] by the action of a cell-cycle kinase.[48]

Since absence of p110[RB1] leads to RB and cancer, the normal function of p110[RB1] must promote non-proliferation. Therefore, the hypophosphorylated form that is present in resting cells is presumed to be the active form. This idea is supported by the observation that the proteins of the DNA tumour viruses that are essential for the virus to transform mammalian cells, do so, by binding to several mammalian proteins including hypophosphorylated, p110[RB1,50,51,52,53,54] presumably preventing the cells from remaining in a resting state.

p110[RB1] functions by interacting with other cellular proteins[55,56] to influence the activity of other genes. For example, activity of proliferation-promoting genes such as *c-fos*,[57] *c-myc*,[58] and *RB1* itself, is influenced by p110.[RB1] The effect of p110[RB1] on other genes may be determined by tissue and development-specific protein complexes that act on specific target DNA sequences[59,60,61,62] in the regulatory regions of the genes whose activity is modified.

Initial reports indicated that replacement of functional p110[RB1] into tumour cells had a dramatic effect on growth and tumour formation in immune-deficient animals.[63,64,65] We have observed different results: there is no change in the growth rate or ability to form tumours of RB or breast cancer cells reconstituted with *RB1*. These reconstituted cells produce p110[RB1] that shows normal features: the correct size, cell cycle-regulated hyperphosphorylation, and binding to the adenovirus transforming protein, Ela.[66] The ability of the cells to grow in adverse conditions and regulation of growth-promoting genes may be altered. All the cell lines tested have the secondary growth promoting mutations in other genes, that are not overridden by p110,[RB1] suggesting that *RB1* is a weak tumour suppressor gene. This would be consistent with the restricted cell types in which *RB1* mutations can initiate cancer. Careful examination of the previously published experiments reveals that the data also supports only a weak tumour suppressor activity for p110.[RB1]

Most of the *RB1* mutations found to lead to RB cause a truncated, unstable, protein[4] (P. Hamel, personal communication), and no p110[RB1] is detected in RB tumours.[67] A minority of *RB1* mutations in RB tumours may produce a near-normal protein,[4,5] but no such cells have been available for study of the protein. In other types of tumours where *RB1* mutations contribute to progression of malignancy, stable truncated p110[RB1] proteins have been observed.[67]
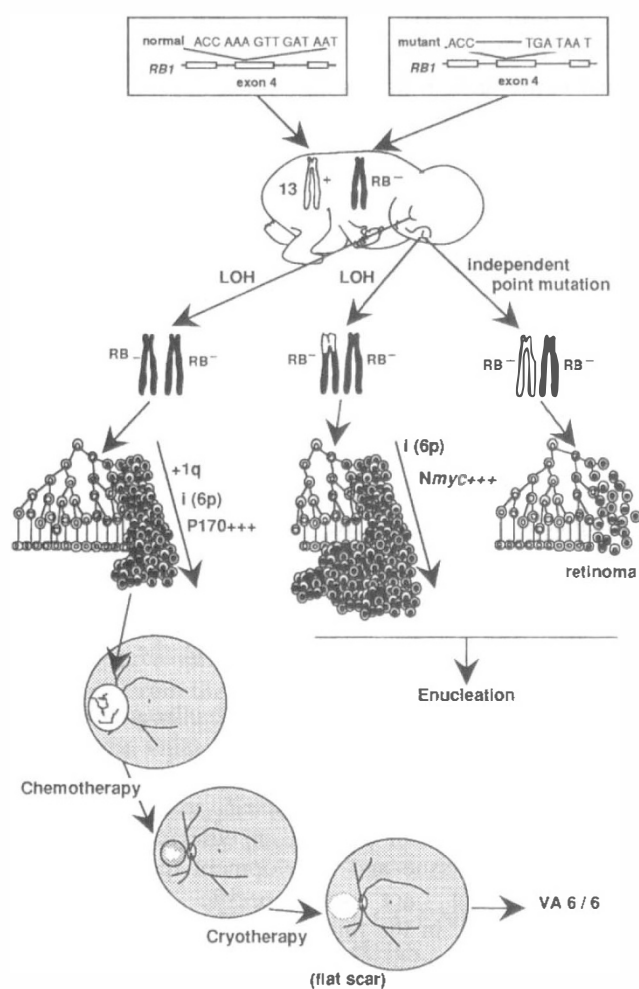
Fig. 1. *The figure illustrates a representative case of RB, illustrating 'How do Retinoblastoma tumours form?' The paternal* RB1 *allele inherited by the baby suffered a 5bp deletion of exon 4. In late pregnancy, loss of heterozygosity (LOH) occurred in individual retinal cells in each eye, prior to full retinal differentiation. In the left eye, another cell suffered a point mutation of the maternal normal allele. Absence of* $P110^{RB1}$ *resulted in unregulated proliferation of the cells. An i(6p) chromosome arose in two of the* RB1-*clones; N-myc oncogene amplification occurred in one, while duplication of 1q occurred in the other. The third* RB1-*clone proliferated only through several cell divisions without other mutations arising, and reached a non-functional but terminal differentiation. Since further cell divisions could not occur, the abnormality formed only a retinoma. In response to the abnormal retina and absense of photoreceptors, underlying pigment epithelial cells also became abnormal.*

The tumour in the left eye proliferated to fill the vitreous cavity, showing to the parents a white pupillary reflex, and leading to diagnosis of RB. The tumour in the right eye was of moderate size, overhanging the optic nerve at diagnosis. Although conventional treatment was radiotherapy, a trial of experimental chemotherapy with VM26, vincristine, and high dose cyclosporin A was initiated. The tumour shrank to show its origin nasal to the optic nerve. After six treatments at 10 day intervals, the residual mass was translucent, partially calcified, and about 1/3 the original size. The residual was treated on three occasions with cryotherapy through a conjunctival incision, freezing up to the nasal rim of the optic nerve. No further tumour grow occurred. Ultimate visual acuity was normal, and radiation therapy was avoided.

Clinical application of RB1 mutation identification

Efficient methods are now required to identify the *RB1* mutation of each family in order to provide accurate genetic counselling. The research technology used so far to identify mutations[3,4,5] has been time consuming, costly and not readily available. Now several labs are identifying mutations in RB families[30] (J. Cowell and B. Gallie, personal communication) by somewhat more efficient methods. The DNA of each exon, amplified by polymerase chain reaction (PCR), is screened by searching for mobility differences of single DNA strands caused by the mutations.[29] Suspect regions of DNA are sequenced to define the mutation.

Once the mutation is known in a family, screening of *relatives is relatively simple and cost effective. The DNA is amplified by PCR with oligonucleotides spanning the mutation. Deletions and duplications are detectable on simple agarose gel electrophoresis by the size of the fragment or by formation of a heteroduplex between mutant and wild type strands, which alters mobility;*[68] some point mutations alter restriction enzyme recognition sites; and some may be best detected by specific oligonucleotide probes. Since the alternative to molecular detection of the *RB1* mutation is the more expensive repeated examinations of infant relatives under anaesthetic for the first three years of life, health care finance must be redirected to molecular mutation identification.

Once a family's *RB1* mutation is identified, prenatal diagnosis can be offered. Since early diagnosed RB is rarely fatal,[69] and in most cases blindness can be prevented, few parents will select termination of pregnancy if the fetus is shown to have the mutation. However, diagnosis of the mutation at 36 weeks gestation has been suggested as justification for premature delivery, in order to treat RB earlier, saving more sight and reducing the morbidity of therapy. Ultimately, such individuals will increase the fraction of RB cases that are inherited, if they continue to reproduce.

ADDITIONAL MUTATIONS ARE RQUIRED FOR MALIGNANCY

Retinoma: too few mutations?

Even if LOH has resulted in absence of any normal *RB1* allele or $p110^{RB1}$ in a developing retinal cell, malignancy may still not result (See Figure 1). Retinoma, the benign equivalent of an RB tumour, appears as a non-proliferating lesion resembling irradiated, inactive tumour, in the untreated retina of individuals with germline *RB1* mutations.[70,71] A retinoma that occurs in the same eye as RB tumour can not be clinically recognised, but may be manifest by the residual translucent mass persisting after radiation treatment.[72]

Several explanations may account for retinoma. If both RB alleles are mutated in a retinoblast that is almost terminally differentiated, limited proliferation may be insufficient for progressive accumulation of the other mutations required for malignancy. RB tumours removed from

patients show abnormalities in other chromosomes besides chromosome 13:[73] a unique rearrangement of chromosome 6,i(6p);[74,75] trisomy of 1q, common in many human solid malignant tumours;[76,77] occasional DNA amplification of the N-*myc* proto-oncogene,[78,79,80] a protein expressed normally in retinoblasts;[81,82] and overexpression of the membrane transport protein, P170 (See below). Retinomas have not been studied for these progressive genetic mutations since there is no clinical need to remove the eyes, and few specimens are available.

Alternately, retinomas may be determined by the *RB1* mutations. The germline mutation of a patient with retinoma is not yet described, but two families have been identified that showed fewer than expected RB tumours and had germline mutations in the regulatory region of *RB1*.[30] It was proposed that such regulatory mutations might result in a small amount of normal $p110^{RB1}$, that would account for fewer than the usual number of tumours. Similar mutations could lead to retinoma.

When retinoma is documented in an individual, repeated clinical observations must be carried out to ensure that a cell within the retinoma does not further mutate to become proliferative, malignant RB.[83]

Resistance to Chemotherapy: too many mutations?

The majority of cases treated by removal of the eye are cured of cancer,[84] and the mortality from RB in countries with modern medicine is only 8%.[85] However, if curative therapy is delayed, the RB tumour may grow into the optic nerve and enter the meningeal space to disseminate throughout the brain and spinal cord. Up to the present time, RB disseminated in the central nervous system has not been curable. Rarely, bone marrow metastases arise without brain involvement, and may be curable by intensive treatment.[86]

Many RB are controlled by radiotherapy, as long as less than half the ocular volume is involved, and no vitreous seeding has occurred.[87] However, patients with germline *RB1* mutations have a several hundredfold risk of developing second primary neoplasms, including osteosarcoma, fibrosarcoma, soft tissue sarcoma, melanoma, brain tumour and others, and this risk is significantly increased by radiotherapy.[88,89,90,91] Therefore, unless radiotherapy is the only way to achieve useful vision, control of the cancer by other therapies such as photocoagulation and cryotherapy is preferable.

Intraocular RB tumours have not been extensively treated by chemotherapy. The drugs available had high toxicity, and systemic potential to also cause second malignancies.[90] Now, however, we have shown good initial responses of intraocular RB tumours to relatively non-toxic, non-alkylating drugs, VM26 and vincristine. Moderate sized tumours can be shrunk to a size treatable by cryo- or phototherapy. If the tumours remain too large for these treatments however, they become resistant to the effects of the drugs.

The membrane glycoprotein, P170, prevents accumulation of multiple classes of chemotherapeutic agents in cancer cells, and results in multidrug-resistance (MDR) of the tumour cells.[92] Increased P170 expression in RB tumours correlates well with an MDR phenotype, both *in vitro* and *in vivo*.[93] Several approaches are being developed to specifically counteract P170 and increase the effectiveness of chemotherapy.

Competition for the P170 drug efflux pump, for example by high levels of drugs such as cyclosporin A,[94] interference with P170 by blockers such as verapamil,[95] or inactivation of P170 by specific agents such as monoclonal antibodies,[92] have potential to improve therapy and cure rates for many cancers, including RB. By understanding 'How RB tumours form', we may be able significantly improve prospects for children with RB.

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