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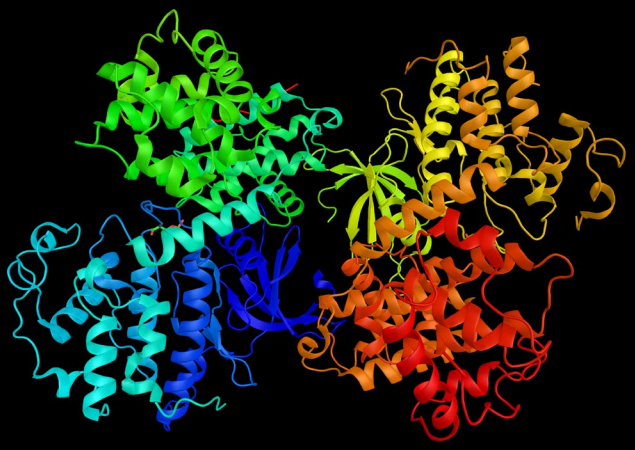
Retinoblastoma: The Hits Just Keep Coming



by
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Part I – White Spots

Chris and Julie were sitting in their living room one evening with their 10-month-old daughter, Kay, between them. She was doing a particularly cute thing, so Chris decided to take a picture. "That's odd," he said, frowning down at the photograph on his phone. He asked his wife, Julie, "What do you think is wrong with her eyes?"

Looking at the photo, Julie saw what Chris was talking about: there was a large, white reflection in Kay's left eye and a smaller one in her right eye. Julie's heart sank as she remembered the pediatrician asking them to look out for this type of thing. "We should take her to the doctor. You know that eye cancers run in my family. I've seen that left eye wander, too. I'll make an appointment in the morning." Chris and Julie didn't sleep well that night.

When she was a baby, Julie was treated for tumors in both of her eyes. Her mother also had this condition as a child, and died last year after a lengthy battle with bone cancer at the age of 58. Julie's uncle (her mom's younger brother) had tumors in both eyes as an infant and was diagnosed two years ago with melanoma at the age of 66; he passed away last spring. Julie's maternal grandmother also had tumors in each of her eyes as a child, but her grandfather did not. Julie's younger brother had no history of cancer. However, Julie's older brother did have eye tumors as a child; his daughter, however, did not.

So a few days later, a nervous Chris and Julie took off work to take Kay to see the family pediatrician, Dr. Whitt. Dr. Whitt explained to Chris and Julie that she was hosting a medical intern that day who would be observing with their consent. Dr. Whitt noted leukocoria in both eyes and strabismus in the left. After Kay's physical exam, Dr. Whitt asked Julie to confirm a few questions about her family history. "Chris," Dr. Whitt asked, "out of curiosity, does your family also have a history of cancers or eye problems?"

"No," replied Chris, with a hopeful look at Julie.

"I see," replied Dr. Whitt. "I'm going to order an MRI. We'll have a better idea of what we're dealing with once we see the results. However, this looks so far like Kay has the same childhood cancer that runs in Julie's family: retinoblastoma. If the MRI confirms this diagnosis, then we'll talk about next steps. Likely, these will involve a pediatric oncologist and possibly a genetic counselor."

Questions

1. Dr. Whitt noted leukocoria and strabismus. Define these terms.

2. Based on what you know so far, create a pedigree that depicts the prevalence of cancer in this family. Indicate Kay as the proband.

3. Consider the pedigree that you just drew. What mode of inheritance seems most likely? Justify your prediction.

4. What is the difference between a sporadic cancer and an inherited (or familial) cancer? Which is most common?

5. Based on the pedigree you drew, do you think that the cancer in this family tree is sporadic or inherited? Why?

6. Briefly, describe the fundamental problem that distinguishes a cancer cell from a non-cancerous cell.

Part II – Not a One-Hit Wonder

Kay's MRI results confirmed a diagnosis of retinoblastoma, so Dr. Whitt called Chris and Julie back into her office the following week. Dr. Whitt's intern listened attentively as Dr. Whitt explained what they'd found out. "The MRI shows a type of tumor called retinoblastoma in both eyes, which is causing her leukocoria and strabismus. The tumor is growing in her retinas, and we need to fix it fast in order to minimize vision loss."

"Is she going to be OK?" asked Chris nervously.

"The MRI suggests that the tumors are still in early stages, and there is no indication that either has spread outside of her eyes. That's a very good sign. We're lucky you paid such close attention to her picture." Dr. Whitt patted Chris comfortingly on the shoulder.

"I don't understand," Julie said with some confusion. "Did we give this to her? It runs in my family … is this my fault?"

Dr. Whitt sighed; she had heard this concern before. "Retinoblastoma is a tumor that arises as a result of a mutation in the *RB1* gene. This gene encodes a very important protein that helps stop cells from dividing under conditions in which they should not, so its mutation can be a driving force in cancer development. Since there are tumors in both eyes, not just one, and since Julie had retinoblastoma as a child and has a family history of this disease, it is likely that the mutant copy of the gene came from you; but, no, it is not 'your fault.' It would be a good idea for both you and Kay to have genetic testing done. I'm going to refer you both to a genetic counselor who can help you navigate these kinds of conversations better than I can."

"But," interjected Chris, "shouldn't I have given her a normal copy of this gene? So, she should be fine, shouldn't she?"

"Yes," replied Dr. Whitt, "Kay has a normal-functioning, or 'wild-type,' copy of the *RB1* gene, which she probably got from you, Chris. In her other body cells, this copy is still intact and protecting those cells from becoming cancerous. However, likely during development, one of the retinal cells in her eye suffered a second mutation. Or, maybe that cell lost the second copy of the gene by some mechanism other that mutation. Either way, the normal copy of the RB1 gene was lost in that cell and all the cells it gave rise to. The mutated cells lost their ability to regulate their growth. Retinoblastoma was virtually inevitable after that. This is a very important gene."

Dr. Whitt gave Chris and Julie a minute to process the information while she stepped outside with her intern, Jamar, to see how he was doing with the case. "It's heartbreaking to see a little kid with cancer," he replied.

Dr. Whitt nodded. "Let's see what you remember from your medical coursework. What makes *RB1* such an important gene?"

Jamar thought a moment, then explained: "Cells cycle through a series of stages: G1, S, G2, and mitosis. The *RB1* gene encodes a protein that keeps the cell stuck in G1. Any cell that wants to progress through the cycle and ultimately divide must inactivate the RB protein; this inactivation is called the restriction (or R) point. To inactivate RB, normal cells depend on extracellular signals from neighboring or distant cells; without these signals, RB remains functional and normal cells do not divide until the tissue needs them to do so. So cancer cells that lack both copies of the *RB1* gene, and therefore the RB protein, have no way to stop themselves from dividing."

"Very good," replied Dr. Whitt. "Cancer cells that have lost RB function can blow through the R point and proliferate unchecked, even in the absence of proliferative signals. As you know, this is a major hallmark of cancer."

Jamar thought a moment and then said, "I want to do some research on this gene to learn more about it. It sounds really interesting!" Dr. Whitt suggested that he visit <https://www.ncbi.nlm.nih.gov/> for more detailed information.

Questions

1. Go to the NCBI's website at <https://www.ncbi.nlm.nih.gov/> (or you can scan the QR code at the right). On the database dropdown menu, select "Gene" and search for "RB1." The first hit should be the *Homo sapiens* version; click the gene name. Then, study the information to answer the following:

a. What is the chromosomal location of *RB1?*

b. Scroll down to the "Expression" data. List two or three examples of normal tissues that typically express the *RB1* gene. What general conclusion can you make about its expression across tissue types?

c. Scroll down to the "NCBI Reference Sequences (RefSeq)" section. Click on the "GenBank" link under NG_009009.1 RefSeqGene. This will take you to the genomic sequence of *RB1*.
   i. How many nucleotides long is the full-length *RB1* gene?

   ii. On the right hand sidebar of the screen, there is a section called "Analyze this Sequence." In that section, click on "Highlight Sequence Features." Scroll up and down to see the highlighted regions, which indicate exons of the *RB1* gene. How does the abundance of exonic DNA compare to that of intronic DNA? Does this proportion vary between genes? Why or why not?

d. Return to the RefSeq section in the Gene Database entry for *RB1*. Click on the link under "mRNA and Protein(s)" listed as NM_000321.3, or the most updated sequence data. This will take you to the mature mRNA sequence data.
   i. How many bases long is the full-length *RB1* mRNA transcript?

   ii. Scroll down to "Features." Click on "CDS." This will highlight the coding sequence region of the RB1 mRNA. This is the sequence that will be translated into a protein.
      α. Indicate the start and stop positions of the CDS. How long is the coding sequence? How many amino acids should this encode for? (Remember that the last codon is a stop codon and does not encode an amino acid.)

      β. Indicate the start and stop positions of the 5′ untranslated region (UTR). (Assume that position "1" is the first base in the transcript.)

      γ. Indicate the start and stop positions of the 3′ untranslated region (UTR).

   iii. Scroll back up to "Features." Count the number of "exon" links there are. How many exons make up this transcript?

   iv. As you scroll through the "Features" list, observe some of the miscellaneous features ("misc_feature") of the transcript. These are examples of annotations, which give more information about the sequence than the just what the order of bases is. For example, "misc_feature 916…918" is a "phosphothreonine, by CDK1."
      α. What is your interpretation of this annotation?

β. What other kinds of information can these annotations give you? List a different example.

e. Use the information you have acquired so far to generate a map for the RB1 mRNA transcript.

f. Go back to the RefSeq section in the Gene Database entry for RB1. Click on "NP_000312.2," or the most updated sequence information, to access the protein's amino acid sequence information.
   i. How many amino acids make up the RB protein? Does this value match your prediction, based on the length of the RB1 mRNA transcript?

   ii. Scroll down to "Features." Note some of the particular "sites" at which different post-translational modifications can occur. List two examples.

(Note: Questions 2 through 5 do not require the use of NCBI.)

2. There are three broad categories of cancer-related genes: proto-oncogenes, tumor suppressor genes, and DNA repair/stability genes. Distinguish between these three groups, then indicate which you think *RB1* belongs to.

3. In 1971, Alfred Knudson noticed that children like Kay, who have retinoblastoma in both eyes (bilateral), presented at earlier ages than those who had unilateral disease. To explain these different disease kinetics, he proposed the "two-hit hypothesis." What does this hypothesis propose?

4. Consider Dr. Whitt's comments about the relationship between the wild-type and mutant *RB1* alleles and what you now know about Knudson's two-hit hypothesis. Do these ideas seem to confirm or contradict your earlier prediction (based on the pedigree) about whether the mutant *RB1* allele is dominant or recessive to the wild-type allele? Explain.

5. Do you think that Kay (and Julie) will be more likely to develop other cancers later in life?

Part III – The Second Hit

Before Dr. Whitt and Jamar returned to the exam room to talk with Kay's parents, Jamar asked Dr. Whitt for some clarification about Kay's father's question: "So, since Kay is heterozygous, having one wild-type and one mutant *RB1* allele, does that mean this mutation is dominant?"

"A loss-of-function mutation in the *RB1* gene is both recessive and dominant," explained Dr. Whitt. "At a cellular level, the *RB1* mutant allele is recessive, because the remaining wild-type copy of the gene continues to synthesize enough RB protein to halt inappropriate cell division, preventing that cell from becoming cancerous. Therefore, it takes two "hits," or inactivating events, to transform the normal cell into a cancer cell. At an organismal level, the *RB1* mutant allele is dominant, because heterozygotes inevitably get the disease. You see, it is virtually certain that a second hit will inactivate the wild-type copy in heterozygous individuals."

"Why is it a given that a second hit will happen?" asked Jamar.

"Well, it's basic statistics. There are more cells in the developing embryonic retina than there are odds of incurring a second mutation. Let's assume, for example, that the likelihood of a mutation that inactivates the wild-type *RB1* allele is one in a million, and that there are three million cells in the developing retina; this embryo should, statistically, suffer a *RB1*-inactivating mutation in three different cell lineages, each of which would initiate a tumor."

"Wow, that's scary," replied Jamar. "So, Kay has the heritable form … but what about kids with the sporadic form of the disease? Would they need both wild-type genes to be hit by a mutation?"

"Yes," replied Dr. Whitt. "However, it is statistically unlikely that two mutations will occur in the same gene in the same cell lineage. Therefore, other mechanisms, which inactivate *RB1* at higher frequencies than mutations do, are more often responsible for one or both of the hits. And, these mechanisms can also inactivate the remaining wild-type *RB1* allele in familial cases; they don't only act in sporadic cases."

Questions

1. A second hit might occur through epigenetic alterations. In the promoter of *RB1*, there is a CpG island. Knowing this, how might you predict that a cell could epigenetically inactivate *RB1* transcription?

2. A second hit might also occur through loss of heterozygosity (LOH). An example of how LOH may occur by reciprocal crossing over during mitosis is diagrammed in Figure 1 (next page). Discuss and interpret this model with your group. Write a brief explanation of (a) what LOH means and (b) how LOH by mitotic reciprocal crossing over can give rise to a cell lineage with functional loss of the wild-type copy of a tumor suppressor gene.

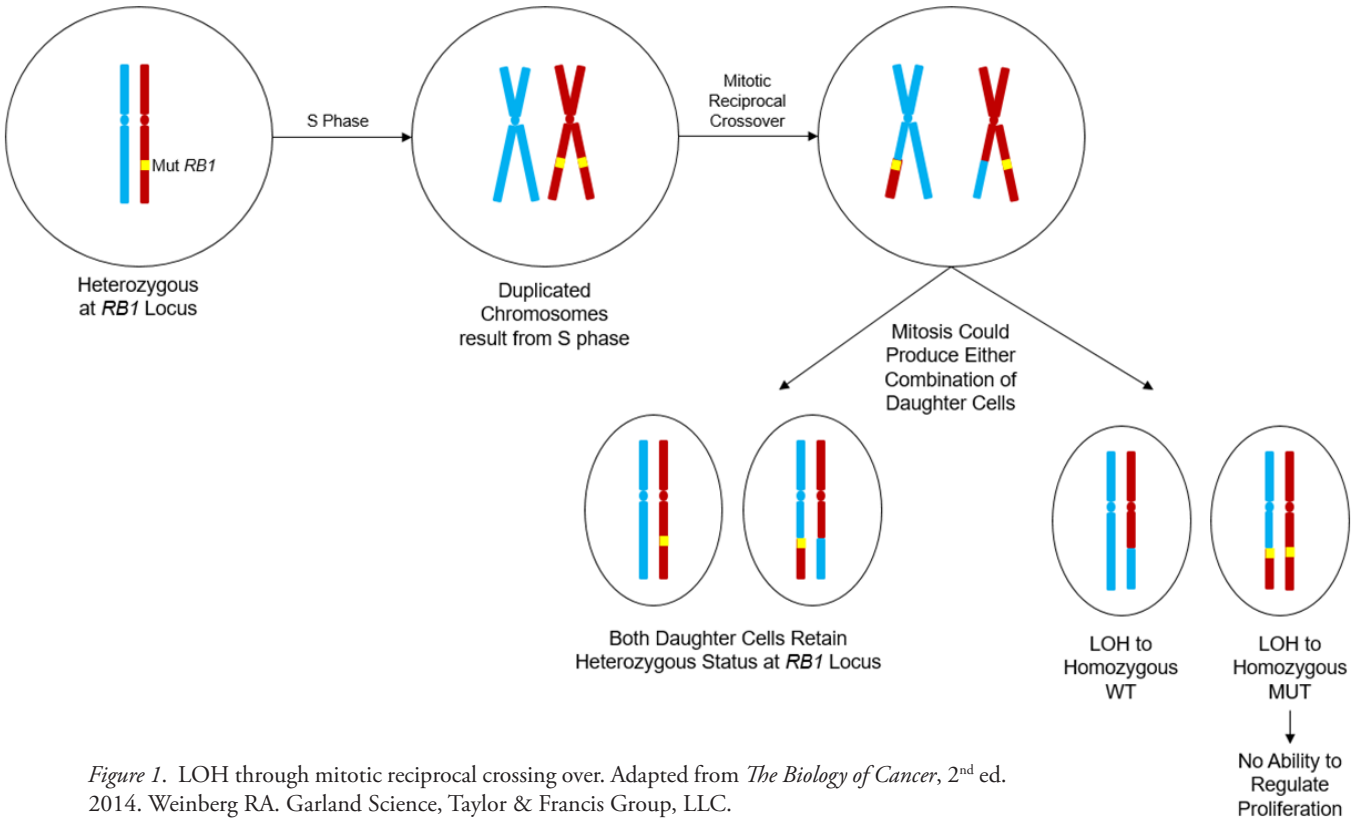

Figure 1. LOH through mitotic reciprocal crossing over. Adapted from *The Biology of Cancer*, 2nd ed. 2014. Weinberg RA. Garland Science, Taylor & Francis Group, LLC.

3. One of the ways that we know what the RB protein does in cells is that its inactivation is a common priority of tumor-initiating viruses.
   a. What advantage would a virus gain by inactivating RB function in its host cell?

   b. One example of a DNA virus (a virus that uses DNA, not RNA, as its genetic material) that causes tumors is human papillomavirus (HPV). Do some research and explain how HPV inactivates the RB protein and indicate with which type(s) of cancer it is associated. Don't forget to cite your sources!

Part IV – Time to Reconvene

Jamar asked Dr. Whitt how a second hit to a tumor suppressor gene like *RB1* might occur other than by an inactivating mutation. Dr. Whitt explained, "Several mechanisms may silence the wild-type *RB1* gene. For example, the *RB1* gene may be abnormally silenced by epigenetic modifications, like DNA methylation in its promoter region. Alternatively, it may undergo something called loss of heterozygosity, or LOH. There are a variety of ways that this can occur, but the main effect is that the person is no longer a heterozygote, meaning they no longer have one wild-type and one mutant allele. Instead, they could become homozygous for the mutation through some kind of recombination event, or hemizygous, in which case they would have only a single (mutant) copy of the gene."

Jamar asked, "How could a cell lose its heterozygosity?"

"Well, one mechanism of LOH is mitotic recombination. In this scenario, the maternal and paternal homologous chromosomes undergo a crossing-over event, much like what happens in meiosis. The result is that mitosis, depending on how the sister chromatids separate, may produce a daughter cell that has two inactivated *RB1* alleles. There are other mechanisms, but this is one of the best-studied."

"What are the other mechanisms?" asked Jamar.

"Well, the most common is something called gene conversion. During DNA replication, it may happen that the DNA polymerase, elongating a DNA strand on the chromosome that has the wild-type allele, for example, 'jumps' to the homologous chromosome—the one with the mutant allele—before eventually jumping back to the original template. Therefore, the polymerase replicates the mutant allele twice and fails to replicate the wild-type allele at all. If this cell later goes on to divide through mitosis, the daughter cells may thus have two mutant alleles, whereas the parent cell was originally heterozygous."

"Oh yeah," replied Jamar, "now I remember; we talked about some of these things in medical school."

Dr. Whitt, getting into lecture mode, continued. "Yes. In fact, it was the first tumor suppressor gene ever identified. Actually, a lot of the early insights into the function of the RB protein came from studying viruses that caused tumors. I suppose the most clinically relevant example is human papillomavirus, or HPV, which inactivates the RB protein through a viral protein named E7; certain strains of HPV, as you probably know, are responsible for virtually every cervical cancer diagnosis on the planet. In a case of bitter irony, HPV infection is largely preventable by vaccination. There are other examples of viruses that inhibit RB, but how exactly they do it—either by structurally altering it or by inducing its degradation—varies from virus to virus."

Jamar was eager to continue their discussion, but Dr. Whitt held up a hand and said kindly, "That's all for now! We have to get back to Chris and Julie."

When they walked in, Chris asked, "Does Julie also have a higher cancer risk since she also has the mutation?"

"The RB protein does the same thing in every cell in the body," Dr. Whitt replied. "Since a person like Kay—or you, Julie—has lost one functional *RB1* gene in every body cell, your other tissues are also at an increased risk of developing cancer. Interestingly, the RB protein's loss is more consequential for some cell types than others. We don't really understand why. But certain types of cancer are more likely."

Chris looked at Julie and asked, "Which ones do we have to worry about?"

"Well, a type of bone cancer called osteosarcoma in particular," Dr. Whitt responded. "There's a 500-fold increase in your risk for that particular cancer. Various other tumors of epithelial tissues, like melanoma, lung and bladder cancers, and others, are also more likely; it's why both of you will need close monitoring going forward. I also want you, Julie, to get tested in order to verify that you and Kay share the same mutation. Some mutations are more serious than others. I'm referring you to a genetic counselor who will help you through the process. In the meantime, I'm also referring Kay to a specialist, a pediatric oncologist who works in a pediatric ophthalmology clinic. They will take over her treatment. We'll be in touch soon about scheduling your appointment with them."

Chris and Julie left feeling the same weight that everyone feels upon learning their child has cancer. On the drive home, they both began to wonder about their plans to have more kids. Julie in particular wanted to know if there was anything they could do to ensure that her future children wouldn't have to go through this. She also resolved to get herself tested, like Dr. Whitt suggested.

Question

1. Since Julie is indeed a carrier of the mutated *RB1* allele and Chris is homozygous for the wild-type allele, what is the likelihood that their next child will inherit Julie's *RB1* mutation?

Part V – A Different Kind of Hit

As Dr. Whitt suggested, Julie met with a genetic counselor, Mrs. Hernández. Based on the recommendations of Dr. Whitt and Mrs. Hernández, Julie underwent genetic testing to determine what specific mutation she harbored in the *RB1* allele. However, it turned out that Julie's mutation was not the same one that Kay had! Julie didn't understand how this could be, so she met with Mrs. Hernandez for an explanation.

In their meeting, Julie asked Mrs. Hernández, "How can Kay have a different mutation than I do?"

Question

1. Chris does not have an *RB1* mutation, and is therefore homozygous wild-type. Julie is heterozygous for the mutation. However, Kay has inherited a different *RB1* mutation than the one her mother carries. Therefore, Kay did not receive her mother's mutant allele. Assuming that Chris really is the father, what other explanation might there be for how she got a germline mutation?

Part VI – Conclusion

Mrs. Hernández paused, clearly surprised. "I'm sorry, you said that you and Kay do *not* have the same mutation?"

"That's right," replied Julie. "How can that be?"

Mrs. Hernández whistled. "Well, that's unusual... How old did you say Chris is?"

"He's 43," replied Julie, growing more concerned. "What is it? What's unusual?"

"Well …" Mrs. Hernández considered a moment and then explained. "Clearly Kay didn't receive the mutant allele from you, because her mutant allele is different from yours; and Chris never had one to give her. The only remaining explanation is that a mutation arose spontaneously in the *RB1* allele during the development of either your egg or Chris's sperm. Most likely, it was an error in the developing sperm; they divide more rapidly than eggs, and paternal age has been linked to retinoblastoma risk."

"So," asked Julie slowly, "Kay's cancer isn't actually the same cancer that runs in my family?"

"As it turns out, no!" replied Mrs. Hernández. "It is still a heritable cancer, because it was caused by a germline mutation that was passed down from a parent. It's just that the mutation arose spontaneously during development of the germ cells. But if Kay has kids, she has the potential to pass this on to them. It seems your pedigree now has two different types of familial retinoblastoma in it."

"What luck," said Julie dryly. Mrs. Hernández nodded silently.

"While I have you," continued Julie, "Chris and I want to have more kids. Since I have the mutation, and his sperm are apparently getting old, is there any way to reduce our next child's risk of getting this awful disease?"

Mrs. Hernández suggested that Chris and Julie think about preimplantation genetic diagnosis (PGD). "This technique is well-established and very safe. Essentially, you will each donate eggs and sperm that will be fertilized in the laboratory; a process called *in vitro* fertilization or IVF. The embryos will be allowed to grow for a few days in small dishes and genetically screened for the presence of the *RB1* mutation. The doctor would then implant only those embryos with two wild-type *RB1* genes back into your uterus. This would ensure your next child does not inherit a germline mutation, either pre-existing or spontaneous.

"How expensive is that?" Julie asked.

"IVF would be somewhere between $10,000 and $15,000," said Mrs. Hernández. "And not all insurance providers offer coverage. The genetic testing would be an additional $2,500 to $5,000."

"OK," replied Julie. "We will look into our options and may need to meet with you further to discuss them. I appreciate all of your help. We have our first visit with the oncologist tomorrow."

"I'll keep my fingers crossed for Kay! I'll be following her treatment."

Kay's tumors were luckily caught early, though the left eye's tumor was significantly larger and more advanced. For treatment, Kay was placed under general anesthesia. The entire left eye was surgically removed in a procedure called enucleation, and an orbital implant made of silicone was attached to the ocular muscles in place of the eyeball. Kay then underwent intra-arterial chemotherapy for her right eye. For this treatment, a catheter was inserted through the femoral artery and carefully threaded up to an artery in the eye to allow direct injection of the chemotherapeutic drug Melphalan into the tumor. This procedure was repeated three times, once every four weeks, with fewer side effects than systemic chemotherapy. Kay had to be placed under general anesthesia for each treatment.

In frequent subsequent visits, Kay's medical team closely monitored her for recurrent and metastatic disease. Kay is now happily tumor-free. Though her left eye has been replaced, she has only moderate visual loss in her right eye at five years old and begins Kindergarten this fall.

Jamar the intern is now a Fellow in pediatric oncology and an expert retinoblastoma physician-scientist. Dr. Whitt continues to brighten the days of sick children and their families and train enthusiastic interns.

Suggestions for Further Reading

RB and the Cell Cycle

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