Medical Genetics

Sheet: Sheet 17 - Cancer Genetics 2

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Please note that italicized statements are for clarification and are not mentioned in the slides or lecture video.

The Two-Hit Theory

Brief Review

- We have previously established that proto-oncogenes promote cell division while tumor suppressor genes (TSGs) inhibit cell division.
- A noteworthy distinction we mentioned was the mechanism by which they drive carcinogenesis:
    - Proteins encoded by proto-oncogenes promote cancer when activated, overexpressed or get a gain-of-function mutation (One mutated allele (one hit) is sufficient to drive carcinogenesis)
    - Variants in TSGs contribute to malignancy by a different mechanism: the loss of function of both alleles of the gene.

TABLE 7-8 -- Selected Tumor Suppressor Genes Involved in Human Neoplasms

Subcellular Locations	Gene	Function	Tumors Associated with Somatic Mutations	Tumors Assocated with Inherited Mutations
Cell surface	TGF-β receptor	Growth inhibition	Carcinomas of colon	Unknown
	E-cadherin	Cell adhesion	Carcinoma of stomach	Familial gastric cancer
Inner aspect of plasma membrane	*NF1*	Inhibition of RAS signal transduction and of p21 cell cycle inhibitor	Neuroblastomas	Neurofibromatosis type 1 and sarcomas
Cytoskeleton	*NF2*	Cytoskeletal stability	Schwannomas and meningiomas	Neurofibromastosis type 2, acoustic schwannomas, and meningiomas
Cytosol	*APC*/β-catenin	Inhibition of signal transduction	Carcinomas of stomach, colon, pancreas; melanoma	Familial adenomatous polyposis coli/colon cancer
	PTEN	PI3 kinase signal transduction	Endometrial and prostate cancers	Cowden syndrome
	SMAD2 and *SMAD4*	TGF-β signal transduction	Colon, pancreas tumors	Unknown
Nucleus	*RB1*	Regulation of cell cycle	Retinoblastoma; osteosarcoma carcinomas of breast, colon, lung	Retinoblastomas, osteosarcoma
	p53	Cell cycle arrest and apoptosis in response to DNA damage	Most human cancers	Li-Fraumeni syndrome; multiple carcinomas and sarcomas
	WT1	Nuclear transcription	Wilms' tumor	Wilms' tumor
	P16/INK4a	Regulation of cell cycle by inhibition of cyclindependent kinases	Pancreatic, breast, and esophageal cancers	Malignant melanoma
	BRCA1 and *BRCA2*	DNA repair	Unknown	Carcinomas of female breast and ovary; carcinomas of male breast

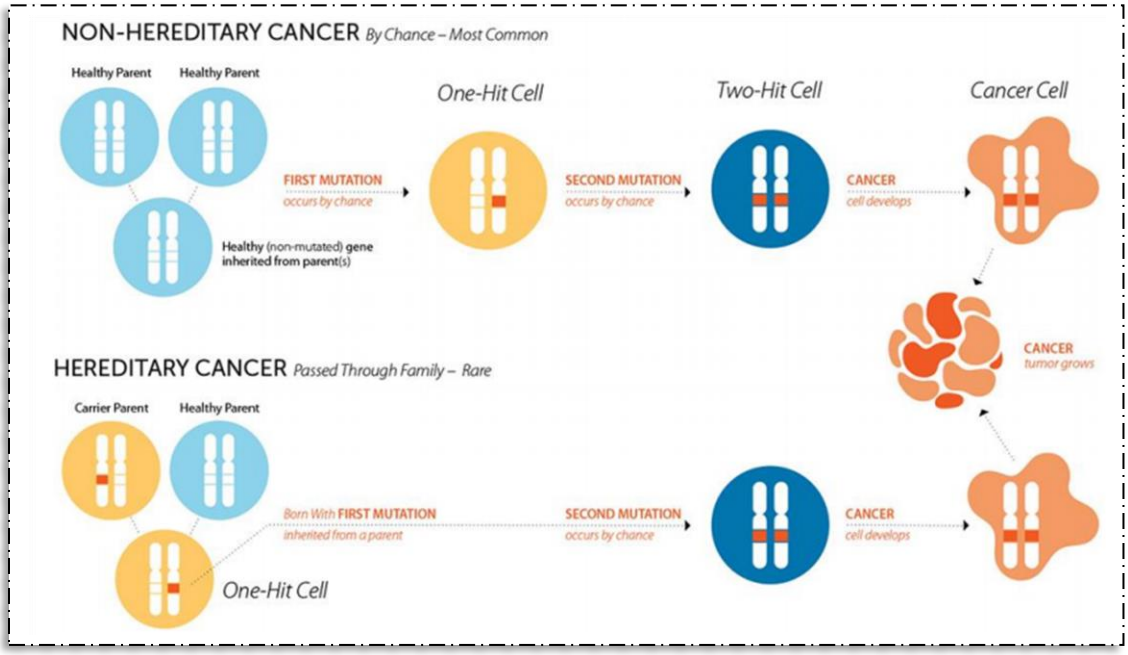
PI3 kinase, phosphatidylinositol 3-kinase.

- The products of many TSGs have now been isolated and characterized. The table above provides numerous examples of TSGs involved in human neoplasms. The variants could be somatic or inherited. Examples: RB1 gene is associated with retinoblastoma, NF1 with neurofibromatosis type 1, NF2 with neurofibromatosis type 2, BRCA1 and BRCA2 are risk genes (susceptibility genes) for breast and ovarian cancer.

The Two-Hit Theory of Tumor Suppressor Gene Inactivation in Cancer

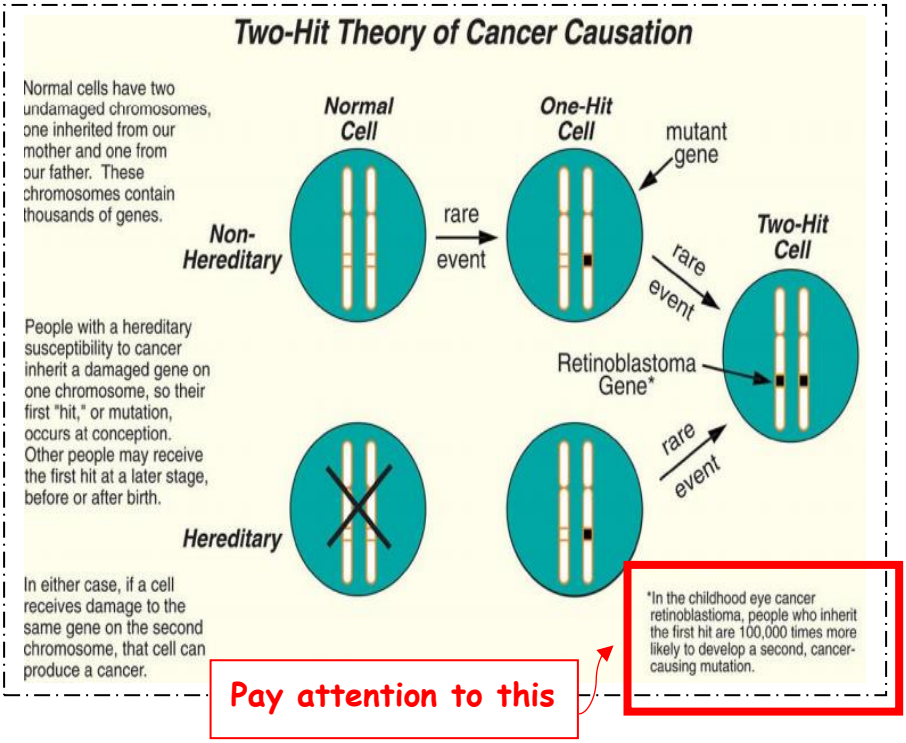
- So far, we know that most tumor suppressor genes require both alleles to be inactivated to cause a phenotypic change.
    - When the first allele is mutated, we call this mutation "the first hit"
    - When the second allele is mutated, we call this mutation "the second hit"
- This is the basis for the two-hit theory; two hits (i.e., two inactivating mutations, one in each tumor suppressor allele) are required for cancer to develop. This is a very crucial theory in genetics and applies to both hereditary and non-hereditary cases of cancer.

- In non-hereditary (sporadic) forms of cancer, the first hit occurs randomly (by chance). The second hit also occurs by chance. (No inherited hits). Once the second hit occurs, the risk increases for developing cancer.
- In hereditary forms of cancer, the story is quite different. Since it is hereditary, a disease-causing allele is inherited from one of the parents, and a normal allele from the other parent. The child's cells will each carry one mutated allele and one normal allele. So, the first hit was inherited from the parent (germline). The second hit, however, occurs by chance (somatic). Both alleles are now mutated, and if this allele is a driver gene for cancer, then it induces cancer cells to form



- So, mutations either occur by chance (randomly), or are inherited from a parent.

- Generally speaking, random events are rare in nature, and thus there is a low probability for one allele to be mutated by chance. You can imagine how even more difficult it would be for two alleles to become mutated by chance, as in sporadic cases (and thus sporadic cases require more time to occur (as they wait for two alleles to be hit), which translates into a later age of onset).



- Hereditary cases, in contrast, occur earlier in life, because the first hit is already present at birth (inherited), and only the second hit occurs by chance. So, again, the timeline to receive one somatic hit (as in hereditary cases) is shorter than the timeline needed for two somatic hits to occur in the same cell (as in sporadic cases), explaining the earlier age of onset in hereditary cases of cancer.
- Now we will apply what we have discussed in the context of retinoblastoma, which was the first disease to be related to the two-hit theory. (Many of the previously discussed points will be repeated, so it will hopefully be easy to go through)

Retinoblastoma

- Retinoblastoma, like many cancers, has both sporadic and hereditary forms. It is the prototype of diseases caused by mutations in a TSG.
- Interestingly, observations regarding retinoblastoma are what actually led to formulation of the two-hit theory.
    - It was suggested that the hereditary form of the childhood cancer retinoblastoma might be initiated when a cell in a person heterozygous for a germline mutation in the retinoblastoma TSG undergoes a second somatic event that inactivated the other retinoblastoma gene allele (in retinal tissue)
    - As a consequence of the second somatic event, the cell loses function of both alleles, giving rise to a tumor.

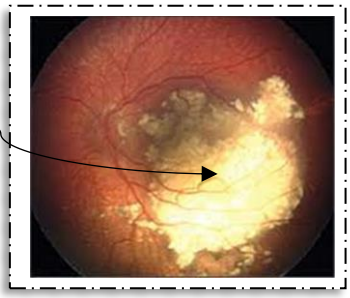
Note the terms "germline" and "somatic". A germline mutation indicates that it was inherited, while a somatic mutation indicates that it was NOT inherited (but instead acquired)

    - In the sporadic form of retinoblastoma, both alleles are also inactivated, but in this case, the inactivation results from two somatic events occurring in the same cell.
- For the sake of inclusiveness, please go through the following points, which are mentioned in the slides (most of the points re-state what was previously described)
    - In 1971, Alfred Knudson performed a statistical analysis on cases of retinoblastoma, a tumor of the retina that occurs both as an inherited disease and sporadically.
    - He noted that inherited retinoblastoma occurs at a younger age than the sporadic disease. In addition, children with inherited retinoblastoma often developed the tumor in both eyes suggesting an underlying predisposition.
    - Kudson suggested that two "hits" to DNA were necessary to cause the cancer. He built the two-hit theory based on a mathematical approach, not a genetic one (TSGs and oncogenes had not been discovered at the time)
    - In children with inherited retinoblastoma, the first mutation in what later came to be identified as the RB1 gene, was inherited, the second was acquired.
    - In non-inherited retinoblastoma, two mutations, or "hits", had to take place before a tumor could develop, explaining the later onset.

- In 1986, the RB gene was the first tumor suppressor gene to be identified in medical history.
- The two-hit model is now widely accepted as the explanation for many hereditary cancers in addition to retinoblastoma, including: familial polyposis coli, familial breast cancer, neurofibromatosis type 1 (NF1), Lynch syndrome, Li-Fraumeni

- Some clinical and epidemiological points regarding retinoblastoma:
  - Retinoblastoma is a rare malignant tumor of the retina in infants, with an incidence of approximately 1 in 20,000 births.
  - Diagnosis of a retinoblastoma must usually be followed by removal of the affected eye, although smaller tumors, diagnosed at an early stage, can be treated by local therapy, so that vision can be preserved, which is why genetic testing and family history is important.
  - Look at the fundus in the adjacent image. If you look through a fundoscope (which is basically a magnifying lens) through the pupil of the eye, you can view the retina. Notice the malignant cells
  - Notice how the young girl in the image has a unilateral retinoblastoma (in one eye). You can see the white area in the center of the eye reflecting light.

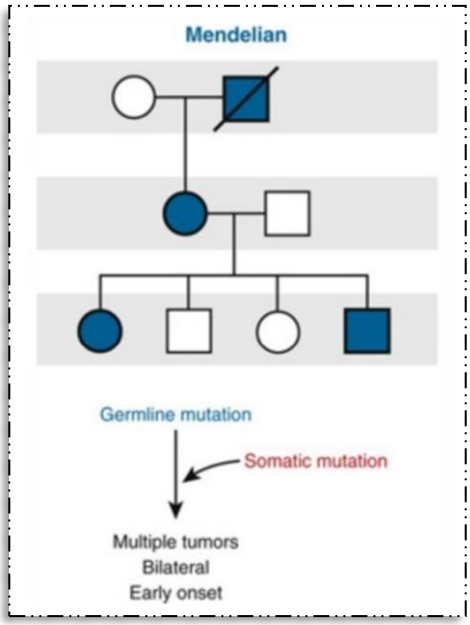
FIGURE 15-7 Retinoblastoma in a young girl, showing as a white reflex in the affected left eye when light reflects directly off the tumor surface. *See Sources & Acknowledgments.*



- Let's focus on each form of retinoblastoma:

Hereditary Retinoblastoma

- Approximately 40% of cases of retinoblastoma are of the heritable form, in which the child inherits one mutant allele at the retinoblastoma locus (RB1) through the germline from either 1. a heterozygous parent 2. or more rarely, from a parent with germline mosaicism for an RB1 variant. Let's discuss each case:
  1. For a heterozygous parent: If we examine cells from the parent's blood, brain, colon, gonads etc., all cells of his body will contain one mutated copy and one normal copy, because all cells originated from mitosis of one ancestral cell (zygote) and their DNA sequence is identical. This parent can give the mutated allele to his offspring.



2. It is also possible to inherit the RB variant from a parent in which mosaicism is present, that is, a small group of cells acquire a mutation during embryonic/fetal development, and the variant is expressed in some, but not all tissues of the parent. For example, if you sequence cells from the blood of the parent, you find two normal alleles, while if you sequence DNA from his/her gonads, you find one mutated allele and one normal allele. So this parent can produce gametes that carry the mutation and transfer it to the offspring. Also, the parent himself may not have the disease if his retinal cells escape the mosaicism (don't have the mutation)

   Note, however, that for the parent with mosaicism to transfer the mutation to his offspring, the mutation should exist in his/her gonads, otherwise the mutation cannot be transferred. For example, if the pancreatic tissue was heterozygous for the RB gene, while the gonadal cells carried both normal alleles, this parent's children cannot inherit the mutation from him/her, since the gametes the parent produces are normal.

- Conclusion: A child may inherit the mutated RB allele from a parent who has a mutation in all of his tissues, or from a parent who doesn't have the mutation in all of his tissues, but has it in his reproductive organs (mosaicism)

- Notice the adjacent image, which shows how the second hit may occur. (+) = a normal RB allele, while (rb) = a mutant RB allele. The first hit is assumed to be inherited.

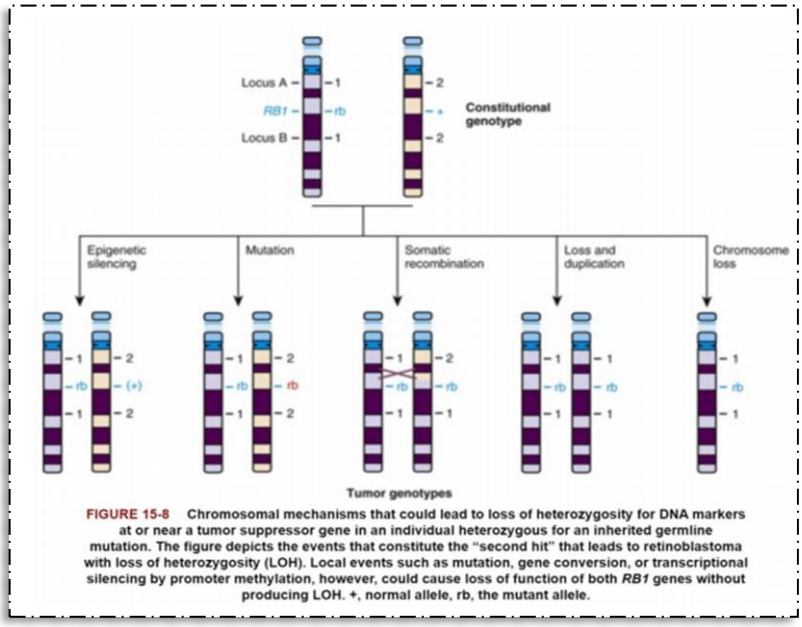


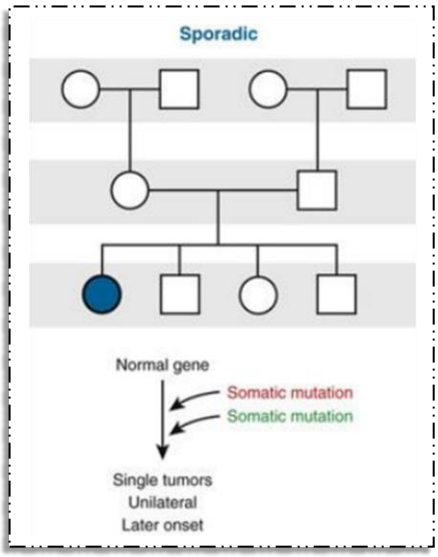
FIGURE 15-8 Chromosomal mechanisms that could lead to loss of heterozygosity for DNA markers at or near a tumor suppressor gene in an individual heterozygous for an inherited germline mutation. The figure depicts the events that constitute the "second hit" that leads to retinoblastoma with loss of heterozygosity (LOH). Local events such as mutation, gene conversion, or transcriptional silencing by promoter methylation, however, could cause loss of function of both *RB1* genes without producing LOH. +, normal allele, rb, the mutant allele.

- In these children, retinal cells, which like all the other cells of the body are already carrying one inherited defective RB1 allele, suffer a somatic mutation or other alteration in the remaining normal allele, leading to loss of function of both copies of the RB1 gene and initiating development of a tumor in each of those cells. The disease will not manifest without a second hit.
- Notice the pedigree for hereditary retinoblastoma in the previous page: Every generation is affected, and so the mode of inheritance is autosomal dominant, even though both alleles need to be mutated for the tumor to develop (clarified later).
  - An individual who inherits one mutated retinoblastoma allele (heterozygote) may or may not develop the second hit. Interestingly, it has been observed that most heterozygotes will actually develop a second hit, that is, most of them will develop a retinoblastoma. This is because the large number of primordial retinoblasts (progenitor cells which give rise to retinal cells) and their rapid rate of proliferation make it very likely that a somatic mutation will occur as a second hit in one or more of the more than 10^6 retinoblasts already carrying an inherited RB1 mutation. Their rapid rate of proliferation causes accumulation of more mutations, ultimately leading to retinoblastoma.

- **Multiplicity of Tumors:** Because the chance of a second hit is so great in hereditary cases, it occurs frequently in more than one cell. Thus, heterozygotes for the disorder often have tumors arising at multiple sites, such as multifocal tumors in one eye, in both eyes (bilateral retinoblastoma), as well as in the pineal gland (referred to as "trilateral" retinoblastoma). The occurrence of a second hit is a matter of chance and does not occur 100% of the time; the penetrance of retinoblastoma therefore, although greater than 90%, is not complete (this means the remaining 10% don't get the second hit).

- **Mode of inheritance:** An important point regarding hereditary retinoblastoma is that this disorder appears to be inherited as a dominant trait.
  - *Recall the definition of an autosomal dominant trait: A trait in which inheriting one allele is sufficient for the phenotype to manifest. Retinoblastoma appears to comply with this definition, as most heterozygotes for the RB gene (who inherit one mutated allele) will express the phenotype (develop a retinoblastoma tumor at some point). So, when observing at the phenotypical level, retinoblastoma "appears" to be inherited in an autosomal dominant fashion.*
  - *But when we further examine the retinoblastoma tumor cells, we notice that the tumor cells that developed in these patients needed two mutated alleles (hits), not one, to occur. This is consistent with the autosomal recessive mode of inheritance. So why is retinoblastoma said to be inherited in an autosomal dominant manner? Since we generally refer to the organismal/phenotypical level when discussing modes of inheritance (unless otherwise specified), and since individuals who inherit one mutated RB allele (heterozygotes) will almost always develop a second hit (and thus develop a tumor), we consider the disorder (or more accurately, the susceptibility to developing the tumor) to be inherited in an autosomal dominant fashion.*

Nonhereditary Retinoblastoma



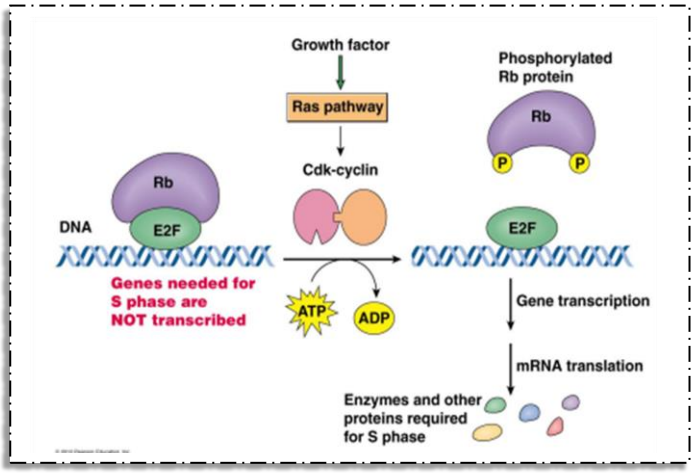
- The other 60% of cases of retinoblastoma are nonhereditary. Both RB1 alleles in a single retinal cell have been inactivated independently by chance. Because two hits in the same cell is a statistically rare event, there is usually only a single clonal tumor, and the retinoblastoma is found at one location (unifocal) in one eye only (unilateral) (Recall that hereditary cases are usually multifocal and bilateral). (The sentence says "clonal". This is how it is written in the slides. It is not completely wrong. Perhaps it meant that tumor cells are clonal for the RB mutation, but as you know, sub-lineages may arise as different tumor cells acquire different mutations along the way)
- Unilateral tumor is no guarantee that the child does not have the heritable form of retinoblastoma, however, because 15% of patients with the heritable type develop a tumor in only one eye (although we said hereditary cases often involve bilateral tumors, they still can be unilateral because the second hit is a matter of chance and it may, by chance, just occur in one eye even though both eyes are at risk. Nevertheless, this unilaterality represents the minority of cases of hereditary retinoblastoma)

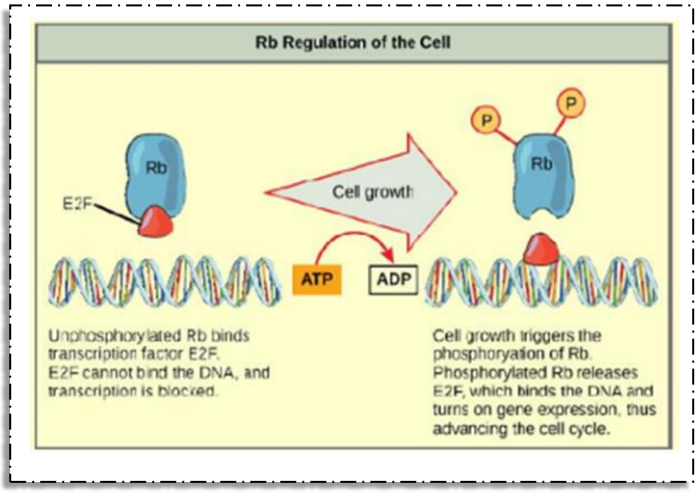
- Another difference between hereditary and sporadic tumors, as previously mentioned, is that the average age at onset of the sporadic form is in early childhood, later than in infants with the heritable form, reflecting the longer time needed on average for two mutations, rather than one, to occur.

- In a small percentage of patients with retinoblastoma, the variant responsible is a cytogenetically detectable deletion or translocation of the portion of chromosome 13 that contains the RB1 gene. Such chromosomal changes, if they also disrupt genes adjacent to RB1, may lead to dysmorphic features in addition to retinoblastoma. So, if a patient comes to you with retinoblastoma and other dysmorphic features, you should suspect that the mutation is at the chromosomal level rather than the genetic level (i.e., genes other than the RB gene are involved).
- In hereditary cases, all cells carry one mutated allele, and theoretically, any cell can undergo a second hit. However, the tumor's exclusive occurrence in retinal tissues is probably attributed to the higher functionality of the RB gene in this particular tissue

Nature of the Second Hit

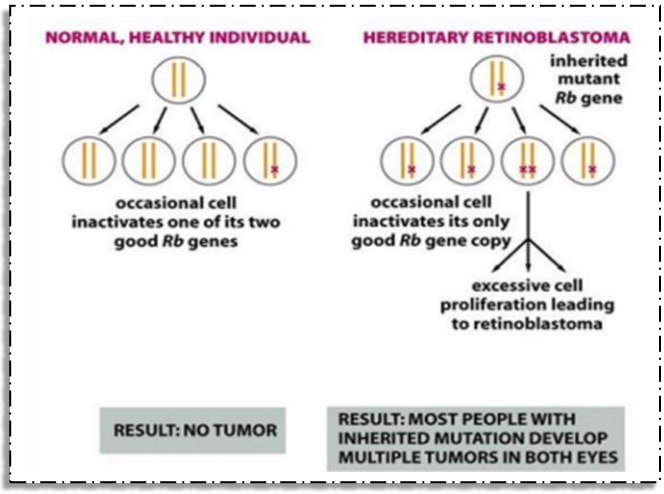
- Typically, for retinoblastoma as well as for the other hereditary cancer syndromes, the first hit is an inherited mutation, that is, a change in the DNA sequence. The second hit, however, can be caused by a variety of genetic, epigenetic, or genomic mechanisms (the second hit can come in different "flavors").
- Although a number of mechanisms have been documented, the common theme is loss of function of RB1 (just like TSGs in general)
- Now, let's dive into more details regarding the function of a normal RB gene:
    - The RB1 gene product, p110 Rb1, is a phosphoprotein that normally regulates entry of the cell into the S phase of the cell cycle.
    - Thus, loss of the RB1 gene and/or absence of the normal RB1 gene product deprives cells of an important checkpoint and allows uncontrolled proliferation.
    - The Rb protein functions as follows: When Rb is unphosphorylated, it binds to a

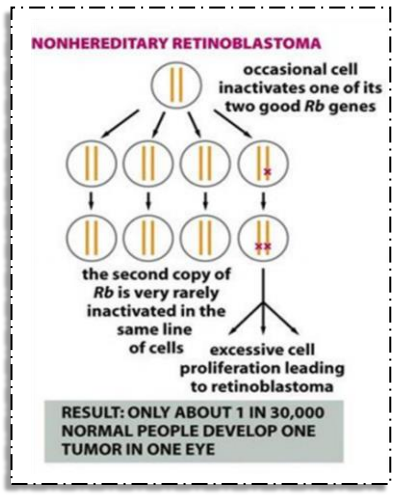




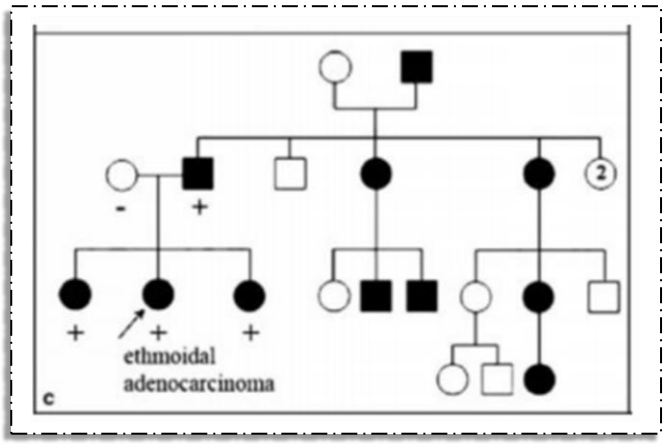
transcription factor known as E2F, preventing it from binding to DNA, and thus preventing gene transcription. When the RB protein becomes phosphorylated, it releases E2F, which then proceeds to bind to DNA, and helps gene transcription, producing mRNAs which are translated into enzymes and proteins required for the S phase. So, if RB becomes non-functional, a checkpoint for regulating the cell cycle is lost, and E2F continuously transcribes genes involved in proliferation causing uncontrolled growth.

- Additional images from the slides. Please go through them

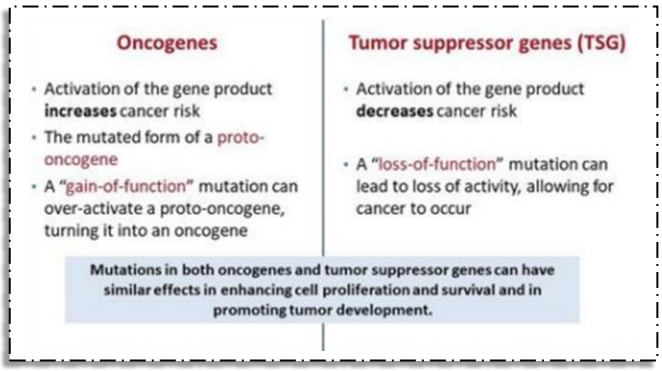




- The adjacent pedigree is for hereditary retinoblastoma. Notice how it is inherited in an autosomal dominant manner.

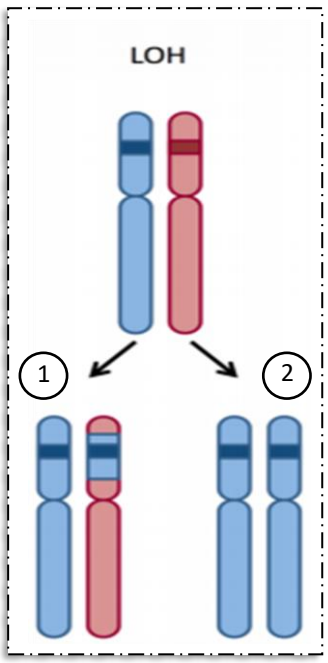


Please don't panic when you move to the the next page. As dense as it looks, we have attempted to simplify the concepts as much as possible.



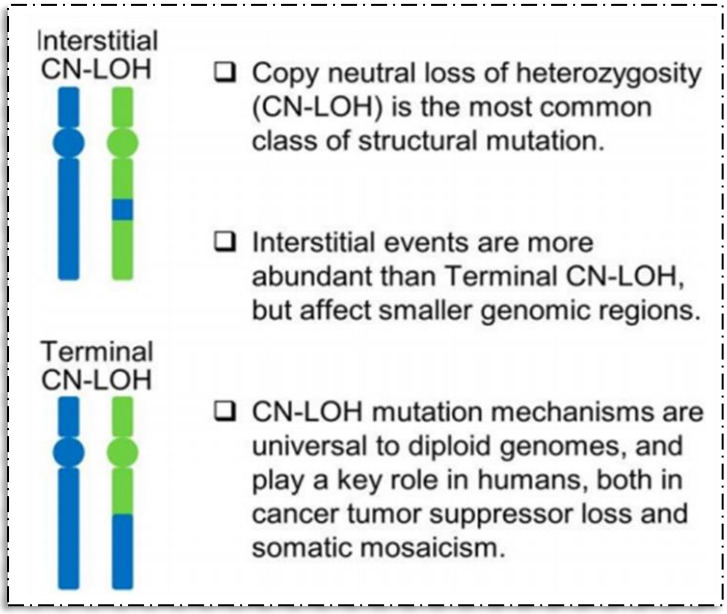
Loss of Heterozygosity (LOH)

- Another important concept to discuss when studying the genetics of retinoblastoma, and perhaps other cancers as well, is loss of heterozygosity (LOH).
- You may find it confusing, but we will approach it in a gradual step-by-step fashion and make it as clear as possible.
    - The RB locus is located on chromosome 13. Within the RB locus, there is the RB gene.
    - Near the RB gene (on both sides of the gene), there are polymorphic markers.
    - Polymorphic markers are regions of DNA which are normally highly variable among individuals, either at the nucleotide level (differences in nucleotides), or the repeat level (differences in the number of repeats)
    - Since they are highly variable among different people, any individual is very likely to have inherited polymorphic markers from his father that are different from the ones he inherits from his mother. So, the individual is often heterozygous for these polymorphic markers. Also, these polymorphic markers are informative, because their high variability makes them unique for each individual and can potentially help identify the person ("mark" the person, hence called a "marker"). They are often used in forensic medicine (crime scenes) or paternity testing.
    - Geneticists noticed the following: If a retinoblastoma patient comes and you sequence cells from his blood (or any normal tissue), you will notice that he is heterozygous for these polymorphic markers around the RB gene (which is expected as we discussed above)
    - If you sequence the same individual's retinoblastoma tumor cells (remember we usually treat retinoblastoma by removing the eye, so we can sequence the tumor cells from the removed eye), would you expect the tumor cells to also be heterozygous for the polymorphic markers? Well, the answer would appear to be yes, because it is the RB gene that is affected in retinoblastoma tumor cells, not the polymorphic markers around it, so polymorphic markers should be the same as any other cell in the body. The polymorphic markers should be heterozygous. But this is where things became unexpectedly interesting: The tumor cells were often HOMOZYGOUS for the polymorphic markers! That is, the RB gene with the polymorphic markers surrounding were all homozygous in the tumor cells. The heterozygosity, which is found in normal tissues, was LOST in tumor cells.
    - This is what we call loss of heterozygosity (LOH).
    - So, in retinoblastoma tumor cells, the second hit is usually in the form of LOH.
    - We will now discuss how this phenomenon could occur (look at the adjacent image):



1. **Scenario 1:** The previously normal RB allele (red) with the region surrounding it were deleted for some reason, and in its place, a new copy was made from the corresponding region on the other chromosome, which carries the mutated RB allele, along with its associated polymorphic markers. The cell became homozygous for this region (LOH). Notice that for the rest of chromosome, however, heterozygosity still exists.
2. **Scenario 2:** The entire chromosome that carries the normal RB gene is lost *(probably due to nondisjunction)* and is replaced by reduplication of the remaining chromosome that carries the mutated RB allele. So now there are two blue chromosomes. The cell becomes homozygous for all the DNA along this chromosome, including the mutated RB alleles and the surrounding polymorphic markers.
3. **Scenario 3:** (not shown in the image) The red chromosome could be lost due to nondisjunction with no duplication (e.g., monosomy 13 due to nondisjunction), leaving one chromosome. *So, in this scenario, the cell has one RB allele, and is thus hemizygous (for the RB gene and the DNA along the entire chromosome). Hemizygous = not heterozygous = LOH.* But this scenario was very briefly mentioned. *So LOH does not necessarily mean the cell becomes homozygous, as the cell could become hemizygous.*

- The LOH in Scenario 1 is considered to be copy neutral LOH (CN-LOH). Because the number of copies for an allele remain the same/neutral (2 copies for the locus) and the copies are identical (LOH) (with no net gain or loss of genetic material). The adjacent image shows two types of CN-LOH: 1. Interstitial (occurs within (in the middle of) the chromosome) 2. Terminal (occurs in a terminal (peripheral) part of the chromosome)



- *Scenario 2 can also be considered CN-LOH, since the number of copies for alleles remain neutral (2 copies)*
- *Scenario 3, however is simply LOH, but not "copy neutral LOH", because we lost a copy with no compensation. The cell is left with one copy (not two, so not neutral) in this case*
- If, in a hypothetical case, there were 3 copies of an allele (maybe a copy was duplicated in a chromosome), this would also NOT be considered copy neutral
- Again, keep in mind that when a region of a chromosome (a relatively large region) is found to be homozygous (100% identical alleles), this is often abnormal. Normally, you'd at least expect several nucleotides to be different, especially in polymorphic regions, so homozygosity in such regions is normally a rare finding, which is why geneticists were baffled to find LOH in the RB gene and surrounding markers in many cases of retinoblastoma tumors.

- This concept may seem very strange, and you will need time to digest it. But it is a very essential concept to understand. The world of cancer is an insanely crazy one.
- We're almost done. Now. Please go through the remaining statements (which were taken from the slides) about LOH. The doctor went through them. They should be easy to understand, they repeat what we already discussed
    - In addition to mutations and epigenetic silencing a novel genomic mechanism was uncovered when geneticists made an unusual but highly significant discovery when they **compared DNA polymorphisms at the RB1 locus in DNA from normal cells to those in the retinoblastoma tumor from the same patient.** Individuals with retinoblastoma who were heterozygous at polymorphic loci flanking the RB1 locus in normal tissues had tumors that contained alleles from only one of their two chromosome 13 homologues, revealing a loss of heterozygosity (LOH) in tumor DNA in and around the RB1 locus. Loss of Heterozygosity
    - Furthermore, in familial cases, the retained chromosome 13 markers were the ones inherited from the affected parent, that is, the chromosome with the abnormal RB1 allele. Thus, in these cases, LOH represents the second hit of the remaining allele.
    - LOH may occur by interstitial deletion, but there are other mechanisms as well, such as mitotic recombination or monosomy 13 due to nondisjunction.

Good Luck