Retinoblastoma Epigenetics

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Abstract

The aim of the present chapter is to give new insights into the pathogenesis of retinoblastoma, by applying the principles of epigenetics to the analysis of clinical, epidemiological, and biological data concerning the disease. As an emerging new scientific approach linking the genome to the environment, epigenetics, when applied to the interpretation of clinical, epidemiological, and biological data in retinoblastoma, can explain not only the inconsistencies of the mutational ("two hit") model, but also open new outstanding scenarios in this fields of diagnosis, treatment and prevention of this eye tumor, and cancer in general. After more than four decades of predominance of the genetic theory, this chapter represents the first attempt to look at retinoblastoma from the point of view of epigenetics. The epigenetic model in the genesis of retinoblastoma, proposed herein, emphasizes the role of environment and the interaction of the environment with the genome, in generating retinoblastoma in young children. Environmental toxicants, including radiations, wrong diets, and infectious diseases, play a major role in conditioning the degree of DNA methylation (one of the leading mechanisms of epigenetic gene modulation) in embryos and fetuses during pregnancy, thus leading to stable, functional alterations of the genome, which, on the other hand, can also be transmitted from one generation to the next, thus mimicking a hereditary

disease. An accurate analysis of the currently available literature on both retinoblastoma and epigenetics, coupled with the knowledge of the variegated phenotypic expression of the disease, can easily lead to the conclusion that retinoblastoma is an epigenetic, rather than a genetic disease.

Introduction

Although rare, retinoblastoma is the most common eye tumor affecting children under the age of 5 years. Knudson (1971), after reviewing a series of 48 cases, formulated a hypothesis, according to which, this eye tumor may be determined by the loss or inactivation of both copies of a single gene. The presumptive gene responsible for tumor development in retinoblastoma was later identified and named as Rb1, and its complete DNA sequence was fully characterized (Friend et al. 1986). Since the beginning, retinoblastoma has been considered a hereditary tumor, and this view has been further reinforced by DNA investigations (polymorphism and conformational DNA analysis followed by DNA sequencing) demonstrating that retinal tumors usually bear mutations on both copies of the Rb1 gene, thus apparently confirming the mechanisms hypothesized by Knudson (1971), and allowing the identification of a new class of cancer genes defined as tumor suppressor genes.

Knudson's mutational model, maintains that two sequential mutations of the Rb1 gene are necessary to develop a retinoblastoma, and the timing and target of these two mutational events determine the clinical phenotype of the disease. Namely, when both the first and second mutations involve the somatic cells, the individual will develop a tumor affecting only one eye (unilateral retinoblastoma), but when the first mutation occurs in the germinal cells of one parent, and the second involves the individual's somatic retinal cells, the disease will affect both eyes (bilateral retinoblastoma). This fundamental diversity in the pathogenesis of the tumor represents the basic distinction between two different clinical retinoblastoma phenotypes:

1. Unilateral retinoblastoma (65–70% of all cases), which is sporadic (i.e., non hereditary), occurs at a later age, and usually presents with a single tumor focus on the retina of the affected eye.
2. Bilateral retinoblastoma (30–35% of all cases), which is hereditary, occurs at an earlier age, involves both eyes, and commonly presents with multiple tumor foci in the retina of at least one eye (Lohmann and Gallie 2010).

The theoretical model proposed by Knudson was accepted and used worldwide to explain some of the most important features concerning the different genetic, clinical, and epidemiological aspects of retinoblastoma, but the mutational model itself has been more recently challenged by evidences showing that both aneuploidy and genetic instability play an essential role in the genesis of cancer (Duesberg 2007). Nevertheless, neither the mutational nor the aneuploidy model seems to be able to explain the variegated phenotypic expression of retinoblastoma, which can be, instead, better understood and explained if the principles of epigenetics are applied to the study of this tumor affecting young children.

While the literature concerning the genetic origin of retinoblastoma has flourished in the last four decades, and the idea that this tumor is determined by two sequential mutations of the Rb1 gene still persists among geneticists and ophthalmologists, evidence is cumulating which clearly argues against the role of DNA mutations in cancer in general and in retinoblastoma in particular (Mastrangelo et al. 2008). The main purpose of the present chapter is to show that according to the currently available evidence, the concept of epigenetic gene regulation offers a totally new and consistent model to understand both etiology and pathogenesis of retinoblastoma, by taking into consideration the complex gene/environment interactions which account for the variable and variegated phenotypic expression of the disease.

What Is Epigenetics?

Epigenetics is a term coined in 1940 by Waddington who defined it as, "the interactions of genes with their environment, which bring the

phenotype into being". Literally, epigenetics means "above" genetics and the term properly designates events which modify gene expression without modifying the structure of the genes themselves. Although epigenetic regulation of gene expression is the basic mechanism through which billions of specialized cells belonging to an organism differentiate (starting from a single embryonic ancestor and one and the same DNA) the idea that gene expression can be stably modified in the absence of structural alterations of the DNA sequence has never been taken into serious consideration in the pathogenesis of cancer.

After Waddington (1940), Holliday and Pugh (1975) proposed the methylation of cytosine-guanine (CpG) dinucleotide rich regions of the DNA as the biochemical basis of epigenetic regulation of gene expression. They indicated that gene expression can be either totally stopped or increased in total absence of evident or detectable changes (mutations) of the basic DNA structure of the genes.

Other mechanisms of epigenetic gene regulation, such as covalent histone modifications (via methylation, acetylation, phosphorylation and ubiquitination) do exist. Also, non-covalent changes such as alterations in nucleosome position and histone variants and miRNAs (Sharma et al. 2010) have been proposed. However, a detailed analysis of all the possible mechanisms involved, is beyond the scope of the present chapter. The discovery that epigenetic (or functional) modulation of gene expression is dependent on the environment, is stable, and can be transmitted from one generation to the next, has opened a completely new perspective in the study of the interactions between environment and human genome and will, ultimately clarify how these interactions lead to the development of many different human diseases, including cancer. This is why one of the most recently reported definition of epigenetics is: "*an emerging branch of investigation in cancer research (but also in other fields of clinical pathology), which studies the interactions between environment and genome in determining disease*" (Jirtle and Skinner 2007).

Epigenomics has shown that environmental exposure to nutritional, chemical, and physical factors may stably modify gene expression through methylation of CpG rich DNA portions, such as the promoter regions of some housekeeping genes, transposable elements adjacent to genes with metastable epialleles, and regulatory elements of imprinted genes. In other words, the methylation state of different regions of the genome determines whether a gene is expressed or not within a cell (Dolinoy et al. 2007a, b). In the following paragraphs, it is explained that the epigenetic control mechanisms of gene expression are active in retinoblastoma, and therefore retinoblastoma can be viewed as an epigenetic rather than a genetic disease.

Retinoblastoma and Methylation of the Promoter Region of Housekeeping Genes

A housekeeping gene is a gene that is expressed at a fairly consistent level throughout the cell cycle and from tissue to tissue because it is usually involved in routine cellular metabolism (i.e., basic cell functions which are common to all different cell types). Moreover, gene expression is regulated by a given DNA region called promoter which, therefore, can be defined as a sequence of DNA needed to turn a gene on or off. Given their functions, housekeeping genes are usually expressed in almost any kind of human cells. The Rb1 gene is one of such genes and its function is to regulate cell growth by preventing cells from dividing too fast or in an uncontrolled way (Hernando et al. 2004). Its key role in the development of cancer has been highlighted in different studies.

It has been shown that in vitro methylation of the promoter region of the Rb1 gene dramatically reduces pRb expression particularly in sporadic retinoblastoma which, on the other hand, is the most commonly accepted form of non hereditary disease. Moreover, methylation of the promoter regions of housekeeping genes is a common mechanism that contributes to inactivating cell cycle control related genes (Rb1, among others) in the early stages of development of glial tumors. Interestingly, as a key gene in cell cycle control,

Rb1 has been found aberrantly methylated, alone or together with other cell cycle regulating genes in different types of cancers (Chinnam and Goodrich 2011). Finally, retinoblastoma frequently shows aberrant methylation of other genes such as HIN-1 (Shigematsu et al. 2005), HIC-1 (Rathi et al. 2003), Caspase 8 and 10 (Harada et al. 2002a), and RASSF1A (Harada et al. 2002b), all of which are commonly considered as key genes in the development of cancer in young children. Current evidence, therefore, suggests that at least DNA methylation, which is a fundamental mechanism in epigenetic regulation of gene expression, plays a major role in all Rb1-dependent cancers investigated so far, including retinoblastoma, thus confirming the epigenetic nature of the processes underlying cancer development in retinoblastoma.

Retinoblastoma, Metastable Epialleles, and Transposable Elements

Metastable epialleles are defined as gene loci that can be epigenetically modified (i.e., modified by the environment) in a variable and reversible manner, such that a distribution of phenotypes can occur from genetically identical cells. Currently, only a few genes with metastable epialleles have been identified, but experiments with these genes have produced very interesting results. For example, it has been shown that in the Agouti mice, maternal dietary exposure to phytoestrogen genistein during gestation shifts the coat-colour distribution of viable yellow offspring towards brown, and that the genistein-induced hypermethylation protects the offspring from obesity in adulthood. Moreover, genistein, when given at a level that is comparable to that consumed by humans with high soy diets, increases DNA methylation even though it is not a methyl-donating compound. The mechanism through which this is accomplished is still unknown. Taken together, these results suggest the interesting possibility that hypermethylating dietary supplements could reduce the effect of environmental toxicants that cause DNA hypomethylation, thereby protecting the epigenome from their deleterious effects (Dolinoy and Jirtle 2008).

Furthermore, regarding genes that can be epigenetically modified in a variable and reversible manner (i.e., genes with no structural DNA alterations), it is of interest to note that the phenotypic expression of retinoblastoma is not only highly variable, encompassing clinical entities such as retinoma, which is considered a precancerous lesion (Nichols et al. 2009), but can also be modulated as if it would depend on variable environmental exposures. At this regard, while it is known that both retinoblastoma and neuroblastoma show the highest rate of spontaneous remission, cases are reported in which a spontaneously regressed retinoblastoma underwent a new malignant transformation (Eagle et al. 1989).

Interestingly, the observation of spontaneously regressed retinoblastoma, dates back to 1956 (Steward et al. 1956), more than a decade before the formulation of the mutational two hit model, and it still represents a theoretical challenge to it. In fact, the mutational model gives no clear cut explanation of how a structurally modified DNA could lead to a whole array of cancer phenotypes, including the spontaneous return to normality, unless the concept of penetrance is adopted. Penetrance, however, is a rather fuzzy and undefined concept which does not correspond to any known biochemical/molecular mechanisms, and is presently viewed as a pure stochastic (but still unexplained) fluctuation in gene expression.

Epigenetics, on the contrary, by looking at gene expression as the result of the functional interaction between genes and the environment (through gene methylation and other mechanisms), acknowledges the possibility that the resulting phenotype could be modulated and consequently exhibit different degrees of variability and plasticity. Variations in phenotypic expression, on the other hand, can also be explained, according to epigenetics, by the presence of transposable elements (Transposons) within the genomic DNA.

Transposons are parasitic, repetitive mobile elements dispersed throughout the mammalian genome. They are remnants of ancestral infections which became fixed in the germline DNA and subsequently increased in copy number. The sequencing of the human genome has shown that

transposons comprise roughly 45% of our genome, and most transposable elements are silenced by CpG methylation, the same biochemical process involved in epigenetic gene regulation. The epigenetic state of a subset of transposable elements is metastable. In other words, these mobile elements are variably expressed in genetically identical individuals due to epigenetic modifications occurring during the early development (Dolinoy et al. 2006). In contrast with other regions of the human mammalian genome, the epigenetic changes occurring at the insertion site of transposable elements are a stochastic event which not only causes individual variation but also accounts for epigenetic cellular mosaicism.

Therefore, given their role in silencing genes and their variability within the same individual, transposons are responsible for both interindividual and intraindividual variations in phenotypic expression of the same genes within different cells of the same organism, thus leading to mosaicism.

Retinoblastoma is not a single cancer phenotype; beyond the above mentioned benign form of the disease, called retinoma, and spontaneously regressed retinoblastoma, other clinical phenotypes do exist, such as diffuse infiltratin retinoblastoma, unilateral and bilateral retinoblastoma, and trilateral retinoblastoma, in which a bilateral disease is associated with intracranial tumors involving the pineal region.

Moreover, somatic mosaicism for Rb1 gene mutations is common in retinoblastoma, in which a high proportion of cases represent *de novo* mutations (Sippel et al. 1998), it can be found in both affected patients and their unaffected parents (Rushlow et al. 2009), and it can involve both the paternal and maternal germline (Barbosa et al. 2008). Both phenotypic variation and cellular mosaicism, although quite common in retinoblastoma, are unexplainable in the light of the mutational model which assumes that when the first mutation is inherited through the germline, all the somatic and germ cells of the individual must carry that mutation (and its phenotypic effects).

On the contrary, by adopting the epigenetic model, phenotypic variation in the clinical expression of the disease is easily explained by the variable exposure of the fetus to environmental toxicants which, in turn, determine the degree of hypomethylation of different key genes. Within this conceptual framework, mosaicism can be viewed as the result of the interaction between the environment and the transposable elements of the genome.

Retinoblastoma and Imprinting

Imprinting is defined as a non-Mendelian, germline inherited epigenetic form of gene regulation involving heritable DNA methylation and histone modification. The human genome is subject to imprinting which represents the consequence of epigenetic inactivation (through methylation) of different genes in either the male or female gametes, so that in the resulting zygote they complement each other, and the normal embryo development proceeds. On the contrary, two male or female derived genomes are incompatible with a normal growth of the embryo or fetus.

Because imprinted genes are epigenetically modified in both the male and female gametes, the expression of different genes in the zygote, embryo, and fetus, derived from the fusion of the two, will depend on the parental environment in which both gametes (male and female) have grown and differentiate. A parentally imprinted gene in one of the gametes is not expressed; therefore the resulting zygote will be functionally haploid, (i.e., only one copy of the gene is functioning) and the consequences may be severe. In Knudson's (1971) hypothesis, inheriting an imprinted Rb1 gene means that one copy of the gene is already functionally inactivated (first hit) and only a single event is further requested for both copies to be inactivated.

Abnormal expression of imprinted genes during development may result in severe pediatric disorders such as Prader-Willi syndrome (PWS), Angelman syndrome (AS), and Beckwith-Wiedemann syndrome (BWS), where epigenetic alterations have an important contributory or causative role. Moreover, imprinted gene dysregulation can also occur in somatic cells, either by epigenetic or genetic mutations, causing cancer; therefore, with specific reference to cancer development, the inheritance of an epigenetically imprinted gene can be equated, as previously

mentioned, to Knudson's first hit, although in this case no structural DNA alteration is involved.

Given all the above findings, the fact that the Rb1 gene can be imprinted in retinoblastoma, may add important clues to the probable epigenetic nature of the disease. In this regard it is important to mention that, according to the most recent evidence with only a few exceptions, hypermethylation of CpG islands is acknowledged as the most relevant epigenetic inactivation mechanism for tumor suppressor genes, representing a major contributor to neoplastic transformation (Feinberg 2007). Accordingly, recent data show that Rb1 gene is imprinted in retinoblastoma with a shift of expression in favor of the maternal allele (Buiting et al. 2010), while previous reports had already significantly shown that hypermethylation with loss of function occurs in 18% of sporadic retinoblastoma (Greger et al. 1989). Imprinting is, by definition, a process by which human genes are functionally inactivated and its detection in retinoblastoma represents another argument against the mutational model, which assumes that gene expression can be altered only in the presence of structural DNA modifications, and in favor of the epigenetic one.

Retinoblastoma: Epigenetics Rather Than Inheritance

As we have seen, with the only exception of familial retinoblastoma (8–10% of all cases), in which the disease is found in the proband and in some of his/her relatives, hereditary retinoblastoma is (according to the "two hit" model) a sporadic retinoblastoma (since no other affected family member can be identified) determined by a germ line mutation. In fact, transgenerational inheritance involves the transmission of biological traits to subsequent generations through the germ line.

Epigenetic alterations of the genome, as it has been shown, can be inherited (transmitted from one generation to another), and because environmental factors can alter the epigenome, their ability to influence the disease risk might involve epigenetic transgenerational inheritance. We can speak of transgenerational inheritance of environmental effects, when the effects themselves are maintained and detectable in at least F3 (third) generation, where F0 is the gestating mother exposed, F1 is the embryo and F2 are the embryo's germ cells. It is clear that, when the gestating female (F0) is exposed to toxicants, both F1 (embryo) and F2 (embryo's germ cells) are also directly exposed. Therefore, disease phenotypes in the F1 and F2 generations might still be due to the direct exposure of F0, F1, and F2 to environmental toxicants.

This line of reasoning alone would be more than sufficient to demonstrate that hereditary (bilateral) retinoblastoma is not a true hereditary disease, but an epigenetic disorder most probably linked to the gestational exposure to environmental harmful agents. Indeed, clinical reports on retinoblastoma are almost invariably limited to retinoblastoma patients (F1) and very rarely to their first generation descendants (F2), while a retinoblastoma occurring in the F3 generation, according to the mutational model proposed by Knudson, belongs to the "familial" group. Notwithstanding the above mentioned considerations, epigenetic alterations of gene expression have been reported up to F4 generations (Franklin and Mansuy 2010), thus demonstrating that the environment may stably imprint its effects on the genome, mimicking a "genetic disease" even though no mutations are detectable, as reported in many cases of "hereditary" retinoblastoma. Moreover, it has been shown that a poor diet and infectious diseases are presently considered risk factors for the development of retinoblastoma in less affluent populations throughout the world, but even radiation may play an important role.

Finally, of extreme interest is the case of the American-Indian Navajo population which has represented the main working force in the uranium mines of South-West America, from World War II until 1971 (Brugge and Goble 2002), and still live in villages located near the mines. The incidence of retinoblastoma among these populations is more than twice when compared to other world populations (Berkow and Fleshman 1983). More importantly, the incidence seems to arise 20 times in the offspring or mothers who had lived in the village of Seascale (UK), situated in

the vicinity of a nuclear reprocessing plant, and best known in epidemiological circles for its longstanding high incidence of malignant diseases in young people (Stiller 1993). All the reported data represent a clear demonstration of the role of environmental factors in the genesis and development of retinoblastoma and, as a consequence, the role of epigenetics rather than genetics in the determinism of this eye tumor.

Concluding Remarks

Epigenetics can be defined as the study of changes that influence the phenotype without causing alterations of the genotype. It involves changes in the properties of a cell, which are inherited in the absence of structural changes of its DNA. Although epigenetic regulation of gene expression is the mechanism through which the extraordinary variety of specialized cells of the body differentiate starting from a single undifferentiated ancestor, the relevance of epigenetic factors in disease in humans was first detected only in 1983 when Feinberg and Vogelstein found that gene hypomethylation could distinguish some human cancers from their normal counterparts.

Presently, deregulation of gene expression is widely considered a hallmark of cancer, and although genetic lesions have been the focus of cancer research for many years, as in the case of retinoblastoma, it has become increasingly recognized that aberrant epigenetic modifications play major roles in cancer development. This represents a great revolution and advancement with respect to the understanding of the pathogenesis of cancer we have gained so far, by applying the concepts and principles of Mendelian (or classic) genetics. In fact, Mendelian genetics has been proven largely insufficient to explain the diversity of phenotypes within a population, nor it explains how, despite their identical DNA sequences, monozygotic twins or cloned animals can have different phenotypes and different disease susceptibilities (Taby and Issa 2010; Costa 2010).

On this line of reasoning, we have tried to show herein and elsewhere that the mutational model is largely inadequate to explain the variegated phenotypic expression of retinoblastoma. Also and more importantly, there is an increasing agreement among researchers worldwide that the mutational ("two hit") model is outdated and that another paradigm has to be adopted for a better understanding of the pathogenesis of retinoblastoma. Epigenetics explains the inconsistencies of the mutational ("two hit") model as applied to the pathogenesis of retinoblastoma, but it also has other important advantages which promise to revolutionize the fields of both ophthalmology and oncology.

The potential reversibility of epigenetic states offers exciting opportunities for novel cancer drugs that can restore epigenetically silenced cancer genes. DNA methyltransferases and histone deacetylases (Poulaki et al. 2009) are the two major drug targets for epigenetic inhibition to date, although others are expected to be added in the near future. Epigenetic changes in cancer cells not only provide novel targets for drug therapy but also offer unique prospects for cancer diagnostics through the study of gene expression, the evaluation of histone modifications, chromatin protein composition, and the analysis of the promoter DNA methylation status. Finally, and more importantly, by shifting the focus on the environment and the complex interactions between the environmental regulation of gene expression and the genome, rather than on the genes themselves, epigenetics stresses the importance of cancer prevention and the changes of most of our common lifestyles, including diet and behavior.

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