Retinoblastoma as an Epigenetic Disease: A Proposal

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ABSTRACT

The aim of the present review 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, as applied to the interpretation of clinical, epidemiological and biological data in retinoblastoma, can not only explain the inconsistencies of the mutational ("two hit") model, but also opens new outstanding scenarios in the fields of diagnosis, treatment and prevention of this eye tumour. This review is both a collection of literature data arguing against the role of the mutational ("two hit") model in the genesis of retinoblastoma, and a documented evaluation of how the Epigenetic, rather than the genetic model fit the variegated phenotypic expression of the disease. 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, among others, all play a major role in conditioning the degree of DNA methylation in embryos and foetuses during pregnancy, thus leading to stable, functional alterations of the genome, which, on the other hand, can be also transmitted from generation to generation, 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.

Keywords: Retinoblastoma, Epigenetics, Genome Imprinting, DNA Methylation, Histone Acetylation/Deacetylation

1. Introduction

Although rare, retinoblastoma is the most common eye tumour affecting children under the age of five years [1]. In 1971, Alfred Knudson [2], by reviewing a series of 48 cases, formulated a hypothesis according to which this tumour may be determined by the loss or inactivation of both copies of a single gene. The presumptive gene responsible for tumour development in retinoblastoma was later identified and indicated as “Rb1”, and its complete DNA sequence fully described and characterized [3]. Since the beginning, retinoblastoma has been considered a hereditary tumour, and this view has been further reinforced by DNA investigations (polymorphism and conformational DNA analysis followed by DNA sequencing) demonstrating that retinal tumours usually bear mutations on both copies of the Rb1 gene [4], thus apparently confirming the mechanisms hypothesized by Knudson in 1971, and allowing the identification a new class of “cancer genes” defined “tumour suppressor genes” [5]. 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 determines the clinical phenotype of the disease. Namely, when both the first and second mutations involve the somatic cells, the individual will develop a tumour 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) [6,7]. This fundamental diversity in the pathogenesis of the tumour represents the basic distinction between two different clinical retinoblastoma phenotypes; e.g.:

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 tumour focus on the retina of the affected eye, and;

2) bilateral retinoblastoma (30% - 35% of all cases),
which is hereditary, occurs at an earlier age, involves both eyes, and commonly presents with multiple tumour foci in the retina of at least one eye [2].

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 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 [8-10]. Nevertheless, neither the mutational nor the aneuploidy model seem to be able to explain the variegated phenotypic expression of retinoblastoma.

We propose herein, that the pathogenesis and clinical expression of retinoblastoma can be better understood and explained if the principles of Epigenetics are applied to the study of this eye tumour affecting young children.

2. Purpose

While the literature concerning the genetic origin of retinoblastoma has flourished in the last four decades, and the idea that this tumour 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 [8-10] and in retinoblastoma in particular (see below). The most relevant arguments raised against the mutational model are reported in Table 1 together with the related references, and although they have been abundantly discussed elsewhere (see References 8-32, in Table 1), and the role of aneuploidy itself, in the genesis of cancer, is now well established, no clear cut “alternative” has been proposed, so far, which consistently explains how and why retinoblastoma develops in human beings.

The main purpose of the present review is to show that according to the currently available evidence, the concept of “epigenetic” gene regulation offers a totally new and consistent explanation of both aetiology 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.

3. What Is Epigenetics

“Epigenetics” is a term coined in 1940 by the developmental biologist Conrad Waddington [20] to define “…the interactions of genes with their environment, which bring the phenotype into being”. Literally, “epi” —genetics means “above genetics” and the term properly designates events which modify gene expression without modifying the structure of the genes themselves [33].

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, Hollyday and Plough proposed, among others, the methylation of Cytosine—Guanine (CpG) dinucleotide rich regions of the DNA [21], as the biochemical basis of epigenetic regulation of gene expression, showing 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 do exist, such as histone acetylation/deacetylation, and micro RNAs, but a detailed analysis of all the possible mechanisms involved, is beyond the scope of the present review.

However, 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, most probably, 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 definitions 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” [34].

Epigenomics has shown that environmental exposure to nutritional, chemical, and physical factors may stably modify gene expression through, among others, 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.

The purpose of the next paragraphs is to show 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 [35,36].
Table 1. Arguments against the mutational ('two hit') model in the genesis of retinoblastoma (and related references).

<table>
<thead>
<tr>
<th>CONSEQUENCES OF THE &quot;TWO HIT&quot; (MUTATIONAL) MODEL</th>
<th>ARGUMENTS AGAINST THE &quot;TWO HIT&quot;</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer depends on gene mutations</td>
<td>Some carcinogens are not mutagens</td>
<td>8 - 15</td>
</tr>
<tr>
<td></td>
<td>No cancer genes can transform normal into cancerous cells</td>
<td>8 - 15</td>
</tr>
<tr>
<td></td>
<td>Cancer should show an unrealistic high rate of mutation</td>
<td>8 - 15</td>
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<tr>
<td></td>
<td>The latency of tumour development after exposure to carcinogens is not explained</td>
<td>8 - 15</td>
</tr>
<tr>
<td></td>
<td>Aneuploidy rather than gene mutations is responsible for cancer development and progression</td>
<td>8 - 15</td>
</tr>
<tr>
<td>Unilateral Rb occurs later than bilateral Rb</td>
<td>There is no difference in the &quot;mean&quot; age at diagnosis between unilateral and bilateral Rb</td>
<td>16 - 19</td>
</tr>
<tr>
<td>Bilateral Rb is hereditary and unilateral Rb is sporadic</td>
<td>Bilateral Rb can occur sporadically and unilateral Rb can be inherited</td>
<td>16 - 19</td>
</tr>
<tr>
<td>Familial Rb should always show the bilateral disease phenotype</td>
<td>As many as 23% of familial Rb are unilaterally affected</td>
<td>16 - 19</td>
</tr>
<tr>
<td>Constitutional mutations of the Rb1 gene are associated with the bilateral phenotype</td>
<td>Only 8 out of 16 constitutional 13q deletions show the bilateral phenotype</td>
<td>18, 19</td>
</tr>
<tr>
<td>Gene expression is modified by mutations in the DNA sequence</td>
<td>Gene expression in human and animal tissues is modulated by methylation/acetylation (&quot;epigenetics&quot;) and no mutations are required</td>
<td>20, 21, 22</td>
</tr>
<tr>
<td>Rb1 gene mutation should always be present in Rb</td>
<td>Finding a mutation is not always possible in Rb</td>
<td>23</td>
</tr>
<tr>
<td>Rb1 mutations should be present only in Rb</td>
<td>Rb1 gene mutations can be found in almost any type of cancer</td>
<td>24, 25, 26, 27</td>
</tr>
<tr>
<td>The incidence of unilateral and bilateral Rb are the same all over the worlds</td>
<td>The incidence of unilateral Rb is highly variable in different countries</td>
<td>28, 29, 30</td>
</tr>
<tr>
<td>The cell of origin in Rb is an undifferentiated retinoblast</td>
<td>The cell of origin in Rb is well differentiated and belongs to the horizontal cell layer of the retina</td>
<td>31, 32</td>
</tr>
</tbody>
</table>

4. 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 [37]. Its key role in the development of cancer has been highlighted by different Authors [24-27,38,39].

As it has been shown, in vitro methylation of the promoter region of the Rb1 gene, dramatically reduces pRb expression [40] particularly in sporadic retinoblastoma [41] 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 tumours [42]. 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 cancer; among others: epithelial odontogenic tumours [43], bladder cancer [44], radon induced rat lung tumors [45], follicular lymphoma [46], SV40 associated B cell lymphoma [47], gastric carcinoma [48], neuroblastoma [49], and pituitary adenoma [50].

Furthermore, aberrant methylation of the Rb1 gene promoter region has been found in 20% of embryos with chromosomal mosaicism that die in utero, thus suggesting that aberrant epigenetic genomic modifications at early stages of human embryonic development, can be one of the mechanisms promoting genomic instability (realized in the form of mosaic abnormalities of the karyotype), that are incompatible with the normal course of embryogenesis [51].

Finally, retinoblastoma frequently shows aberrant methylation of other genes such as HIN-1 [52], HIC-1 [53], Caspase 8 and 10 [54], and RASSF1A [55], all of which are commonly considered 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.

5. Retinoblastoma, Metastable Epialleles, and Transposable Genetic 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, Jirtle and coll. have 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 [56].

Furthermore, talking about 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, for example, “retinoma”, which is considered a precancerous lesion [57], but can also be “modulated” as if they would depend on variable environmental exposures. At this regard it must be outlined that the literature refers of cases of spontaneously regressed retinoblastoma [58,59], which underwent a new malignant transformation [60].

Interestingly, the observation of spontaneously regressed retinoblastoma, dates back to 1956 [61], more than a decade before the formulation of the mutational, “two hit” model, and it still represents a theoretical challenge to it. As a matter of fact, the mutational model has no clear cut explanation of how a structurally modified DNA leading to a cancer phenotype may spontaneously revert to normality, although the concept of “penetrance” has been largely abused, for this purpose, by the supporters of the mutational model. “Penetrance” is, in fact, a rather fuzzy 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 [62].

Epigenetics, on the contrary, by looking at gene expression as the result of a 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 be also 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 [63]. The sequencing of the human genome has shown that Transposons comprise roughly 45% of our genome [64], and most transposable elements are silenced by CpG methylation [65], 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 occurred during the early development [66]. 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 of both inter-individual and intra-individual variations in phenotypic expression of the same genes within different cells of the same organism, thus leading to mosaicism.

Retinoblastoma is a cancer showing phenotypic variation, and a number of “variant” phenotypes of the disease are currently known; among others: retinoma (a form of pre-retinoblastoma) [57,67], spontaneously regressed retinoblastoma [58-61], diffuse infiltrating retinoblastoma [68,69], unilateral and bilateral retinoblastoma [2,4], and also “trilateral retinoblastoma” [70,71].

Moreover, somatic mosaicism for Rb1 gene mutations:
1) is common in retinoblastoma, in which a high proportion of cases represent de novo mutations [72];
2) can be found in either affected patients and their unaffected parents [73], and
3) can involves both the paternal and maternal germ-
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, finding that the Rb1 gene can be imprinted in retinoblastoma may add important clues to the probable epigenetic nature of the disease. At 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 a major epigenetic inactivation mechanism for tumour suppressor genes, representing a major contributor to neoplastic transformation [78]. Accordingly, recent data show that Rb1 gene is imprinted in retinoblastoma with a shift of expression in favour of the maternal allele [58,79], while previous reports had already significantly shown that hypermethylation with loss of function occurs in 18% of sporadic retinoblastoma [80].

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 instead assumes that gene expression can be altered only by structural DNA modifications.

7. 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 germline mutation.

As a matter of fact, transgenerational inheritance involves the transmission of biological traits to subsequent generations through the germline. Epigenetic alterations of the genome, as it has been shown, can be inherited (transmitted from one generation to another), and since 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 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 [33-36].

This line of reasoning alone would be more than sufficient to demonstrate that hereditary (bilateral) retino-
blastoma, is not a true hereditary disease, but an epigenetic disorder most probably linked to the gestational exposure to environmental harmful agents. As a matter of fact, 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 all the above mentioned considerations, epigenetic alterations of gene expression have been reported in F3 generations [81-85], thus demonstrating that the environment may stably imprint its effects on the genome, thus mimicking a “genetic disease” even if no mutations are detectable, as reported in many cases of “hereditary” retinoblastoma [23].

As we have seen, 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. Of extreme interest, at this regard, is the case of the American-Indian Navajo population who has represented Of extreme interest, at this regard, is the case of the American-Indian Navajo population who has represented.

The incidence of retinoblastoma among these populations is more than twice when compared to other world populations [87].

More importantly, the incidence seem to arise to twenty times in the village of Seascale, 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 [88].

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 tumour.

8. 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 that are inherited in the absence of structural changes of its DNA [89]. 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 [90] found that gene hypomethylation could distinguish some human cancers from their normal counterparts [91].

Today, 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 [92,93].

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 [94].

On this line of reasoning, we have been able to show herein and elsewhere [16-19] that the mutational model is largely inadequate to explain the variegated phenotypic expression of retinoblastoma, and there is an increasing agreement among researchers worldwide that the mutational (“two hit”) model is outdated and another paradigm has to be adopted for a better understanding of the pathogenesis of retinoblastoma [95].

Epigenetics allows to explain the inconsistencies of the mutational (“two hit”) model as applied to the pathogenesis of retinoblastoma (see Table 1), but it also has other important advantages which promise to revolutionize the field of both ophthalmology and oncology [96]. We refer herein to the following:

1) 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 are the two major drug targets for epigenetic inhibition to date, although others are expected to be added in the near future [97-100].

2) Epigenetic changes in cancer cells not only provide novel targets for drug therapy but also offer unique prospects for cancer diagnostics [100], through the study of gene expression, the evaluation of histone modifications and chromatin protein composition, and also the analysis of the promoter DNA methylation status;

3) 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 [101] and the changes of our lifestyles, including diet and behaviour [53,63,66,102,103].

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