The Epigenetic Origin of Retinoblastoma

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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 study of clinical, epidemiological and biological data concerning the disease. As an emerging new scientific approach linking the genome to the environment, epigenetics, can not only explain the inconsistencies of the mutational (‘two hit’) model in the genesis of retinoblastoma, but also open new outstanding scenarios in the fields of diagnosis, treatment and prevention of this eye tumour and cancer in general. After more than four decades of predominance of the ‘genetic’ theory, this review represents, to the authors’ knowledge, the first attempt to look at retinoblastoma from the point of view of epigenetics. The epigenetic model in the genesis of retinoblastoma, proposed herein, emphasises the role of environment and its interactions with the genome, in generating retinoblastoma in young children. Environmental toxicants, including, among others, radiations, wrong diets and infectious diseases, all play a major role in conditioning the degree of DNA methylation (one of the leading mechanisms of epigenetic gene regulation) in embryos and foetuses during pregnancy, thus leading to stable, functional alterations of the genome, which can 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.

Keywords
Retinoblastoma, epigenetics, DNA methylation, histone acetylation/deacetylation

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About ‘Causation’ in Retinoblastoma
Since the formulation of the ‘two hit’ hypothesis in the genesis of retinoblastoma (Rb),1 there has been widespread agreement, among researchers in the field of ocular oncology, that this eye tumour is determined by the loss, mutation or inactivation of both copies of one and a single gene. The gene was later identified and denominated Rb1,2 and this discovery opened the “hunting” to the inactivating mutation/s ‘causing’ Rb.

The ‘causative’ role of the Rb1 gene mutations in Rb, is no longer a matter of discussion, among the vast majority of researchers worldwide, as it is clearly stated in both the most recent scientific papers3–10 and institutional information11–15 concerning the disease. However, it is widely recognised that the ‘cause’ of most (if not all) cancers is still unknown and the ‘mutation theory’ only tells us about a possible pathogenetic mechanism (the alteration of the DNA structure of the genome) presumably involved in the genesis of Rb. Nevertheless, the question about what ultimately ‘causes’ the mutations, which lead to cancer development in Rb (and in cancer, in general), remains unanswered.

Moreover, the role of gene mutations in the genesis of cancer, has been largely questioned by scientists who have remarked, among others, the fact that carcinogens do exist, as pesticides,16 phenobarbital and clofibrate,17 tumour promoters (1,4-dichlorobenzene), endocrine-modifiers (17β-estradiol), receptor-mediators (2,3,7,8-tetrachlorodibenzo-p-dioxin), immune suppressants (cyclosporine) or inducers of tissue-specific toxicity and inflammatory responses (metals such as arsenic and beryllium), among others, which are not genotoxic and therefore must be assumed to induce cancer without producing gene mutations.18–22 These non-genotoxic (or ‘epigenetic’) carcinogens are well-known chemicals which induce cancer without modifying the DNA structure and nucleotide sequence of the human genome, and represent the strongest argument against the role of gene mutation as the only pathogenetic mechanism leading to cancer.

However, the arguments against the role of gene mutations in cancer abound in the literature, even though a detailed discussion of this matter goes beyond the scope of the present review.

In summary, the awareness that gene mutations are only part of the process through which normal cells become cancerous, the other being represented by epigenetic (and ‘non-structural’) gene modifications, should suggest more caution in attributing a causative role to a mutation affecting one and a single gene, as it has been the case of Rb, after the first formulation of the two hit hypothesis, proposed by Knudson in 1971.

Epigenetics – The New Frontier in Cancer
The term ‘Epigenetics’ was coined in 1940 by Conrad Waddington to designate, “… the interactions of genes with their environment which bring the phenotype into being”,23,24 thus clearly indicating that
explain how tumours develop and grow in human beings: it is not disease. But, as we have seen in the previous paragraphs, the formulation of the two hit hypothesis, 31 Rb has, for a long time, represented an exception. As reported above, mutations are not the only mechanism through which the environment transforms normal cell into cancerous ones, the other being represented by non-structural modifications of gene expression.29–32 These modifications are realised through different mechanism, including, among others, DNA methylation, covalent histone modifications, nucleosome positioning and histone variants, and microRNA, all of which have been already investigated in great detail.29,33

Literally, ‘epi’ means ‘above’ and therefore the term epigenetics (i.e. above genetics) designates events which modify gene expression without modifying the structure of the genes themselves.29–32 Once again, from the standpoint of cancer research, this peculiar aspect of epigenetics reminds us that gene mutations are only one of the possible pathogenetic mechanisms through which the environment transforms normal cell into cancerous ones, the other being represented by non-structural modifications of gene expression.29–32 These modifications are realised through different mechanism, including, among others, DNA methylation, covalent histone modifications, nucleosome positioning and histone variants, and microRNA, all of which have been already investigated in great detail.29–32

For the purposes of the present discussion, it will be important to mention that through hypermethylation of certain portions of the DNA, the function of entire genes (including tumour suppressor genes) can be suppressed,39 and through hypomethylation, imprinted and/or ‘silenced’, as well as growth promoting genes can be activated.39 In other words, the DNA methylation ‘landscape’ of cancer is profoundly distorted if compared to that of the normal cell, even if the DNA structure itself is intact.39

Furthermore, the work in the field of epigenetics, has shown that the inheritance of DNA sequences is not the only mechanism underlying the transgenerational transmission of physical, behavioural, and emotional traits in mammals.43 An epigenetically modified DNA methylation landscape, is stable and can be transmitted from one generation to the next.

More importantly, the ‘epigenome’ (i.e. a genome-wide map of epigenetic modifications)44 can be modulated by a variety of environmental factors, including chemicals, nutrition, and early environment, as well as by ageing. The epigenome, therefore, provides an important interface between genes and the environment and may be viewed as a potential mechanism for a rapid form of environmentally driven transgenerational adaptation.45–49

Finally, a growing body of evidence suggests that epigenetic gene regulation, including DNA methylation and histone modifications, are not only modulated by the environment, but may play a role in the foetal basis of adult disease.49–53

Epigenetics – Relevance to Retinoblastoma

Although cancer is commonly viewed as a disease linked to the environment,8 Rb has, for a long time, represented an exception. As a matter of fact, since the formulation of the two hit hypothesis, cancer is generally viewed as a genetic, rather than an epigenetic disease. But, as we have seen in the previous paragraphs, the gene mutation in itself is neither necessary nor sufficient to explain how tumours develop and grow in human beings: it is not necessary, because epigenetics tells us that gene expression can be stably modified by the environment, in the total absence of structural changes (mutations) of the genomic DNA, and it is not sufficient because:

- A – cancer is a multistep process;44
- B – multiple and more complex phenomena (such as, for example, aneuploidy) must take place before cancerous transformation and progression take place;50,51,54
- C – the mutation in itself must have some underlying, though yet unknown, cause/s, and as such, cannot be considered causative, in cancer.

In a series of previously published papers,55–59 the authors have argued against the mutation model in Rb, by highlighting the discordance between some of the predictions made by the two hit model and the available clinical data, but did not propose a clear cut alternative to the mutation theory.

Herein the authors propose a few arguments, which in the authors’ opinion, demonstrate that epigenetics (and environment) play a crucial role in the genesis of Rb.

The Methylation Landscape of Retinoblastoma

Aberrant retinoblastoma 1 (pRb) pathway activity, resulting from defects in pRb (the protein produced by the Rb1 gene), cyclin-dependent kinase inhibitor 2A (p16 INK4a), cyclin D1 (CCND1), or cyclin-dependent kinase 4 (CDK4), is observed in the majority of human sporadic cancers.59–62 This pathway is commonly altered early in cancer development, indicating an ability to predispose cells to tumourigenesis.60

Given its ubiquitous expression in human tissues and its function of cell cycle control,64–66 the Rb1 gene fits the requisites to be included in the group of so called ‘housekeeping’ (HK) genes.63,65

As reported above, mutations are not the only mechanism through which the Rb1 gene is inactivated in human cancer; namely, in vitro methylation of the promoter region of the Rb1 gene, has been shown to dramatically reduce pRb expression67 particularly in sporadic Rb which, on the other hand, is the most commonly known form of nonhereditary disease.

Moreover, methylation of the promoter regions of HK 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.68

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, such as among others; glioblastoma70 and other nervous system tumours,71 epithelial odontogenic tumours,72 bladder cancer,73 radon-induced rat lung tumours,74 follicular lymphoma,75 SV40 associated B cell lymphoma,76 gastric carcinoma,77 neuroblastoma,82 pituitary adenoma82 and malignant fibrous histiocytoma.83 Finally, Rb frequently shows aberrant methylation of other genes such as HIN-1,84 HIC-1,84 Caspase eight and 1085 and RASSF1A,86 all of which are commonly considered key genes in the development of cancer in young children. What does all this mean?
There is little doubt that DNA methylation, a key mechanism in epigenetic gene modulation, together with other mechanisms, such as histone acetylation, is involved in the genesis of cancer and the finding of an altered methylation landscape of the Rb1 and other important genes, in Rb, represents a clear indication of the role of epigenetics and environmental influences, on the genesis of this tumour.

Phenotypic ‘Plasticity’ and Retinoblastoma Epigenetics

Rb is not a single clinical entity, but rather a group of neoplastic affections of the retina showing various degrees of involvement of one or both eyes and also a sometimes extraordinarily different clinical behaviour.

As an example, ‘retinoma’ is considered a benign form of Rb or, in other words, a benign proliferation of an early retinal cell, caused, according to the mutational model, by the same mutation/s of the Rb1 gene which is also responsible for the full blown disease.69

Although different hypotheses have been made to explain the ‘unreliable’ expression of the mutation affecting the Rb1 gene (including, among others, the fuzzy concept of ‘low penetrance’), advances in Rb genetic research have demonstrated that even severe inherited mutations segregating in families with bilateral Rb patients, can cause retinoma.93

But, in the light of the genetic theory, the ‘spontaneously regressed’ Rb represents an even more challenging puzzle. The complete, spontaneous tumour necrosis leading to ‘cure’, is a well described phenomenon which is said to occur more frequently in neuroblastoma and Rb than in any other known malignant cancer.94 The observation of spontaneous regression of retinoblastoma, dates back to 1956,95 (i.e. more than a decade before the formulation of the mutational (‘two hit’) model). Further evidence has shown that ‘spontaneously regressed retinoblastoma’ is a rather common event and actually malignant transformation of a spontaneously regressed tumour is also reported,96 thus indicating the inherent plasticity of the cancer phenotype in Rb.

Other clinical phenotypes of the disease include: diffuse infiltrating Rb, unilateral and bilateral Rb, unilateral and bilateral Rb, and also ‘trilateral retinoblastoma’, in which a bilateral Rb is associated with intracranial tumours involving the pineal region.

How a mutation affecting one and a single gene (Rb1) could produce such a variety of clinical phenotypes, remains unexplained, on the basis of the mutational theory, unless the conceptual trick of ‘penetrance’ is used.

Penetrance is defined as “the proportion of individuals of a particular genotype that express its phenotypic effect in a given environment”,111 as remarked by Opitz111 and Lenz, before him, “When the frequency of a trait in close relatives of probands does not agree very well with the assumption of [segregation] of a dominant gene, the discrepancy is addressed with the word ‘penetrance’”. However this word does not convey any special physiological or genetic explanation. It is nothing more than a verbal circumscription of the discrepancy between observation and assumption.”

Although penetrance has always been matter of discussion, in Rb, and various speculative hypotheses have been proposed for low penetrance Rb, penetrance in itself is a rather 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.112

A more appropriate term, to describe the variable expression of a given cancer phenotype, is that of ‘phenotypic plasticity’, usually defined as a property of individual genotypes to produce different phenotypes when exposed to different environmental conditions.113

In Rb, as we have seen, the same (presumed) mutation is responsible for sometimes extremely different clinical phenotypes and the reversibility of a pathological into normal phenotype is also a rather common event. This behaviour can be ascribed to phenotypic plasticity, which is not explained by the genetic theory, but is, instead, an integral part of the epigenetic theory of cancer.114

As a matter of fact, epigenetics, by linking the variable and potentially reversible effects of the environment to disease in general,115 and cancer in particular,116,117 represents the only possible explanation of phenotypic variation in health and disease and also offers important new tools to understanding how the interaction between the environment and the genome, leads to disease.42

Trans Generational Inheritance of Epigenetic (Acquired) Alterations and Retinoblastoma

There is cumulating evidence that chemical toxicants have detrimental effects not only on individuals directly exposed to the toxicant but also on their offspring.

Diethylstilbestrol, a synthetic non-steroidal oestrogen prescribed in the 1970s to prevent miscarriage in women with prior history, helped pregnancies to go to term, but also induced severe developmental abnormalities and increased the risk for breast cancer and a rare form of adenocarcinoma in girls whose mothers were exposed to the drug during the first trimester of pregnancy;118 in other words, the risk of cancer appeared to be transmitted to the following generation.

Similar, transgenerational effects were obtained in mice, in which perinatal exposure to the drug, produced abnormalities in uterine development and uterine cancer in first and second generations. These abnormalities were suggested to result from aberrant DNA methylation of a gene controlling normal uterine development.119

Vinclozolin, a fungicide commonly used for agricultural fruit crops, increases the incidence rate of tumour formation in ageing males exposed to it prenatally (F1) and their offspring (F2–F4).120

But the transgenerational extension of the detrimental effects of the environment on the epigenome (basically the DNA methylation landscape), also seem to apply to diet (either low protein or high fat), and poor early environment (childhood trauma or abuse), thus showing that the environment can stably impress its effects on the genome of several generations in the offspring of the first individual exposed; changes in the epigenome, established by the environment during early development, may be reversed by environmental stimuli even in adults, emphasising the plasticity of DNA methylation (and cancer phenotypes) in the adult.121

Regarding Rb, it is known that, with the only exception of the familial group (8–10 % of all cases), in which the disease is found in
the ‘proband’ and in some of his/her relatives, the hereditary (bilateral) form of the disease is, according to the two hit model, a sporadic Rb (since no other affected family member can be identified) determined by a germline mutation.

In terms of epigenetic gene regulation therefore, Rb is still to be viewed as an inherited disease, but the inheritance concerns, in this case, the transmission of acquired environmental negative effects on the genome, through several generations.

More importantly, transgenerational inheritance of environmental effects, is maintained and detectable in at least the F3 generation, where F0 is the gestating mother exposed, F1 is the embryo and F2 are the embryo’s germ cells. In other words, 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.29–32,52,53

Regarding the transgenerational transmission of environmental effects on the genome, in Rb, it is extremely significant that clinical reports on the disease are almost invariably limited to retinoblastoma patients (F1) and 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. All this implies that the (presumed) transgenerational effects of a mutation on the expression of cancer phenotype, in retinoblastoma, are indistinguishable from those produced on the epigenome by the environment.

In conclusion, Epigenetics tells us that the ‘inheritance’ of the cancer phenotype, does not necessarily depend on mutational events affecting one or more genes, but may rather be the result of more complex and still incompletely understood (functional) interactions between DNA and the environment.

Retinoblastoma, Inheritance and Environment – The Navajo Paradigm

The incidence of hereditary (bilateral) Rb is rather invariant throughout the world, but that of the sporadic form of the disease, shows wide variations, being more frequent in less industrialised countries and in less affluent populations, thus indicating that the appearance of a sporadic retinoblastoma is most probably conditioned by non-genetic factors such as poor living conditions and other environmental agents in utero.117–122

Environmental factors, such as diet127 and infectious agents,128 as well as ionising radiation129 have been reported to play a role in the genesis of Rb. However, the mechanism through which this is achieved is not a direct structural DNA damage, but rather a stable modification of the epigenome, which is transmitted through the germ cells.130–132

Regarding the role of low level radiation, of extreme interest is the case of the American Indian Navajo population who has represented a focus of cancer research for many years, as in the case of Rb, it has

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.130 The paper published by Stiller, on this subject, is of great interest since, although radiation is known to induce DNA mutations, it clearly argues against the mutational genesis of Rb. In particular it refers to a group of five unilateral sporadic Rb, born to mothers who had lived in Seascale, a village situated near the Sellafield nuclear reprocessing plant, in the region of Cumbria. The puzzling feature of these five cases is that none of them have been diagnosed among children who were themselves resident in Seascale, although the observed incidence among children of mothers who had previously lived there has been calculated to be about 20 times expected. Interestingly, “… even if the risk is 20 times that in the rest of Britain”, observes the author, “only one case of Rb would be expected in Seascale every 40 years”.

The above data, further confirmed by other short reports133–135 on the same subject, represent a rather typical circumstance for epigenetics which explains the ability of certain chemical compounds (or low levels radiation) to initiate biological perturbations that can lead to malignancy, despite being weak mutagens or lacking mutagenic activity altogether.136–137

Concluding Remarks and Future Perspectives

Traditionally, cancer has been viewed as a genetic disease, although recent advances in the field of epigenetics show that cancer is instead the manifestation of both genetic and epigenetic modifications.138

Classic genetics alone has been shown largely insufficient to explain the diversity of phenotype within a population and why, despite their identical DNA sequences, monozygotic twins or cloned animals show different phenotypes and disease susceptibilities.139

Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene sequence. This is most commonly (but not exclusively) achieved through DNA methylation, which is one of the most commonly occurring epigenetic events in the mammalian genome.90

Alterations of the gene methylation profile in cancer include either hypo- or hypermethylation, which in turn, encompass both global and/or gene specific modifications. Global hypomethylation is responsible of the increased chromosomal instability and tumour frequency with age, while gene-specific hypomethylation leads to oncogene activation. In addition, the silencing of tumour-suppressor genes is associated with promoter DNA hypermethylation and chromatin hypoacetylation, which affect diverse genes, including Rb1.140 Although epigenetic regulation of gene expression is the mechanism through which the extraordinary variety of specialised cells of the body differentiate starting from a single undifferentiated ancestor,141 the relevance of epigenetic factors in disease in humans was first detected only in 1983 when Weinberg and Vogelstein found that gene hypomethylation could distinguish some human cancers from their normal counterparts.142

Today, deregulation of gene expression is widely considered a hallmark of cancer143–146 and although genetic lesions have been the focus of cancer research for many years, as in the case of Rb, it has
increase becoming increasingly recognized that aberrant epigenetic play major roles in all cancers, including RB.145

As the authors have shown herein, the mutational model is largely inadequate to explain both the variegated expression and inherent plasticity of the clinical phenotype in RB, while epigenetics offers a more coherent and reliable paradigm. Also and more importantly, this paradigm shift (from genetic to epigenetic), in RB, opens up other important avenues in cancer research which promise to revolutionise the fields of both ophthalmology and oncology, namely:

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 inhibitions to date, although others are expected to be added in the near future.146

Epigenetic changes in cancer cells do not only allow novel prospects for drug therapy but also offer unique prospects for cancer diagnostics, 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.147,148

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 and diet.

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