REVIEW ARTICLE

THE EPIGENETICS CHANGES IN CHILDHOOD SOLID CANCER: A REVIEW

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Abstract

Epigenetics is the study of heritable changes in gene expression that do not involve changes to the underlying DNA sequence. It is a change in phenotype without changing in genotype which in turn affects how cells read the genes. The epigenetic change is a regular occurrence but can also be influenced by several factors including age, environment, lifestyle, and disease state. It may have damaging effects that result in diseases like cancer. At least three systems including DNA methylation, histone modification and RNA-associated gene silencing are currently considered to initiate and sustain epigenetic change. New and ongoing research is continuously uncovering the role of epigenetics in a variety of diseases including in childhood solid cancer such as Ewing sarcoma, neuroblastoma, Wilms tumours, brain tumours and rhabdomyosarcoma. A better understanding of epigenetic changes in childhood cancers can guide towards future therapy and diagnosis.

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Introduction

The behaviour of an individual's genes does not just depend on the genes' DNA sequence but also affected by so-called epigenetic factors. Changes in these factors play a critical role in disease, especially cancer. Why do we inherit some traits from our father and others from our mother? How do our experiences and our environment influence us? These questions are addressed by a scientific field called epigenetics, which studies heritable changes in the absence of alterations in our DNA. Epigenetic changes can switch genes on or off and determine which proteins are transcribed.

Literally, epigenetics means *around* genetics. It is the study of cellular and physiological traits that could be inherited but are not caused by actual changes to our DNA or genetic code. Epigenetics refers to natural control mechanisms that influence

gene expression. Their role is often compared to computer software whereby the DNA and the genes that we are born with is a hard drive in which controlling the cells. But, this hard drive cannot work without software. Epigenetics is then, the software package. Researchers believe that every cancer may have 50 to several hundred genes that have working (hard drives), but their epigenetic (software) is causing them to act in a way that can lead to cancer development.

In the world of cancer, epigenetics is considered an emerging field, but its study is not new. Many researchers have done intense research on epigenetics. This article mainly to emphasise the role of epigenetics including childhood cancer.

How do epigenetics affect genes?

Epigenetics is involved in many normal cellular processes. Consider the fact that our cells all have the same DNA, but our bodies contain many different types of cells such as neurons, liver cells, pancreatic cells, inflammatory cells and others. How can this be? In short, cells, tissues and organs differ because they have certain sets of genes that are expressed, as well as other sets that are inhibited. Epigenetic silencing is one way to turn genes off, and it can contribute to differential expression. This silencing might also explain why genetic twins are not phenotypically identical. Besides, epigenetics is important for X-chromosome inactivation in female mammals, which is necessary so that females do not have twice the number of X-chromosome gene products as males [1]. Thus, the significance of turning genes off via epigenetic changes is readily revealed. Within cells, three systems can interact with each other to silence genes: DNA methylation, histone modifications, and RNA-associated silencing [1].

DNA Methylation

DNA methylation is a chemical process that adds a methyl group to DNA. It is highly specific and always happens in a region in which a cytosine nucleotide is located next to a guanine nucleotide that is linked by a phosphate. This is called a CpG site [1, 2, 3]. CpG sites are methylated by one of three enzymes called DNA methyltransferases (DNMTs) [1, 3]. Inserting methyl groups changes the appearance and structure of DNA, modifying a gene's interactions with the machinery within a cell's nucleus that is needed for transcription.

There are stretches of DNA near promoter regions that have higher concentrations of CpG sites which is called CpG islands. These CpG islands become excessively methylated or hypermethylated in cancer cells. Nevertheless, the term hypomethylation can be applied to describe the unmethylated state of most CpG sites in a specific sequence that is normally methylated, or as a general phenomenon affecting the bulk of the genome. DNA demethylation is the process of removal of a methyl group from nucleotides in the absence of methylation of newly synthesised DNA strands by DNMT1 during several replications rounds, usually in response to treatment, leading to dilution of the methylation signal.

Histone Modifications

Histones are proteins that are the primary components of chromatin, which is the complex of DNA and proteins that makes up chromosomes. When histones are modified after they are translated into protein, they can influence how chromatin is arranged, which, in turn, can determine whether the associated chromosomal DNA will be transcribed. If chromatin is not in a compact form, it is active and the associated DNA can be transcribed. Conversely, if chromatin

is condensed (creating a complex called heterochromatin), then it is inactive and DNA transcription does not occur.

There are two main ways histones can be modified: acetylation and methylation. These are chemical processes that add either an acetyl or methyl group, respectively, to the amino acid lysine that located in the histone. Acetylation is usually associated with active chromatin, while deacetylation is generally associated with heterochromatin. On the other hand, histone methylation can be a marker for both active and inactive regions of chromatin. For example, methylation of a particular lysine (K9) on a specific histone (H3) that marks silent DNA is widely distributed throughout heterochromatin. This is the type of epigenetic change that is responsible for the inactivated X-chromosome of females. In contrast, methylation of a different lysine (K4) on the same histone (H3) is a marker for active genes [1].

RNA-Associated Silencing

Genes can also be turned off by RNA when it is in the form of non-coding RNAs (ncRNA), antisense transcripts or RNA interference. RNA might affect gene expression by causing heterochromatin to form, or by triggering histone modifications and DNA methylation [1]. NcRNA is employed for RNA that does not encode a protein, for example microRNA and small nucleolar RNAs (snoRNAs), many of which remain to be identified and contain information as it alternatively spliced and/or processed into smaller products. NcRNAs are involved in epigenetic processes, playing a role in the regulation of gene expression at the transcriptional and post-transcriptional levels. Natural antisense transcripts (NATs) are the oldest group within the family of ncRNAs. These are transcribed in the opposite direction to protein coding transcripts and are widespread in eukaryotes.

Epigenetics in cancer

As previously mentioned, DNA methylation occurs at CpG sites and the majority of CpG cytosines are methylated in mammals. However, there are stretches of DNA near promoter regions that have higher concentrations of CpG sites (CpG islands) that are free of methylation in normal cells. These CpG islands become excessively methylated (hypermethylated) in cancer cells, thereby causing genes that should not be silenced to turn off. This abnormality is the trademark epigenetic change that occurs in the early development of most cancer [1,2,3]. This hypermethylation also leads to shutting off tumour-suppressor genes. In fact, these types of changes may be more common in human cancer than DNA sequence mutations.

Furthermore, although epigenetic changes do not alter the sequence of DNA, they can cause mutations. About half of the genes that cause familial or inherited forms of cancer are turned off by methylation. Most of these genes normally suppress tumour formation and help repair DNA, including O^6-methylguanine-DNA methyltransferase (MGMT), MutL homolog 1 (MLH1), cyclin-dependent kinase inhibitor 2B (CDKN2B), and ras association domain family protein 1 (RASSF1A). For example, hypermethylation of the promoter of MGMT causes the number of G-to-A mutations to increase.

Hypermethylation can also lead to instability of microsatellites, which are repeated sequences of DNA. Microsatellites are common in normal individuals and they usually consist of repeats of the dinucleotide CA. Too much methylation of the promoter of the DNA repair gene MLH1 can make a

microsatellite unstable and lengthen or shorten it. Microsatellite instability has been linked to many cancers, including colorectal, endometrial, ovarian and gastric cancers [2].

Which solid cancers in childhood affected by epigenetic changes?

Ewing sarcoma

Ewing sarcoma is aggressive bone cancer in children and adolescents. It is due to a single genetic defect; the Ewing sarcoma-E26 transformation-specific (*EWS-ETS*) gene fusion, but the tumours carry very few DNA mutations that could explain the observed differences in the disease course. Ewing sarcoma showed unique characteristics that differ markedly from other cancers and the DNA methylation patterns also varied between patients. Moreover, the researchers found that Ewing sarcoma tumours appear to retain part of the characteristic DNA methylation patterns of their cell-of-origin. Thus, the diverse clinical courses observed among Ewing sarcoma patients may be explained epigenetically. Since DNA methylation influences gene activity, the combination of Ewing sarcoma-specific and cell-of-origin specific patterns can lead to different outcomes. The epigenetic diversity also appears to correlate with the tumours' aggressiveness and metastatic state. These new insights into the biology of Ewing sarcoma provide the basis for developing epigenetic biomarkers that can reliably predict disease course and therapy response.

In 85% of cases, the resulting chimeric protein is a fusion between EWS and friend leukaemia integration 1 transcription factor (FLI1) [4]. EWS-FLI1 functions as an aberrant transcription factor and is believed to be an initiating tumorigenic event. The very primitive neuroectodermal histology of Ewing family of tumours (EFT) and their diverse presentation in bones and soft tissues suggest an early stem or progenitor cell of origin. Indeed, recent experimental evidence supports this hypothesis and bone progenitors, mesenchymal (MSC) and neural crest stem cells (NCSC) are now all implicated as potential cells-of-origin [5-9].

Initial investigations into EWS-FLI1 function focused mainly on identifying genes that were induced by the fusion. During these studies, several promising candidates were identified and genes such as NK2 homeobox 2 (*NKX2.2*) [10], nuclear receptor subfamily O, group B member 1 (*NR0B*) @ (*DAX1*) [11], and enhancer of zeste homolog 2 (*EZH2*) [12] are now well-established direct transcriptional targets of EWS-FLI1. However, EWS-FLI1 represses as many genes as it induces [8,13,14] and the mechanisms by which EWS-FLI1 suppresses transcription and the contribution of gene repression to the EFT phenotype have become the focus of intense investigation.

EWS-FLI1-mediated induction of *EZH2* blocks differentiation of MSC and leads to the repression of genes involved in neuroectodermal and endothelial differentiation [12, 15]. In established EFT, tumorigenicity is dependent on continued over-expression of EZH2 and B-cell-specifics moloney murine leukaemia virus integration site-1 (BMI-1) [6, 12, 16,17]. To exploit this, cell therapy with cytotoxic T-cells directed against EZH2 was recently proposed as a novel approach to EFT therapy [18] demonstrating that the abnormal cancer epigenome could be a source of novel antigenic targets for immunotherapy [19].

Apart from that, regulation of polycomb group-family (PcG) protein expression and function is mediated by a complex network of miRNAs [20] and by long non-coding

RNAs (lncRNAs). EWS-FLI1 modulates the expression of both miRNAs [21] and lncRNAs [22], so it will be interesting to discover whether EWS-FLI1-mediated deregulation of ncRNA transcripts contributes to PcG protein dysfunction. Besides, the contribution of altered DNA promoter methylation to EFT pathogenesis is beginning to come into focus. Global analysis of DNA promoter methylation has revealed that the CpG islands of PcG target genes are aberrantly hypermethylated in EFT and the promoters of transcription factors involved in neural differentiation become progressively methylated over time in EWS-FLI1-expressing NCSC. Thus, EWS-FLI1 drives EFT pathogenesis by invoking global deregulation of the epigenome through diverse mechanisms. Direct pharmacologic targeting of transcription factors continues to be an immense therapeutic challenge. In EFT targeting the epigenome may be an effective way to circumvent the oncogenic activity of EWS-FLI1 without having to target the oncogene itself directly.

Neuroblastoma

Neuroblastomas (NB) are neural crest-derived tumours, expressing genes characteristic of sympathoadrenal cell lineages. Mutations in paired-like homeobox 2b (*PHOX2B*), a major regulator of sympathoadrenal development, have been identified in 6% of familial neuroblastoma [23]. Functional studies contributed to the concept that the cells corresponded to different stages of sympathoadrenal development retained potential to differentiate into a number of neural crest cell lineages and thus represented a multi-potent sympathoadrenal progenitor cell with distinct tumorigenic potential [24, 25]. Consistent with these concepts, aggressive tumours are characterised by increased expression of cell cycle genes [26], while ganglioneuroma and ganglioneuroblastoma express genes associated with neural development [27].

EZH2 is elevated in poor prognosis, undifferentiated NB and is associated with enrichment of H3K27me3 at the promoters of tumour suppressor genes such as castor zinc finger 1 (*CASZ1*), runt related transcription factor 3 (*RUNX3*), nerve growth factor receptor (*NGFR*) and neutrophic receptor tyrosine kinase 1 (*NTRK1*) [28]. What leads to elevated EZH2 levels is still unknown. BMI-1 is a direct transcriptional target of N-myc proto-oncogene protein (MYCN) [29] and is also over-expressed by and essential for tumorigenicity of NB [30]. In addition, the lysine-specific demethylase 1 (LSD1) is over-expressed in poorly differentiated NB [31]. In embryonic stem cells, LSD1 preferentially demethylates H3K4me3 thus favouring PcG-associated H3K27me3 silencing at bivalent loci and maintenance of stemness. This physiologic function of LSD1 and EZH2 over-expression most likely contribute to the poor prognosis that is associated with LSD1 over-expressing NB compared to more differentiated forms of a tumour [31].

The relationship between PcG protein dysregulation and DNA methylation in NB tumours is tenuous but intriguing. The chromodomain helicase DNA binding protein 5 (*CHD5*) tumour suppressor gene, part of a family of chromatin remodelling proteins, is in a chromosomal region that is subject to loss of heterozygosity (LOH) in many tumours including NB [32]. *CHD5* contains a bivalent chromatin mark in stem cells and in NB cells with LOH the remaining allele is silenced via DNA methylation [33]. It is not clear whether silencing of a single gene or an entire pathway is needed for NB initiation or

progression but number of genes, including a commonly hypermethylated gene in cancer (*HIC1*), putative tumour suppressor genes (*RASSF1A, PRKCDBP*), differentiation genes (*HOXA9*) and apoptosis genes (*CASP8, APAF1, TMS1*) have been consistently identified [32, 34-37]. In addition, emerging data from unbiased, global approaches have confirmed established loci and identified novel DNA methylated regions (telomeric silencing of 1p, 3p, 11q and 19p) and a "methylator" phenotype that is associated with a worse clinical outcome [35,37, 38].

There is also an intriguing association of MYCN amplification with DNA methylation. This was first noted with *CASP8* [40] and has now been associated with additional loci [36, 37]. In NB cell lines genome-wide MYCN DNA binding is significantly associated with the binding of methyl-CpG binding protein 2 (MeCP2) [41]. At gene promoter, transcription is relatively high when MYCN has bound alone, intermediate when both MYCN and MeCP2 are present and low with the only MeCP2. This suggests that MYCN may serve as an initial but transient focus to recruit components required to mediate DNA methylation [41].

There are many shreds of evidence to support the use of epigenetic modifiers in NB therapy. First, the ability of retinoids to restore differentiation indicates that despite the genetic alterations associated with high-risk tumours the regulatory signalling pathways controlling growth and differentiation are intact but dysregulated. Indeed, it is becoming increasingly evident that retinoids function to relieve epigenetic suppression. Second, histone deacetylase (HDAC) inhibitors, either alone or in combination with cis-retinoic acid, inhibit NB growth *in vitro* and *in vivo* [42,43].

Third, reactivation of caspase 8 in drug-resistant NB cells by exposure to DNA methylation inhibitors restores sensitivity to standard cytotoxic agents [40]. Fourth, inhibition of EZH2 by 3-deazaneplanocin A (DZNep) leads to re-expression EZH2 silenced tumour suppressor genes and resulted in decreased growth and differentiation of NB cells [28]. Finally, the most aggressive NB is driven by aberrant activation of MYCN. Recent studies have shown that JQ1, a novel small molecule inhibitor of the chromatin-modifying co-factor, bromodomain-containing protein 4 (BRD4), dramatically inhibits proliferation and promotes terminal differentiation of acute myelogenous leukaemia cells and it achieves this, in part, by suppressing MYC function [44]. Whether epigenetic therapy with JQ1 can be used to block the oncogenic activity of MYCN in NB remains to be determined but provides an exciting new opportunity for investigation.

Brain tumours

Epigenetic deregulation is emerging as a fundamental process underlying the pathogenesis of paediatric brain tumours. Hypermethylation of the *RASSF1A* tumour suppressor gene has been identified in nearly 90% of medulloblastomas and ependymomas [45] and upregulation of PcG proteins, particularly BMI-1 and EZH2, is common and is associated with worse clinical outcomes [46, 47]. Intriguingly, recent next-generation sequencing studies of paediatric medulloblastomas have discovered that, although tremendous genetic diversity exists between individual tumours, there is a marked over-representation of disruptions in genes that encode for chromatin modifiers and epigenetic regulators [48]. Specifically, deletions in individual genes that encode H3K9 methyltransferases (i.e. *EHMT1*, *SMYD4*) were detected in 2%

of cases in a series of 212 tumours while H3K9 demethylase genes were selectively amplified in another 2% [48]. In total, mutations, deletions or amplifications in genes that converge on modulating H3K9 methylation were detected in 19% of tumours examined in this study and absence of nuclear staining for methylated H3K9 was confirmed in 41% of cases in an independent cohort [48]. Demethylation of H3K9 is required for silencing of proliferative genes and successful terminal differentiation of progenitor cells in the external granule layer [48]. Mutations that disrupt H3K9 methylation likely contribute to malignant transformation by blocking normal differentiation [48]. In a second study, inactivating mutations of lysine methyltransferases, *MLL2* and *MLL3* were detected in 16% of primary and mutations in SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily A, member A (*SMARCA4*) and AT-rich interaction domain 1A (*ARID1A*) in another 4%. Thus, mutations in epigenetic regulators figure prominently in paediatric medulloblastoma, and cumulatively these lesions contribute to tumour initiation and progression by repressing normal developmental pathways while promoting maintenance of a more stem-like state.

Next-generation sequencing studies of gliomas have also uncovered a basis for epigenetic deregulation with the discovery that 70–80% of grades II and III astrocytomas harbour a mutation in either the *IDH1* or *IDH2*, isocitrate dehydrogenase genes [49]. IDHs produce α -ketoglutarate, an obligatory co-substrate for histone demethylases and ten-eleven translocation (TET) family proteins. Mutated IDH proteins produce D-2-hydroxyglutarate which acts as a competitive inhibitor of α -ketoglutarate and thereby inhibits histone and DNA demethylation [50]. Thus, IDH-mutated gliomas are epigenomically abnormal, and this contributes to an abnormal DNA methylator phenotype [51]. Importantly, however, mutations in IDH genes are associated with younger age and an improved prognosis indicating that epigenetically driven tumours may be less aggressive and more responsive to therapy than tumours that are characterised by genetic instability, such as those that occur in older patients.

Retinoblastoma

Retinoblastoma is a rare type of eye cancer of the retina that commonly occurs in early childhood and mostly affects the children before the age of 5. It occurs due to the mutations in the retinoblastoma gene (RB1) which inactivates both alleles of the RB1. RB1 was first identified as a tumour suppressor gene, which regulates cell cycle components and associated with retinoblastoma. Previously, genetic alteration was known as the major cause of its occurrence, but later, it is revealed that besides genetic changes, epigenetic changes also play a significant role in the disease. Initiation and progression of retinoblastoma could be due to independent or combined genetic and epigenetic events. Remarkable work has been done in understanding retinoblastoma pathogenesis concerning genetic alterations, but not much in the context of epigenetic modification. Epigenetic modifications that silence tumour suppressor genes and activate oncogenes include DNA methylation, chromatin remodelling, histone modification and non-coding RNA-mediated gene silencing. Epigenetic changes can lead to altered gene function and transform a normal cell into tumour cells. This review focuses on crucial epigenetic alteration which occurs in retinoblastoma and its current state of knowledge. The critical role of epigenetic regulation in retinoblastoma is now an

emerging area, and a better understanding of epigenetic changes in retinoblastoma will open the door for future therapy and diagnosis.

Although the precise cell of origin of retinoblastoma tumours remains controversial, their clinical presentation before birth and in early infancy leaves no doubt as to their embryonic origin. Interestingly, a recent study found that developmental pathways that are normally expressed in a mutually exclusive fashion during normal retinal cell development are abnormally co-expressed in single retinoblastoma cells [52]. Although the mechanisms underlying this observation remain to be elucidated, it is highly probable that disruption of normal epigenetic regulation within developing retinal cells contributes to this oncogenic developmental abnormality.

Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma of childhood, with skeletal muscle presumed origin because of its myogenic phenotype. However, unlike normal myoblasts, RMS cells differentiate poorly both *in vivo* and in culture [53]. Experiments in the late eighties showed that the treatment of the RMS cell line RMZ-RC2 with the demethylating agent 5-azacytidine resulted in an increased differentiation capacity, suggesting that aberrant DNA methylation was repressing differentiation genes in this RMS cell line [54]. Also, the comparison of DNMTs levels between normal skeletal muscles and RMS tumours showed significantly higher expression of DNMTs in both alveolar and embryonal RMS subtypes, which have distinct etiologic and clinical behaviours [55]. Candidate gene approaches identified DNA methylation changes in RMS tumours in *MyoD* [55], *p21WAF1* [56], *RASSF1*[57], *PAX3* [58], *plakoglobin* [59], *FGFR1* [60], *JDP2* [61], *BMP2* [62] and *CAV1* [63] genes. Recently, a genome-wide analysis of promoter CpG island methylation between RMS subtypes and skeletal muscles revealed RMS-specific hypermethylation in genes associated with tissue development, differentiation and oncogenesis [64]. Besides, cluster analysis showed that embryonal and alveolar subtypes had distinct methylation patterns, with the alveolar subtype being enriched in DNA hypermethylation of polycomb target genes [64]. Importantly, the different DNA methylation signatures between RMS subtypes might aid to define tumour subtype, clinical prognosis and treatment response of RMS tumours.

The connection between miRNAs, tissue differentiation and malignant transformation emerged long time ago [65, 66]. Although some miRNAs can act as oncogenes, miRNAs identified as de-regulated in cancer are more commonly tumour suppressors, that are down-regulated by several mechanisms including epigenetic silencing [67]. In the last few years, the list of miRNAs undergoing promoter hypermethylation has been rapidly expanded in many human tumours, highlighting the transcriptional repression of miRNAs by DNA methylation as a common feature in human cancer [68]. Recent evidence has shown low expression of miR-206 [69], miR-1 and miR-133a [70], and miR-203 [71] in RMS cells which have been correlated with higher proliferation rates, impaired differentiation and poor overall survival. Notably, it was recently demonstrated that miR-203 was frequently down-regulated in both RMS cell lines and RMS biopsies by promoter hypermethylation and importantly, it can be reactivated by DNA-demethylating agents inhibiting tumour growth and migration

capacity and promoting terminal differentiation [71]. All these data allow envisioning the use of combined treatment including epigenetic drugs for the treatment of the aggressive RMS tumours.

Wilms tumour

The majority (95%) of Wilms tumours are sporadic; which is not due to inherited genetic alterations, but instead developing due to genetic alterations that occur in just a few cells in the body. Familial Wilms tumour is defined as either bilateral disease or a family history of Wilms tumour that accounts for approximately 5% of cases. The WT1 gene located at 11p13 was identified in 1989. However, only about a third of patients carry detectable mutations. Hence, the development of Wilms tumour is complex and is likely to involve several other genetic loci. Many of other genes on chromosome 11p have also been implicated in Wilms tumour, including the putative WT2 gene (11p15).

Genome-wide studies of Wilms tumour chromatin and DNA methylation have been extraordinarily informative and have identified global epigenetic aberrations beyond the well-established hypermethylation of H19 that contributes to loss of imprinting and overexpression of insulin like growth factor 2 (IGF2) [72]. Notably, the chromatin landscape of Wilms tumours has been shown to be highly related to embryonic stem cells and is associated with upregulation of PcG activity, abnormal retention of bivalent marks and silencing of genes that direct early renal differentiation [73].

Conclusion

The great mysteries of genes are now uncovered. Some have said the “ghost in our genes,” referring to epigenetic mechanisms’ ability to alter gene expression without leaving a permanent mark on DNA. Now, what was once an outcast in cancer research is being heralded as one of the most promising fields of study in cancer medicine.

In the era of precision medicine, understanding the causes and consequences of tumour heterogeneity will be crucial to developing personalised therapies. Only with precise knowledge of the molecular mechanisms underlying each tumour, we can hope to treat in a targeted way and with far fewer side effects.

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