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Action at a distance: epigenetic silencing of large chromosomal regions in carcinogenesis

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Despite the completion of the Human Genome Project, we are still far from understanding the molecular events underlying epigenetic change in cancer. Cancer is a disease of the DNA with both genetic and epigenetic changes contributing to changes in gene expression. Epigenetics involves the interplay between DNA methylation, histone modifications and expression of non-coding RNAs in the regulation of gene transcription. We now know that tumour suppressor genes, with CpG island-associated promoters, are commonly hypermethylated and silenced in cancer, but we do not understood what triggers this process or when it occurs during carcinogenesis. Epigenetic gene silencing has always been envisaged as a local event silencing discrete genes, but recent data now indicates that large regions of chromosomes can be co-coordinately suppressed; a process termed long range epigenetic silencing (LRES). LRES can span megabases of DNA and involves broad heterochromatin formation accompanied by hypermethylation of clusters of contiguous CpG islands within the region. It is not clear if LRES is initiated by one critical gene target that spreads and conscripts innocent bystanders, analogous to large genetic deletions or if coordinate silencing of multiple genes is important in carcinogenesis? Over the next decade with the exciting new genomic approaches to epigenome analysis and the initiation of a Human Epigenome Project, we will understand more about the interplay between DNA methylation and chromatin modifications and the expression of non-coding RNAs, promising a new range of molecular diagnostic cancer markers and molecular targets for cancer epigenetic therapy.

INTRODUCTION

Both genetic and epigenetic lesions contribute to heritable changes in gene expression in a cancer cell. Genetic changes that are associated with cancer include gene mutations in critical tumour-associated genes, as well as gene amplification, deletion or loss of heterozygosity (LOH) of larger regions harbouring tumour suppressor genes. In addition to genetic changes, we now know that epigenetic changes are also a common hallmark of cancer DNA, with changes in DNA methylation, histone modifications of the CpG island regions spanning the promoters of tumour associated genes and altered expression of non-coding RNAs (1–10). However, we do not still fully understand the extent and nature of these epigenetic changes in cancer cells, nor do we understand what triggers epigenetic lesions during carcinogenesis.

Delving deeper into biology frequently uncovers a complexity greater than predicted. For nearly two decades research into the cancer epigenome has been blinkered by experimental approaches and the concept of 'one methylated CpG island equals one silent gene'. However, recent data from epigenomic and transcription profiling studies has challenged this underlying assumption and demonstrated that epigenetic changes in cancer are not always focal, but can be global encompassing large chromosomal regions, resulting in Long Range Epigenetic Silencing (LRES). Similar to gross genetic changes such as gene deletion or loss of heterozygozity (LOH), LRES results in the concordant repression of large regions of DNA. This review will discuss what is currently known about the mechanisms involved in triggering aberrant epigenetic silencing in cancer and what defines which genes in cancer are susceptible to DNA methylation and epigenetic inactivation and conversely why some genes are protected from DNA methylation, and how this may operate in LRES.

THE MYSTERY OF DNA METHYLATION

In mammals, methylation of cytosine residues at CpG dinucleotides occurs by the addition of a methyl group to the carbon-5 position of cytosine (5MeC). In human somatic

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cells, 5MeC accounts for ~1% of total DNA bases and therefore affects 70–80% of all CpG dinucleotides in the genome (11). However, CpG sites and therefore 5MeC are also not evenly distributed in the genome. The bulk of the mammalian genome is CpG depleted and methylated, but short stretches of CpG-dense DNA, known as CpG islands also exist (12,13). There are at least 29 000 CpG islands in the human genome, according to DNA computational estimates (14,15). Approximately 60% of gene promoters are associated with CpG islands and intriguingly most of these CpG island promoters are unmethylated in a normal cell at all stages of development and in all tissue types and are often associated with active gene transcription (16). However, some CpG islands associated with tissue specific genes also remain essentially unmethylated regardless of expression during differentiation (17). A limited proportion of CpG islands, for example CpG islands located on the inactive X chromosome and imprinted genes, can become methylated during development and for these genes methylation is associated with gene silencing.

The methylation profile of the cell is exquisitely controlled during development. The methylation pattern is transmitted to the daughter DNA strand during DNA replication by the action of the maintenance methyltransferase enzyme *DNMT1* (18,19). Methylation patterns are established in the early embryo with initial demethylation of the parental DNA in the first few cell divisions after fertilization, followed by *de novo* methylation of specific CpG sites between the eight-cell stage and blastocyst implantation by methyltransferase enzymes *DNMT3A* and *DNMT3B* (20–27). It was initially thought that DNA methylation patterns, once established, were faithfully maintained at each cell division but with the advent of bisulphite genomic sequencing (28), it has become clear that the methylation state of any one CpG site is not always maintained. An apparent interplay between *de novo* methylation and demethylation at each cell division gives rise to a heterogeneous pattern of methylation for any one molecule (29–31). It has been estimated that failure of maintenance occurs at ~5% per CpG site per cell division (32). However, even though heterogeneity can occur at individual CpG sites, the actual domains or regions that become methylated are maintained during development, suggesting that it is more than just the *DNMT1* maintenance enzyme that dictates the methylation pattern. Intriguingly, CpG island and allele-specific imprinted methylation are maintained in the absence of *DNMT1* (33,34).

The origin of genomic DNA methylation patterns is a still mystery. It is especially puzzling that even though the *de novo* DNA methyltransferase enzymes *DNMT3A* and *DNMT3B* are highly expressed in early embryonic cells (35–37), when most programmed *de novo* methylation events occur, CpG islands still remain unmethylated, even though they are rich in target CpG sites. Either the bulk of the genome is methylated by 'default' and CpG islands are strategically protected from methylation or global methylation is directed by additional factors such as chromatin-associated proteins (17,38,39), and/or targeted to certain sequences such as DNA methylation centres (40), DNA sequence signatures or DNA structures (41–43). During differentiation, DNA methylation of the promoter regions of tissue-specific genes can occur, but it is unclear if this follows gene inactivation (44) or directly causes gene silencing (31,45,46). Intriguingly, double-stranded RNA (dsRNA) has also been shown to direct *de novo* methylation in plants (47–49); however, there is no clear demonstration as yet that mammalian dsRNA is involved in sequence-specific *de novo* methylation (50,51). It remains to be determined what dictates the distinct genomic methylation pattern, but it is clearly essential for mammalian development as deletion of any of the DNMT genes in mice results in severe developmental defects and early embryonic lethality (35,52).

THE COMPLEXITY OF CHROMATIN

The active or repressed condition of the DNA does not depend solely on its DNA methylation status. In fact, DNA methylation by itself generally does not directly repress transcription (53), but it orchestrates the assembly of different proteins to form chromatin, and it is the state of the chromatin that determines the functional state of a gene. Chromatin structure around unmethylated CpG island promoters facilitates the access of proteins that promote transcription. Alternatively, when CpG islands are methylated, the chromatin is tightly compacted preventing gene expression (17). Chromatin consists of nucleosomes, each containing 147 base pairs of DNA, wrapped around an octamer of the core histones proteins H3, H4, H2A and H2B. The structural state of chromatin is associated with a myriad of post-translation covalent modifications of the histones. Well-described histone modifications include acetylation of the lysine residues and methylation of lysine and arginine residues on the histone tails as well as serine and threonine phosphorylation, glutamic acid ADP-ribosylation and lysine ubiquitination and sumoylation and combinations of histone modifications result in different chromatin states which define the so-called 'histone code' (54,55). On a broad level, acetylation of histone tails is associated with active genes and unmethylated CpG islands, whereas deacetylation of histone tails is associated with silent genes and methylated CpG islands. Methylation of lysine residues in histone H3 however can have opposite effects; methylation of lysine 9 (H3K9) is associated with silent genes and methylation of lysine 4 (H3K4) is characteristic of active genes, reviewed in (4). Though, as usual in biology, it is more complex and currently there is an explosion of information about the control for organizing and maintaining the chromatin structure of the normal nucleus and how the modifications relate to controlling gene expression. Each modification is in delicate balance and small changes in a given parameter may have major consequences for transcription (10).

In addition to histone modifications, there are also a myriad of histone-associated proteins that are involved in regulating gene transcription. Proteins containing bromodomains recognize acetylated lysine residues and proteins containing chromodomains bind to methylated lysine residues (56,57). Polycomb group proteins play an essential role in development through establishing long-term gene silencing as initially recognized in Drosophila, but now shown to be a conserved function across the animal and plant kingdoms (58).

THE NOVELTY OF NON-CODING RNA

One area of epigenetics that is still in its infancy is the role of non-coding RNA in epigenetic silencing. It is clear that non-coding RNA plays a key role in X-inactivation, with the expression of Xist and its antisense partner Tsix (59), and in imprinting, with for example, the expression of IGF2R and its antisense partner AIR (60). Antisense transcription leading to DNA hypermethylation has also been reported to be involved in a patient with alpha-thalassemia (61). It is therefore intriguing to speculate that similar processes of interaction between sense and antisense expression are important in establishing or maintaining normal methylation patterns and may play a critical role in triggering hypermethylation in cancer. Equally as interesting is the epigenetic role of MicroRNAs (miRNAs) that are an abundant class of small non-protein-coding RNAs that function as negative gene regulators. miRNAs are single-stranded and they regulate diverse biological processes; bioinformatic data indicates that each miRNA can control hundreds of gene targets, underscoring the potential influence of miRNAs on almost every genetic pathway (62). Recent evidence has shown that miRNA mutations or mis-expression correlates with various human cancers and indicates that miRNAs can function as tumour suppressors and oncogenes. miRNAs have been shown to repress the expression of important cancer-related genes and might prove useful in the diagnosis and treatment of cancer (63).

EPIGENETIC CHANGES IN CANCER

In a cancer cell, gene expression is commonly altered and is caused by a combination of genetic and epigenetic lesions. Epigenetic changes include alteration of the genomic DNA methylation and histone modification profile. Generally, the overall genomic level of methylation is decreased or hypomethylated; in particular, the normally hypermethylated and silent regions containing the repetitive elements are substantially demethylated (64). Conversely, many normally unmethylated CpG island-containing genes often become hypermethylated and inactivated. Unlike genetic mutations, hypermethylation affects multiple genes in the one cancer cell (65), in fact many hundreds of genes are predicted to be concordantly hypermethylated in a single cancer cell (66). Genes involved in cell cycle regulation, DNA repair, drug resistance, detoxification, differentiation, apoptosis, angiogenesis and metastasis have all been identified as being susceptible to hypermethylation in different cancers (67,68). Many of these genes are associated with familial forms of human cancer when mutated in the germ-line, and thus the selective advantage for loss of function is very clear. However, the majority of the hypermethylated genes in cancer are not defined tumour suppressor genes and for some of these genes the promoter methylation may be the only type of inactivation found in cancers. This suggests that abnormal hypermethylation is often a result of deregulation of the methylation machinery in the cancer cell rather than the result of a selective advantage of a single stochastic methylation event resulting in inactivation of a critical tumour suppressor gene.

LONG RANGE EPIGENETIC SILENCING

A driving force that has underpinned much of the recent work in cancer epigenetics has been the quest to identify CpG island associated genes that are commonly methylated in cancer or methylated in specific cancer types or stages of cancer progression. Identification of methylated genes or sets of methylated genes provide the promise of novel biomarkers for cancer detection or prognosis. Many of the previous studies have used candidate gene approaches or global array surveys and found that hundreds of different and discrete CpG islands can be methylated in any tumour type; some of these are commonly methylated in several tumour types whereas others are methylated in a tumour-specific manner. In the candidate or global studies, the location of the methylated genes or the potential influence of CpG island methylation on the expression of neighbouring genes was not addressed. However, recent studies have found DNA hypermethylation in cancer is not always restricted to discrete CpG islands or single genes but can encompass multiple adjacent CpG rich regions resulting in concordant gene silencing across large chromosomal domains. Frigola *et al*. (69) identified a 4 Mb region on chromosome 2q14.2 in colorectal cancer that contained three distinct methylated zones of neighbouring CpG islands. The largest continuously methylated region, spanned nearly 1 Mb and encompassed 12 CpG islands, including the gene coding for the developmentally regulated homeobox-containing gene, Engrailed-1 (*EN1*). Surprisingly, the neighbouring genes spanning the hypermethylated zones across the 4 Mb region were also suppressed in colorectal cancer even though the genes themselves remained unmethylated. Gene suppression correlated with H3K9 methylation and global remodelling of the chromatin across the 4 Mb cytogenetic band and gene suppression could be relieved by demethylation and de-acetylation drugs (69).

Similar concordant methylation of adjacent CpG island gene promoters, but on a more restricted scale, has been reported for a number of other gene regions including the three human leukocyte antigen (HLA) class I genes located on chromosome 6p21.3 in esophageal cancer (70), the TRAIL receptor gene pairs DCR1 and DRC2 and DR4 and DR5 located on chromosome 8p21 in neuroblastoma (71), the PAX5α and PAX5β genes located on chromosome 9p13, in breast and lung cancer (72) and the HOXA gene cluster on chromosome 7p15.2 in breast cancer (73). The HOXA gene cluster included HOXA1-11 that spanned a 100 kb hypermethylated CpG island zone that was also associated with histone repressive marks. Treatment with a combination of demethylation and de-acetylation agents resulted in activation of the HOXA1-11 genes as well as HOXA13 the flanking unmethylated gene (73). While the genes in these examples are functionally as well as geographically related, they are indicative of the potential for coordinate epigenetic control over larger regions suggesting that LRES may be a more common phenomenon in cancer (Fig. 1). Indeed recent genome scale analysis have found a large chromosomal region on 6q23–q24 that contains several CpG islands that are commonly methylated in lung and head and neck cancer (74), and a study correlating copy number and deregulation of gene transcription has found groups of neighbouring

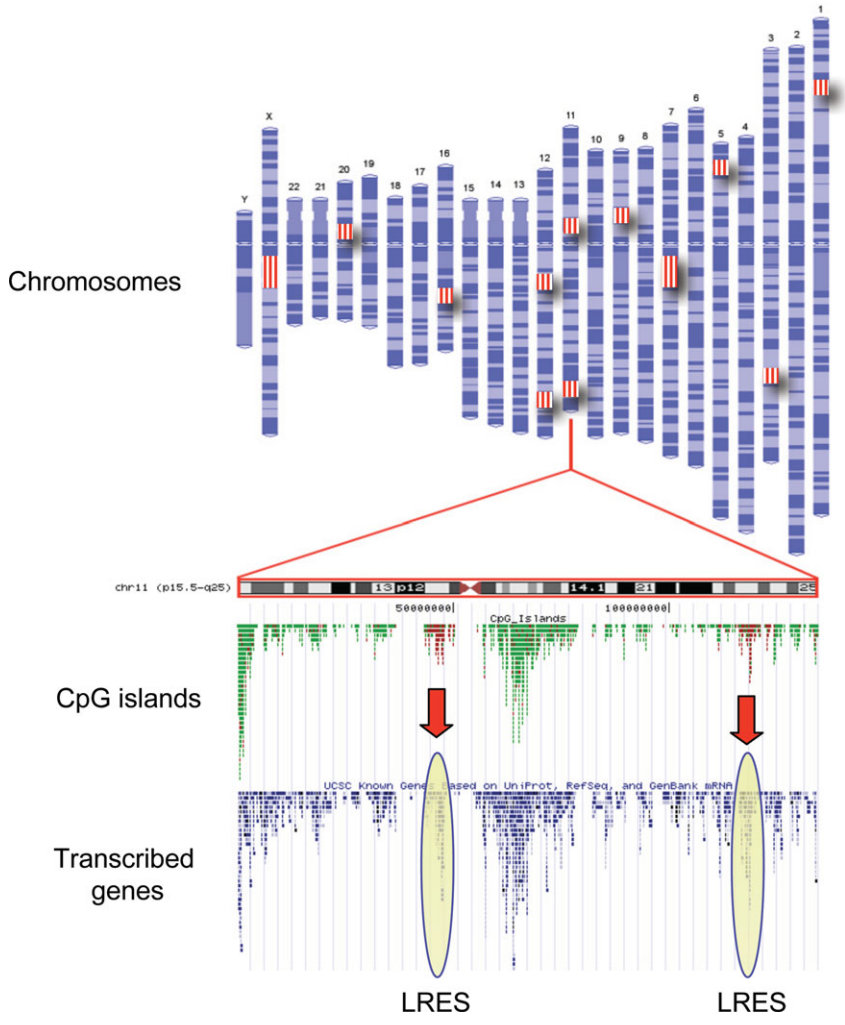

Figure 1. A hypothetical view of LRES in cancer. The chromosome map depicts hypothetical regions of LRES that could be identified by combining gene transcription and DNA methylation and histone H3K9 methylation ChIP on chip tiling array studies (boxed in red stripes). The lower panels show an expanded view of a single chromosome (chromosome 11). In the middle panel, the distribution of CpG islands are indicated, those in green are unmethylated, while those in red are methylated. Notably, there are two DNA hypermethylated zones consisting of concordantly methylated CpG islands, as well as individually methylated CpG islands dispersed across the chromosome. The lower panel shows the location of identified genes across the chromosome, with the ovals indicating regions of LRES that contain sets of genes that are down regulated and are associated with histone methylation.

genes with reduced expression levels (75). One such region on chromosome 3p22.3 in bladder cancer, spanning a 130 kb region and containing four genes, was found to display coordinated loss of expression that was epigenetically controlled by cancer-specific histone H3K9 methylation, but no DNA methylation (75). Interestingly, this region, which is downstream of MLH1, is commonly methylated in colorectal cancer, and therefore the suppression could be influenced by the neighbouring genes. In fact, many of these genes on 3p22.3 are coordinately methylated in colorectal cancer (M. Hitchins and R. Ward, unpublished results).

Long-range epigenetic regulation is a common phenomena in normal cells, in particular in relation to X-chromosome inactivation (76,77) and imprinted gene clusters (78). In X inactivation, it is clear that gene silencing is not restricted to individual genes but incorporates multiple genes in zones across the X chromosome where gene silencing is associated with DNA methylation and H3K9 methylation (76,77). Since both X-inactivation and regulation of imprinting are associated with the expression of non-coding RNA or micro-RNA transcripts, it is intriguing to consider that a similar mechanism may be involved in LRES, especially considering that many CpG islands are not associated with known genes, leaving the possibility that they may be associated with promoters of non-coding RNAs. Indeed, small dsRNA can induce transcriptional silencing in mammalian cells that is associated with H3K9 methylation and can be activated by demethylation and de-acetylase drugs (79).

WHAT TRIGGERS EPIGENETIC SILENCING?

It is clear that DNA hypermethylation is a significant change in the cancer genome; however, the mechanism responsible for eliciting this change is still not well understood. It is difficult to address the question of what triggers hypermethylation

of CpG island associated genes in tumour tissue because DNA methylation is an early event and therefore once the tumour is large enough to detect, the aberrant methylation process has already occurred. However, even though hundreds of genes may be hypermethylated in a given cancer cell, there appears to be two classes of genes; those that are commonly methylated in all cancer types and may represent innocent bystander genes and those that are cancer-type specific. This suggests that CpG island hypermethylation is either a stochastic process that continues to accrue over tumour progression in combination with growth selection for tumour specific targets, or that there is a program that dictates which CpG islands are susceptible to hypermethylation in the different cell-types.

In considering the epigenetic silencing of individual genes a number of intriguing if not overlapping or conflicting hypotheses have been put forward:

1. Hypermethylation of CpG island genes causes inactivation of tumour-associated genes early in carcinogenesis, and it is the DNA methylation that triggers repressive chromatin by recruiting chromatin modifying proteins, such as histone deacetylase and methylase enzymes. However, in this scenario, it is unclear what triggers the initial aberrant DNA hypermethylation. Early studies simply suggest that this could be a consequence of deregulation of the methylation machinery, such as over expression of DNMTs in cancer cells, as it has been demonstrated that over expression of *DNMT*1, 3A and 3B in cell lines results in an increase in CpG island methylation (80–83).

2. Hypermethylation is triggered by prior gene silencing in the cancer cell (84,85). If active gene transcription plays a role in protecting CpG islands from methylation in early development then programmed gene suppression during differentiation may result in these genes showing an increase susceptibility to stochastic *de novo* methylation, which in turn may act as 'methylation seeds' to trigger more extensive hypermethylation and repressive chromatin modification (84). In normal cells, epigenetic silencing of the *c-fms* locus during B-lymphopoiesis was found to occur in discrete steps with gene silencing followed by progressive CpG methylation and H3K9 de-acetylation (44). In cancer cells, CpG island methylation of a transfected *GSTP1* gene construct could be only be triggered after the construct was 'pre-seeded' with a low level of MeC and this initiated a cascade of sequential events including a spreading of the DNA methylation and histone H3K9 de-acetylation and H3K9 methylation (86,87). In addition, silencing of transgene transcription was found to precede methylation of promoter DNA and H3K9 methylation (88). Moreover, loss of *DNMT1* in human cancer cells is followed by a loss of H3K9 methylation (89). It is therefore conceivable that abnormal methylation in cancer effects both developmentally silenced genes, as well as tumour-specific genes that had been stochastically inactivated in individual cells, but where inactivation provided the cell with a selective growth advantage.

3. Aberrant histone modification causes gene silencing in the cancer cell which in turn catalyses DNA methylation by recruitment of the DNMT enzymes. It has been demonstrated that in human cancer cells, histone H3K9 deacetylation and H3K9 methylation precede re-silencing and re-DNA methylation of the p16 gene, which occurs after drug induced DNA demethylation and re-expression (90). However, in this experimental system 'seeds' of methylation would remain thus providing a platform to trigger the repressive histone modifications. Interestingly though are the observations that, mouse ES cells that were null for H3K9 histone methyltransferase displayed impaired DNA methylation, whereas *DNMT* deficiency in mouse ES cells did not impair H3K9 methylation (91).

4. Polycomb-induced H3K27 methylation is involved in gene silencing during development, but in cancer targets programmed DNA hypermethylation. A number of new reports have recently presented compelling evidence that polycomb proteins are involved in epigenetic silencing in cancer (92–97). *EZH2*, a member of the polycomb complex is elevated in many cancers and is responsible for histone H3K27 methylation (98,99). Interestingly, it has now been shown that in ES and progenitor cells, CpG island genes associated with pluripotency of stem cells are expressed at a low level and are marked by H3K27 methylation. Intriguingly, it is these polycomb target genes that constitute a significant fraction of genes also commonly found to be hypermethylated in cancer cells leading to a possible model that suggests that H3K27 methylation may trigger aberrant hypermethylation in cancer cells by recruitment of DNA methylation machinery. This may have relevance to the origin of cancer stem cells in that they may have lost their plasticity through aberrant methylation of the polycomb-repressed genes. Loss of plasticity could result in permanent repression of critical genes involved in the prevention of cell proliferation giving the cell a selective advantage (10).

WHAT TRIGGERS LONG RANGE EPIGENETIC SILENCING?

The mechanism that is involved in LRES of contiguous genes is open for debate. LRES predicts that even if some CpG island genes are targeted for methylation, neighbouring genes may also be affected by default. Are these the innocent bystander genes silenced in cancer? One possibility is that it is the density of CpG islands in the region that results in LRES. Interestingly, this was evident for the 1 Mb contiguously methylated zone on 2q14.2 and the 100 kb HOXA cluster on 7p15.2, which both contained 'hot spots' of CpG islands (69,73). It is intriguing to speculate that if 'seeds' of CpG methylation trigger hypermethylation of neighbouring sites, then maybe methylated CpG islands 'seed' or promote the spread of hypermethylation to the neighbouring CpG islands within the enriched CpG island zone. Similarly, it could be the density of subsequent DNA hypermethylation that recruits the histone methylation machinery and results in a global heterochromatinisation spreading between the CpG rich zones.

A significant question still remains as to why some CpG islands are highly susceptible to DNA methylation and epigenetic inactivation and yet the other CpG associated genes are protected from DNA methylation. In a normal cell, most of the CpG islands that remain unmethylated are actively transcribed (17) and conversely active transcription promotes demethylation if random sites are stochastically methylated (86). It is

therefore possible that the genes that are initially methylated in cancer are those genes that are expressed only at basal levels in normal cells and the genes resistant to methylation are expressed at higher levels in the normal cells, supporting the hypothesis that the level of active transcription is a key determinant in protecting CpG islands from hypermethylation in cancer. It is interesting to note that the genes that remain unmethylated in the 2q region in colorectal cancer are expressed at high levels in normal colon cells, but the genes that become methylated are quiescent in normal cells (69). In addition, to expression levels, CpG islands that became methylated in the cancer cells also appear to display a low level of methylation or 'seeds' of methylation in normal cells, whereas the CpG islands that were not methylated in the cancer were essentially devoid of 'seeding' methylation. Together these findings support the model that the genes that are methylated in cancer are those that are silent and seeded and gene repression alone is insufficient to promote DNA methylation (84,86), and the critical density of seeded DNA methylation may be important to permit subsequent spreading (100) and chromatin remodelling (101).

In contrast, to the 'silencing & seeding' model for promoting DNA hypermethylation, it has been suggested that the propensity for methylation is based on a 'sequence signature' that is associated with protection from or susceptibility to aberrant methylation in cancer (41–43). In addition, genome-wide analyses indicate that tumour-specific methylated genes belong to distinct functional categories, have common motifs in their promoters and are found in clusters on chromosomes, suggesting that *de novo* methylation occurs through an instructive mechanism (102). This model may also work by the signature sequence providing a platform for the spread of methylation 'seeds' due to the CpG density.

The observation of LRES suggests the presence of a control system for autosomal genes that can operate over a defined chromosomal region. In the established systems of programmed epigenetic silencing in X-inactivation and imprinting non-coding RNAs are critically involved in the process. As a working hypothesis it therefore is possible that non-coding RNAs could be involved in spreading a regional epigenetic state in LRES. Participation of such species in triggering transcriptional repression through chromatin modification is currently being defined in yeast and plant systems (103–106). Interestingly, non-coding RNA can target histone modifying enzymes. It is notable that 2q14.2 contains numerous CpG islands that are associated with non-coding RNA, therefore it will be interesting to determine if these play a role in the process of LRES and global heterochromatinisation.

CONCLUSION

The area of epigenetic silencing in cancer is indeed in a state of great enthusiasm because much of the epigenetic machinery is now being unravelled and there are exciting new genome approaches to epigenetic analysis which heralds the birth of the Human Epigenome Project (107,108). In the next few years, the temporal and spatial relationship between DNA methylation, chromatin structure and gene expression should become clearer and the role of epigenetic gene control in normal development and in deregulation in carcinogenesis will be less of a mystery. This knowledge is important as it underpins the success of the new breed of epigenetic drugs that act by releasing silent genes and gene regions from epigenetic suppression.

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