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Review

Epigenetic lesions causing genetic lesions in human cancer: promoter hypermethylation of DNA repair genes☆

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Abstract

The existence of genetic alterations affecting genes involved in cellular proliferation and death, such as *TP53* and *K-ras*, is one of the most common features of tumour cells. Recently, gene inactivation by promoter hypermethylation has been demonstrated. Methylation is the main epigenetic modification in mammals and abnormal methylation of the CpG islands located in the promoter region of the genes leads to transcriptional silencing. Examples include the $p16^{INK4a}$, $p15^{INK4B}$, $p14^{ARF}$, Von Hippel-Lindau (*VHL*), the oestrogen and progesterone receptors, *E-cadherin*, death associated protein (*DAP*) kinase and the first tumour suppressor gene described, retinoblastoma (*Rb*) gene. In most cases, methylation involves loss of expression, absence of a coding mutation and restoration of transcription by the use of demethylating agents. However, is there a linkage between genetic and epigenetic alterations? Our results show one side of this puzzle demonstrating that epigenetic lesions drive genetic lesions in cancer. Four specific epigenetic lesions, promoter hypermethylation of the DNA mismatch repair gene *hMLH1*, the DNA alkyl-repair gene O(6)-methylguanine-DNA methyltransferase (*MGMT*), the detoxifier glutathione S-transferase P1 (*GSTP1*) and the familial breast cancer gene *BRCA1* may lead to four specific genetic lesions, microsatellite instability, G to A transitions, steroid-related adducts and double-strand breaks in DNA. This is probably only the beginning of an extensive list of epigenetic events that change and make the genetic environment of the transformed cell unstable.

Keywords: DNA methylation; Tumour suppressor genes; DNA repair genes; hMLH1; MGMT; Epigenetics

1. Introduction: Gene inactivation by promoter hypermethylation in human cancer

The cancer cell differs from a normal cell in its genotype [1]. Gross alterations such as the aneuploidy state, more discrete deletions (loss of heterozygosity) or gains (gene amplification) of genomic material, and small changes (point mutations, small insertions or deletions) in multiple genes are scattered through the genome of a neoplastic cell. In fact, most of the cancer research in the last 20 years has focused on this aspect. However, the malignant cell has also acquired a different epigenotype. The inheritance of information based on gene expression levels is known as epigenetics, as opposed to genetics, which refers to information transmitted on the basis of gene sequence. The main epigenetic modification in mammals, and in particular in humans, is the methylation of the cytosine nucleotide residue. In a healthy cell, the DNA methylation patterns are conserved through cell divisions allowing the expression of the particular set of cellular genes necessary for that cell type and blocking the expression of exogenous-inserted sequences.

In a cancer cell, there is a clear distortion in the expression profiles and the presence of a dramatic change in the methylation patterns is one of the guilty parties. First of all, there is a dysregulation in the methylating enzymes, mainly DNA methyltransferases 1 (putatively responsible for the 'maintenance' activity), 3a and 3b (probably responsible for the '*de novo*' activity) in the malignant cell. Secondly, there is a global hypomethylation when compared with a normal cell; this is achieved due to a generalised demethylation in the CpGs scattered throughout the genes and may be involved in causing global genomic fragility [2] and re-expression of inserted endogenous retroviral sequences

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[3]. Finally, there are local and discrete regions normally devoid of methylation that suffer an intense hypermethylation [4]. In this paper, I am going to focus on this last event. Approximately half of gene promoters, and typically 'housekeeping genes', contain a region (from 0.5 kb to several kb) very rich in the dinucleotide CpGs, in contrast with the rest of the human genome that is depleted of CpG. These regions, called 'CpG islands', are methylation-free in normal cells, except in the imprinted genes and the inactive X chromosome in women, allowing the expression of the adjacent gene if the corresponding transcription factors are present. In cancer, several of these CpG islands became hypermethylated, shutting down the expression of the contiguous gene, such as a tumour suppressor gene and thereby enabling malignant growth. The process described is illustrated in Fig. 1. Examples of genes suffering this aberrant methylation include *p16INK4a*, *p14ARF*, *p15INK4b*, adenomatous polyposis coli (*APC*), death associated protein (*DAP*) *kinase*, *p73*, *E-cadherin*, von Hippel-Lindau (*VHL*), Retinoblastoma (*Rb*), *LKB1*/*STK11* and the oestrogen and progesterone receptors [4–6]. Furthermore, the silencing by promoter hypermethylation of a particular set of genes, DNA repair (*hMLH1*, O-6-methylguanine-DNA methyltransferase (*MGMT*) and *BRCA1*) and carcinogen detoxifiers glutathione S-transferase P1 (*GSTP1*), may change the whole genetic environment of that cell. Below, I will outline four different scenarios for the phenomenon of epigenetic lesions causing genetic lesions.

2. How *hMLH1* promoter hypermethylation solves 'the enigma' of microsatellite unstable sporadic tumours

A subset of human tumours displays a bizarre genetic phenotype defined by the microsatellite instability (MSI) phenomena. MSI+ tumours are defined because they show aberrant insertions or deletions of mono- or dinucleotides repeats when the tumours are compared with their normal counterparts [7]. These 'shifts' are only a measurable reflection of a 'defect' in the DNA mismatch repair (MMR) pathway that has been well characterised in the yeast model. MSI is a typical feature of tumours from patients with hereditary non-polyposis colorectal carcinoma (HNPCC). The tumour types mainly involved in the disease are colorectal, endometrial and gastric carcinomas. In these HNPCC

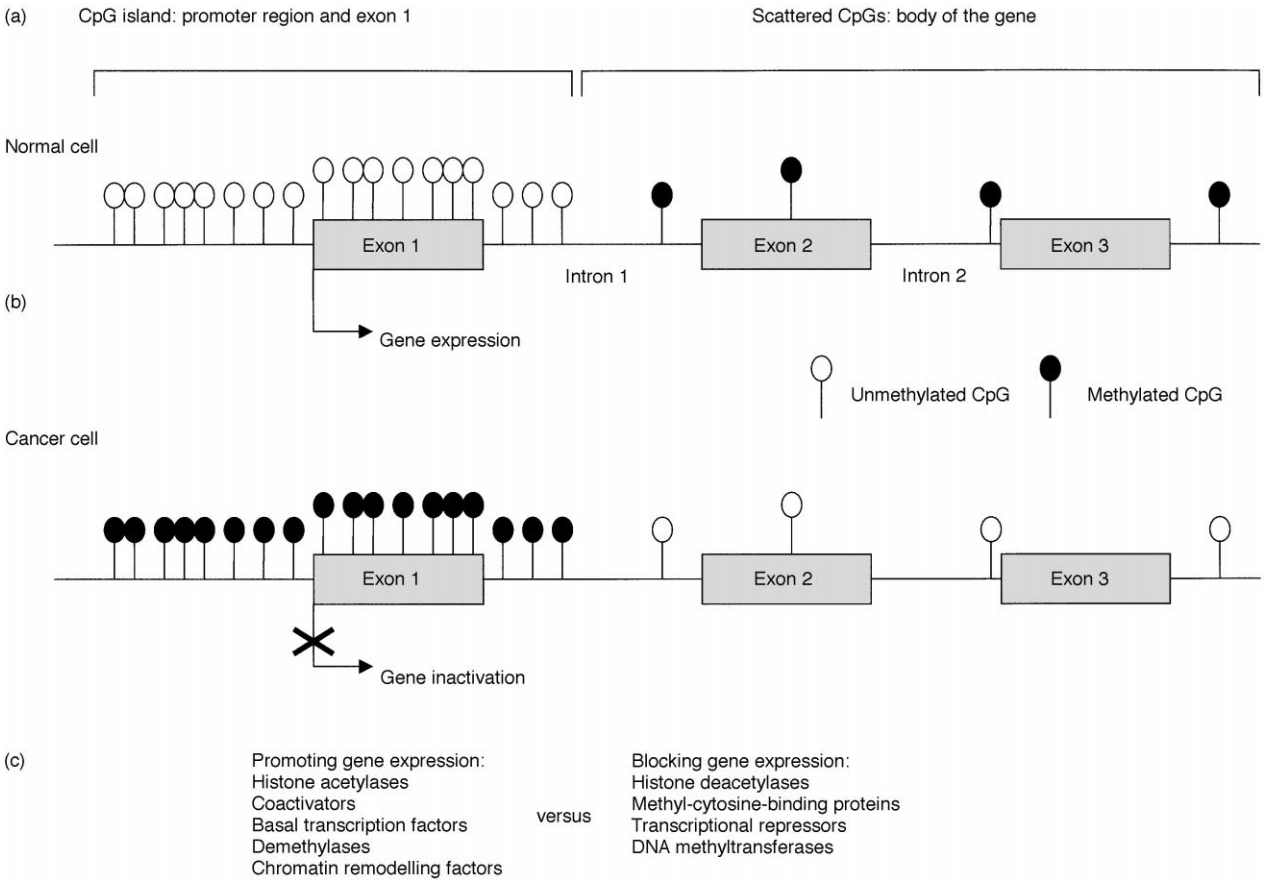

Fig. 1. Graphical representation of a typical gene with a CpG island. (a) In a normal cell the CpG island is devoid of methylation allowing gene expression. (b) In a cancer cell, the CpG island becomes hypermethylated preventing gene transcription. (c) Depiction of the multiple factors involved in the balance between gene expression and repression.

families, the defect is attributed to germ line mutations in the DNA mismatch repair genes, mainly *hMLH1* and *hMSH2* [8], while other components of the MMR pathway such as *hMSH3*, *hMSH6*, *hPMS1* and *hPMS2* seem to play a minor role in the disease. Because MSI+ tumours were also observed in spontaneous cases, genetic mutations in those same MMRs were assumed to be the cause for MSI in the sporadic cases. However, surprisingly, MMR mutations were found in less than 10% of sporadic MSI+ tumours [9]. The reason for this lack of MMR mutations is that the main cause of MSI in the sporadic cases is the transcriptional inactivation of *hMLH1* by promoter hypermethylation.

We and others have demonstrated a tight correlation between the presence of *hMLH1* hypermethylation and MSI+ tumours in colorectal [10,11], endometrial [12,13] and gastric tumours [14], the three tumour types common in HNPCC patients, while *hMLH1* methylation is absent in other tumour types [12]. In fact, among the studies described, 90% of MSI+ tumours are methylated at *hMLH1* (the remaining 10% probably have somatic mutations in either *hMLH1* or *hMSH2*), while the MSI− tumours are unmethylated in 95% of cases [11–14]. From the molecular epidemiological point of view, assuming that approximately 20% of sporadic colorectal, endometrial and gastric tumours present with MSI, we would then expect to find *hMLH1* promoter hypermethylation in 18% of unselected consecutive specimens of these tumour types.

The presence of *hMLH1* hypermethylation, documented by methylation-specific polymerase chain reaction (PCR), restriction cut analysis and sequencing, occurs in the context of a *hMLH1* gene without mutations and correlates with the lack of *hMLH1* expression at the RNA and protein level. No evidence of aberrant methylation of other MMR genes such as *hMSH2*, *hMSH3* and *hMSH6* has been found so far [12,13]. Further proofs of causality between *hMLH1* epigenetic silencing and the MSI+ phenotype have come from studies in cancer cell lines, where demethylating agents are not only able to reactivate the *hMLH1* gene, but are also capable of restoring the mismatch repair activity [11]. Finally, as expected, *hMLH1* promoter hypermethylation is an early alteration, appearing in the premalignant stages of processes such as atypical endometrial hyperplasia [12] and in ulcerative colitis lesions [15]. A third interesting observation is the existence of a certain degree of *hMLH1* hypermethylation in those tumours that present microsatellite instability, but only in a few loci [14,15]. These cells are perhaps caught in a 'temporal window' and, although MMR-deficient, are still progressing to a more apparent unstable phenotype. Supporting this notion, the existence of cancer cells defective in MMR without MSI has been demonstrated [16]. Thus, the data overall suggest that transcriptional inactivation by promoter hypermethylation of the mismatch repair gene *hMLH1* leads to MMR deficiency, further microsatellite instability and to inactivating mutations in multiple target genes such as *BAX* or transforming growth factor beta receptor II (*TGFBRII*).

3. The epigenetic defect in the DNA repair gene *MGMT* and the first mutation in an oncogene

In recent years we have seen a growing list of publications dealing with the description of 'which' oncogenes and tumour suppressor genes are mutated or altered, and 'where' it happens, providing us with a detailed 'molecular geography'. One question still remains almost untouched: 'how' does it happen? Very little information is known about the causes of mutations in key cancer genes. Interesting data in this area came from the tumour suppressor gene *TP53* [17]. Where benzopyrene, a strong carcinogen member of the polycyclic aromatic hydrocarbons and abundantly present in each cigarette, induces a typical G to T mutation in bulky covalent adducts formed in the DNA. Thus, not surprisingly, the majority of *TP53* mutations in lung tumours are G to T transversions [17].

What about the oncogenes? We have another base, guanine, that can undergo a chemical modification, the addition of a methyl or alkyl group. Chemical donors for this aberration may be the dietary nitrates reduced in the proximal colon by bacteria and nitrosated amines and amides derived from protein catabolism. The abnormally generated O^6-methylguanine is recognised as an adenine by the DNA polymerases and thus may generate G to A mutations. Cells are protected against this mutation by the DNA repair gene *MGMT* [18]. *MGMT* removes the promutagenic O^6-methylguanine from the DNA by transferring the methyl group to an active cysteine in its own sequence in a reaction that inactivates one *MGMT* molecule for each lesion repaired. *In vitro* mutational assays using the adenine phosphoribosyltransferase (*APRT*) gene, and *in vivo* experiments using transgenic mice overexpressing *MGMT* or knock-out mice defective in *MGMT* function, also demonstrate this genoprotective effect of *MGMT* [18].

In recent years, the loss of MGMT activity in a subset of human tumours, mainly brain malignancies, has been reported. However, the *MGMT* gene remains intact without any apparent mutations. Early experiments in cancer cell lines suggested that aberrant methylation was one mechanism involved in *MGMT* inactivation [19,20]. Following those data, we have recently shown that the DNA repair gene *MGMT* is transcriptionally silenced by promoter hypermethylation in primary human tumours [21]. An example of *MGMT* methylation and expression analysis is shown in Fig. 2. The pattern of epigenetic inactivation is very suggestive, affecting

approximately one-third of gliomas and colorectal tumours, and to a lesser degree, lymphomas, head and neck and non-small cell lung tumours [21]. As these cancer cells are deficient in *MGMT* DNA repair, they might accumulate many G to A transitions, some of them affecting key genes, in a similar way that loss of the *hMLH1* mismatch repair gene by methylation targets other genes. Where can we look first?

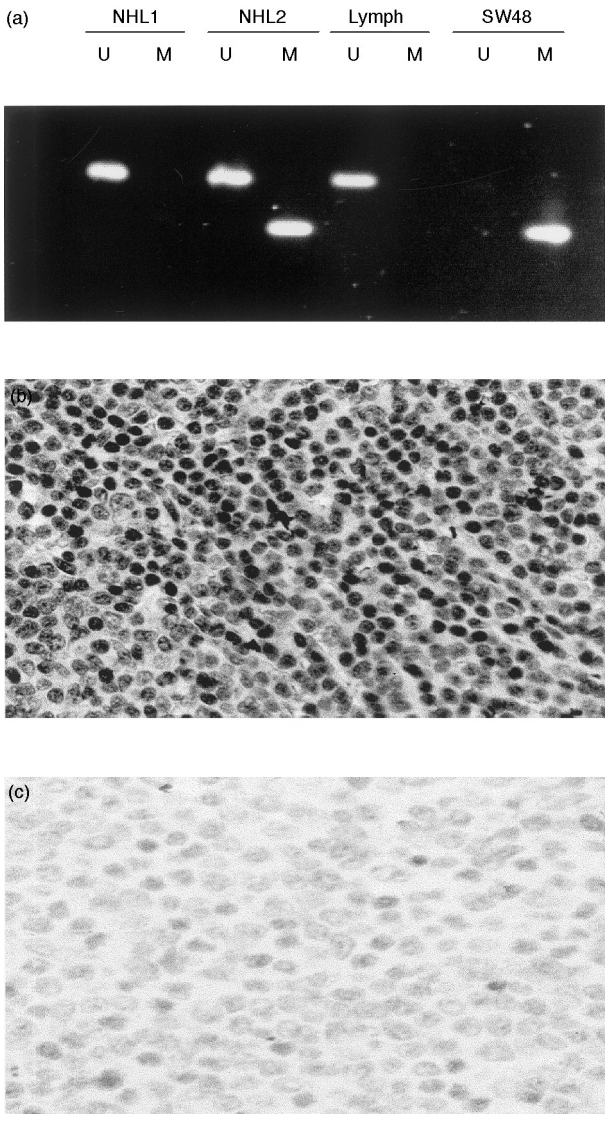

Fig. 2. Gene inactivation of the DNA repair gene O(6)-methylguanine-DNA methyltransferase (*MGMT*) by promoter hypermethylation. (a) Analysis of the methylation status of the *MGMT* CpG island by methylation-specific polymerase chain reaction (PCR) [21,23] in non-Hodgkin's lymphomas (NHL). The presence of a visible PCR product in lane *U* indicates the presence of unmethylated genes of *MGMT*, the presence of product in lane *M* indicates the presence of methylated genes of *MGMT*. Sample NHL2 is hypermethylated at *MGMT* while NHL1 remains unmethylated. Normal lymphocytes (Lymph) and the colorectal cancer cell line SW48 were used as negative and positive controls for *MGMT* methylation, respectively. (b) The sample NHL1, unmethylated at *MGMT*, shows strong expression of the MGMT protein. (c) The sample NHL2, hypermethylated at *MGMT*, shows loss of MGMT expression.

A clue is provided by some of the early work in oncogenesis activation. Simple methylating–alkylating agents such as nitroso-methylureas (i.e. MNU) were shown to induce mammary tumours that harbour a G to A mutation in the *H-ras* oncogene [22]. The *ras* oncogenes were the first transforming factors shown to be mutated in a human tumour. However, curiously no *ras* mutations have been described in breast primary human tumours. The most commonly mutated member of the *ras* family *K-ras*. Approximately 40% of colorectal tumours have *K-ras* mutations (most being G to A changes) and the DNA repair gene *MGMT* is also inactivated by promoter hypermethylation in 35–40% of colonic neoplasms. It is possible that the two events are linked. Our study [23] shows that the vast majority (71%) of colorectal tumours with G to A mutations in *K-ras* are deficient in *MGMT* repair due to epigenetic silencing, while in those tumours with other types of *K-ras* mutation, not involving the G to A change, or in tumours with wild-type *K-ras*, the *MGMT* promoter remains unmethylated in 70% of cases. The detection of *MGMT* aberrant methylation in small adenomas [23] preceding the appearance of *K-ras* mutations, again supports a cause–consequence model.

Thus, some questions are answered, but other new questions are raised. What is going in brain tumours? *MGMT* promoter hypermethylation is observed in 40% of gliomas, but is completely absent in meningiomas. In gliomas, neither G to A mutations in *K-ras* nor any other types of *K-ras* mutation are observed, suggesting that the direct activation of *K-ras* is not a genetic defect selected in the natural history of this tumour type. However, *MGMT* deficiency due to transcriptional inactivation by methylation still occurs. Which gene important for the biology of gliomas is the target? Importantly, we also have to consider the different kind of carcinogen exposure sensitivities that exist in brain and colon tumours, the first being more sensitive to the action of agents causing O6-ethylguanine. We should also remember that the MGMT protein has different repair capacities dependent on the type of radical added to the guanine [24]. Although further clarification is needed, *MGMT* epigenetic inactivation is likely to be a key element in tumorigenesis, causing multiple nucleotide changes in the genomic material.

4. Other candidates, other types of DNA damage: the cases of *GSTP1* and *BRCA1*

Finally, two more genes related to potential DNA lesions that undergo inactivation by promoter hypermethylation are discussed: the *GSTP1* and the breast cancer familial gene *BRCA1*.

GSTP1 does not repair DNA, but may prevent DNA damage. *GSTP1* is a member of the glutathione-S-transferase superfamily that catalyses the conjugation of the peptide glutathione with electrophilic compounds including carcinogens, resulting in less toxic and more readily excreted metabolites [25]. In the scientific literature, changes of *GSTP1* expression have been widely documented, but the cause of these changes was unclear until aberrant methylation of the *GSTP1* CpG island in prostate carcinoma was reported [26]. Following that study, we investigated approximately 400 primary tumours, demonstrating that *GSTP1* promoter hypermethylation is linked to loss of its expression in breast and renal tumours [27], but the vast majority of other tumour types do not show this epigenetic alteration ([27], and unpublished observations). Recently, *GSTP1* aberrant methylation has also been shown to be a common event in liver tumours [28]. Is there any linkage between prostate, breast, kidney and liver? One plausible explanation is that all the tumours derived from these tissues are related, although at different levels, to steroid hormone exposure. A putative pathway involving DNA damage induced by *GSTP1* promoter hypermethylation may be outlined as follows: the metabolism of oestrogens to catechol oestrogen quinones generates electrophilic intermediates [29] that are neutralised by their binding to glutathione in the presence of *GSTP1*. Epigenetic inactivation of *GSTP1* might lead to the accumulation of these dangerous compounds that covalently bind to DNA, forming apurinic stable adducts and perhaps mutations. Nevertheless, further confirmation of this work is necessary. The correlation between the presence of oestrogen-related adducts and the *GSTP1* epigenetic lesion and promising approaches to prevent tumours from these sites by stimulating the expression of *GSTP1* are currently under study.

BRCA1 is responsible for almost half of the cases of inherited breast cancer and the majority of familial ovarian cancer, but the scientific community was puzzled by the absence of *BRCA1* somatic mutations in sporadic breast and ovarian tumours. However, loss of *BRCA1* mRNA and protein does occur in these tumour types [30,31]. Our group [32] and others [33,34] have demonstrated that *BRCA1* promoter hypermethylation leading to loss of *BRCA1* function is present in breast and ovarian primary tumours and cell lines. We have also shown that biallelic inactivation of *BRCA1* is achieved in many cases by retention of one allele silenced by methylation in association with loss of the other allele by genomic deletion in this region [32]. Furthermore, particular breast histological subtypes overrepresented in the familial tumours are also especially rich in aberrant methylation [32]. What are the consequences of losing *BRCA1*? The strict cellular function of *BRCA1* is still unclear. A function in transcriptional regulation is suggested by the interaction of *BRCA1* with the RNA helicase A and the histone deacetylase complex, both key elements in the transcriptional machinery. But a role for *BRCA1* in DNA repair is also strongly supported. *BRCA1* is part of protein supercomplexes, where we can find other partners such as RAD51, BARD1, ATM, BRCA2, hMLH1, hMSH2, hMSH6 and ATM , that activate double-strand break repair and initiate homologous recombination [35,36]. The existence or not of a genomic context of chromosomal chaos (abnormal segregation, high aneuploidy, mutator phenotype) in those sporadic tumours with an epigenetic defect in *BRCA1* should be the focus of future investigations. Furthermore, the suspected reciprocity of *BRCA1* alterations with mutations in the 'guardian of the genome', *TP53*, in both familial and sporadic tumours is also worthy of further study.

5. Conclusions, perspectives and cross-talk: epigenetics and genetics

Each coin has two sides. I have outlined how epigenetic changes, promoter hypermethylation of DNA repair genes, cause genetic changes, from specific point mutations to gross genomic alterations. But it is likely that this is also a two-way road. Genetic alterations affecting genes involved in the establishment and maintenance of methylation patterns, such as the DNA methyltransferases (*DNMT1*, *DNMT3a* and *DNMT3b* are those currently known), putative demethylases (the proposed *MBD2*) and methyl-binding proteins (i.e. *MeCP2*); the histone acetylases (HATs such as *p300*, *pCAF* and *CBP*) and deacetylases (*HDAC1* and *HDAC2*); and the chromatin-remodelling factors (such as the SWI/SNF family) are first-choice candidates in this scenario. Supporting this hypothesis, germ line mutations in *DNMT3b* observed in the immunodeficiency, centromeric instability and facial abnormalities (ICF) syndrome are associated with abnormal methylation patterns in satellite DNA [37].

The picture that has emerged in recent years has shown us that cancer is a polygenetic disease, but also a polyepigenetic disease, where genes involved through multiple pathways from the cell cycle to apoptosis, from cellular adhesion to hormonal response are inactivated by promoter hypermethylation [4,5]. The patterns of epigenetic lesions are extremely specific in human cancer and reflect the idiosyncrasy and needs of each cell type as depicted in Fig. 3. This knowledge can be used as a tool for the monitoring of cancer patients [38–40] and the design of therapeutic approaches reversing the process are currently being studied. Nevertheless, when aberrant methylation shuts down the expression of DNA repair genes, such as *hMLH1*, *MGMT*, *GSTP1* and *BRCA1*, the cellular DNA is left unprotected in the

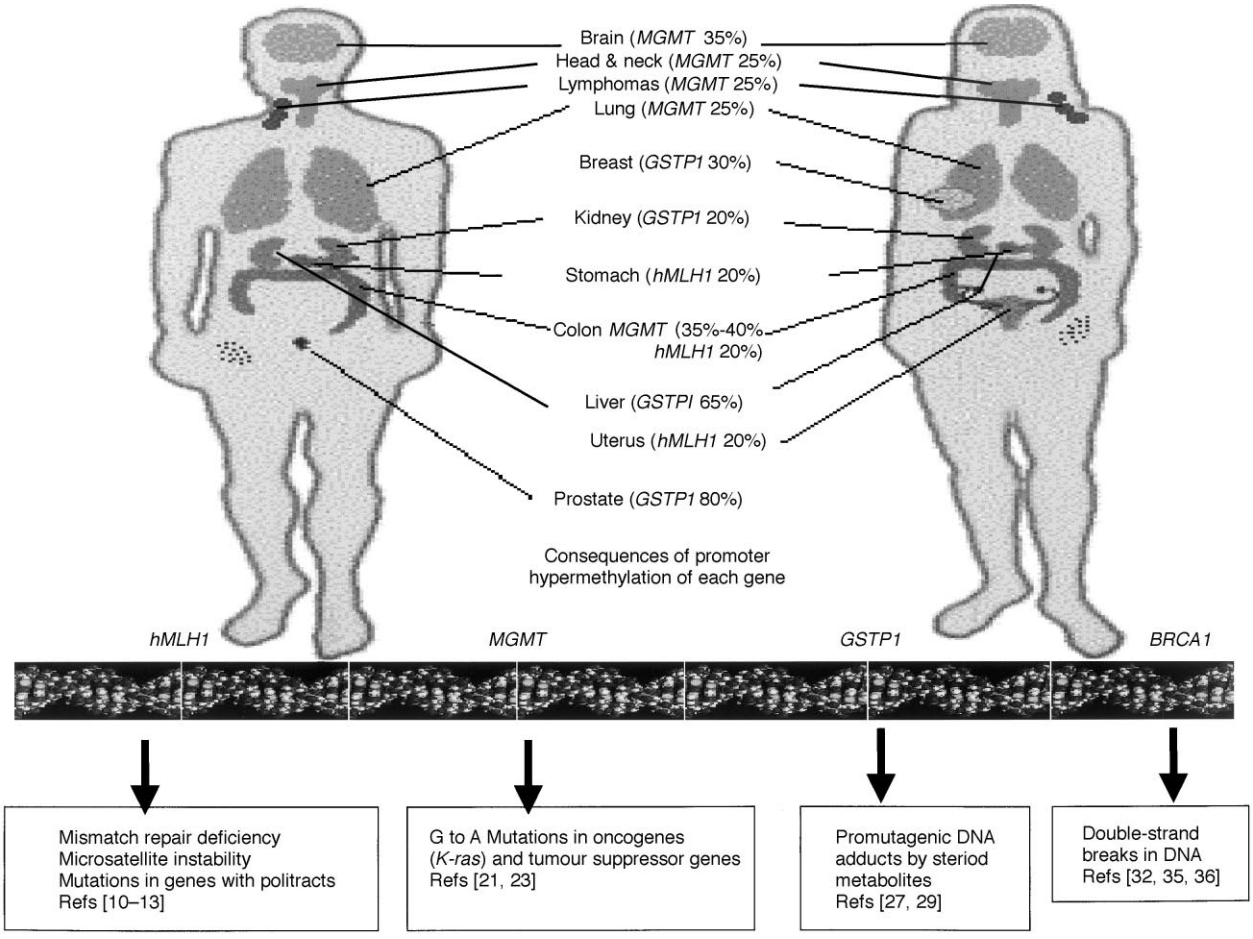

Fig. 3. Patterns of aberrant methylation. Distribution of promoter hypermethylation of DNA repair genes in human cancer and the consequences of their epigenetic inactivation.

face of exogenous chemical exposures and the reactive compounds produced endogenously. That cell will now acquire new genetic aberrations that will push it forward towards and along the non-returnable tumorigenic road.

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