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Review

DNA methylation patterns in lung carcinomas

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ABSTRACT

The genome of epithelial tumors is characterized by numerous chromosomal aberrations, DNA base sequence changes, and epigenetic abnormalities. The epigenome of cancer cells has been most commonly studied at the level of DNA CpG methylation. In squamous cell carcinomas of the lung, CpG methylation patterns undergo substantial changes relative to normal lung epithelium. Using a genome-scale mapping technique for CpG methylation (MIRA-chip), we characterized CpG island methylation and methylation patterns of entire chromosome arms at a level of resolution of ~100 bp. In individual stage I lung carcinomas, several hundred and probably up to a thousand CpG islands become methylated. Interestingly, a large fraction (almost 80%) of the tumor-specifically methylated sequences are targets of the Polycomb complex in embryonic stem cells. Homeobox genes are particularly overrepresented and all four *HOX* gene loci on chromosomes 2, 7, 12, and 17 are hotspots for tumor-associated methylation because of the presence of multiple methylated CpG islands within these loci. DNA hypomethylation at CpGs in squamous cell tumors preferentially affects repetitive sequence classes including SINEs, LINEs, subtelomeric repeats, and segmental duplications. Since these epigenetic changes are found in early stage tumors, their contribution to tumor etiology as well as their potential usefulness as diagnostic or prognostic biomarkers of the disease should be considered.

1. Mammalian DNA methylation

The only known enzymatic modification of DNA bases in mammalian cells is the post-replicative addition of a methyl group to position 5 of cytosines. The methylated cytosines are almost exclusively formed at the CpG (5′) dinucleotide sequence. CpG methylation is catalyzed by DNA methyltransferase proteins (DNMTs). DNA methyltransferase 1 (DNMT1) is responsible for faithful copying of the preexisting cellular DNA methylation patterns following DNA replication. DNMT3A and DNMT3B are primarily thought of as de novo DNA methyltransferases responsible for methylation of previously unmethylated CpG sites [1] although all DNMTs are generally important for the maintenance of methylation patterns [2]. The DNMT2 protein was initially characterized as a DNA methyltransferase [3] but more recently has been shown to mediate tRNA methylation [4]. Removal of methyl groups from DNA cytosines can be accomplished by a passive 'dilution' process involving DNA replication in the absence of DNMT proteins. Alternatively, the methyl group or the entire methylated base may be removed in an active enzymatic pathway. However, the exact nature of the putative mammalian DNA demethylase has remained obscure and controversial [5]. The distribution of CpG sequences along mammalian chromosomes is not uniform. Sequences near transcription start sites, and often including the first exon and intron of a gene, have a much higher frequency of CpG dinucleotides than the rest of the genome. These sequences are called CpG islands [6]. Only about half or less of all CpG islands, however, are associated with protein-coding genes leading to the assumption that CpG islands may have other regulatory roles. CpG islands are thought to remain completely unmethylated in the germ line thus avoiding mutational erosion and CpG loss due to methylation-associated mutagenic mechanisms [7]. Methylation of CpG islands near promoters leads to gene inactivation by several known mechanisms. The binding of certain transcription factors is directly prevented by DNA CpG methylation [8]. Methylated DNA sequences are bound by specialized proteins that have a high affinity for methylated DNA. Examples are MeCP2, MBD1, and MBD2. These methyl-CpG binding proteins have the ability to recruit histone deacetylase complexes upon binding to mCpG DNA [9]. The CpG-methylated DNA is often associated with inactive chromatin marks, including deacetylated histones H3 and H4, histone H3 lysine 9 (H3K9) methylation and histone H3 lysine 27 (H3K27) methylation, chromatin configurations, which reinforce the inactive gene expression state.

2. DNA methylation changes in cancer

Changes in DNA methylation patterns are one of the most frequent events that occur in human tumors, and altered CpG

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methylation patterns discriminate tumor tissue from its nonmalignant counterpart tissue or normal adjacent tissue [10]. Two types of methylation changes are most commonly observed: hypermethylation of CpG islands and a more global hypomethylation of DNA in tumors. The literature now contains thousands of reports that have documented methylation of CpG islands associated with hundreds of different genes, including almost every type of human solid tumor or hematological malignancy. It is unlikely that all of these methylation changes play a causative role in tumorigenesis, and it is a challenge today to pinpoint those crucial genes that are susceptible to methylation-associated gene silencing and are functionally important in preventing tumorigenesis. Tumor-specific methylation may provide a means for detection and early diagnosis of cancer. Identification of methylated CpG islands in easily accessible biological materials such as serum, sputum or urine has the potential to be useful for the early diagnosis of lung cancer and other malignancies [11–13]. If methylation of CpG islands were a critical parameter in tumor maintenance or progression, it would be desirable to reverse DNA hypermethylation. This can be accomplished, at least transiently and in vitro, by treatment of cells with inhibitors of DNA methylation. The prototype of such inhibitors is 5-azacytidine [14]. The potential clinical use of 5-azacytidine and other more recently developed DNA methylation inhibitors as anti-cancer drugs is now being explored by many investigators.

Repetitive DNA elements, such as short and long interspersed nuclear elements (SINEs and LINEs) and other repeat sequences are often hypomethylated in tumors [15–23]. While it seems plausible that methylation-induced silencing of a critical tumor suppressor gene can be an important event in tumorigenesis, the biological significance of tumor-associated DNA hypomethylation is less clear [17,22]. Mouse models of DNA hypomethylation have suggested that loss of methylation can lead to tumor formation. Mice carrying a hypomorphic allele of DNMT1 are susceptible to the development of aggressive T cell lymphomas [24]. One hypothesis that links hypomethylation mechanistically to tumorigenesis is the induction of genomic instability by hypomethylation, either by reactivation of transposable elements or by chromosome rearrangement events directly associated with hypomethylation [25–28].

The mechanisms of CpG island hypermethylation in cancer are mostly unknown. Specific DNA sequences within CpG islands may be associated with the methylation process [29,30]. Whether these sequences are associated with DNA binding proteins in vivo that somehow attract methylation is not known. Others have proposed that gene inactivity imposed by changes in chromatin structure or histone modification predisposes to DNA methylation [31–34]. Specific chromatin configurations may either protect from methylation or may promote DNA methylation at CpG islands. Trimethylation of histone H3 lysine 4 (H3K4me3) is associated with active or potentially active genes and unmethylated CpG islands [35]. This modification interferes with binding of the de novo DNA methyltransferase DNMT3L/DNMT3A complex [36,37]. Trimethylation of histone H3 lysine 27 (H3K27me3), the histone modification mark established by Polycomb repressive complexes [38], is often associated with repression of developmentally regulated genes. Presence of this mark in stem cells has been associated with DNA methylation of the same sequences in human cancers [39–42] (see also below). Although this connection is mostly based on indirect comparisons between different cell types, the striking coincidence of H3K27me3 and DNA CpG methylation suggests a mechanistic connection that could explain methylation patterns in tumor cells. Our lack of understanding of these mechanisms is at least in part related to a lack of a comprehensive picture of genome-wide chromatin structure and DNA hypermethylation events in tumors, somatic stem cells and tumor progenitor cells.

3. DNA methylation and lung cancer

Lung cancer is the leading cause of cancer death in the United States and most other countries [43]. Its causation by cigarette smoking is unquestionable [44]. Lung cancer accounts for about 30% of all deaths from cancer and at least 1.5 million annual deaths from lung cancer are projected worldwide by 2010. The high (>80%) mortality rate associated with lung cancer is at least in part related to suboptimal therapeutic strategies and the lack of an efficient screening approach for early detection. In comparison, breast, colon or prostate cancers, for which early detection approaches exist, have much higher survival rates. Lung cancers are divided into small cell (SCLC) and non-small cell lung carcinomas (NSCLC) depending on histology and cellular origin. Non-small cell lung cancers are further classified on the basis of histological parameters into three subtypes: squamous cell carcinoma (SCC), adenocarcinoma (ADC) and large cell carcinoma (LCC). Squamous cell carcinomas often affect the central airways while adenocarcinomas arise in the peripheral areas of the lung.

During tumorigenesis, both alleles of a tumor suppressor gene need to be inactivated, for example by chromosomal deletions or loss-of-function mutations in the coding region of a gene. As an alternative mechanism, hypermethylation of CpG islands spanning the promoter regions of tumor suppressor genes (for example, *RB*, *p16*, *VHL*, *APC*, *MLH1*, *RASSF1A* and *BRCA1*) is a common and important mechanism in carcinogenesis [45–50]. Since hypermethylation generally leads to permanent inactivation of gene expression, this epigenetic alteration is considered to be a key pathway for long-term silencing of tumor suppressor genes.

The importance of CpG island methylation in functional inactivation of lung cancer suppressor genes is becoming increasingly recognized. From initial analysis of a subset of genes, it has been estimated that between 0.5% and 3% of all genes carrying CpG-rich promoter sequences may be silenced by DNA methylation in advanced stage lung cancer [46,51]. We recently reported that several hundred (~200–800) CpG islands are methylated in individual stage I squamous cell carcinoma of the lung [23]. Some of the hypermethylated genes may be *bona fide* tumor suppressor genes, but in other cases the methylation event may be a consequence of preexisting tissue-specific gene silencing or may somehow be associated with tumor formation rather than being a cause of tumorigenesis. Several specific CpG-island-associated genes are methylated in lung cancer including, for example, *p16*, *RASSF1A*, *RARbeta*, *MGMT*, *GSTP1*, *CDH13*, *APC*, *DAPK*, *TIMP3*, and many others [52–58]. The methylation frequency (i.e., the percentage of tumors analyzed that carry methylated alleles) ranges from only a few percent to more than 80% for these genes. These methylation frequency numbers often differ substantially depending on the study population, tumor histology, and/or methodology used to assess CpG island methylation. In our laboratory, the tumor suppressor gene *RASSF1A* has been identified and characterized [47]. *RASSF1A*, which is localized at 3p21.3 in an area of common deletion or heterozygous loss in lung cancer, is inactivated by promoter methylation in about 30–40% of non-small cell lung cancers (37% of squamous cell carcinomas) and in close to 80% of small cell lung cancers [47,59–61].

4. DNA methylation detection methods

The field of DNA methylation analysis is moving fast towards genome-wide characterization rather than studying methylation of individual genes in tumors. Diverse technical approaches for large-scale methylation analysis have been developed [62]. The first group of techniques is based on methylation-sensitive restriction endonuclease cleavage of the target sequences (e.g., HpaII, NotI) [63,46,64]. These techniques are useful but are limited by the occurrence of the