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Prostate Cancer Epigenome

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Abstract

Prostate cancer is a major health burden within the ever-increasingly aging US population. The molecular mechanisms involved in prostate cancer are diverse and heterogeneous. In this context, epigenetic changes, both global and gene specific, are now an emerging alternate mechanism in disease initiation and progression. The three major risk factors in prostate cancer: age, geographic ancestry, and environment are all influenced by epigenetics and additional significant insight is required to gain an understanding of the underlying mechanisms. The androgen receptor and its downstream effector pathways, central to prostate cancer initiation and progression, are subject to a multitude of epigenetic alterations. In this review we focus on the global perspective of epigenetics and the use of recent next-generation sequencing platforms to interrogate epigenetic changes in the prostate cancer genome.

Keywords

Androgen receptor; Epigenome; Methylation; Prostate cancer

1 Prostate Cancer

Prostate cancer (PCa) is a complex, multifactorial disease that continues to be a significant factor in morbidity and mortality in the USA. Age, geographic ancestry, family history of (PCa), and more recently environmental factors are the only established risk factors. Many putative risk factors [1–3] have also been implicated, but their role in PCa etiology remains unclear (reviewed in ref. 4). Familial PCa is a genetically heterogeneous disease suggesting that many gene loci rather than a specific major susceptibility gene are responsible for its predisposition ([5] and reviewed in ref. 6). The highest incidence rates for PCa in the world are among African-American men [7]. Mortality from PCa among African-American men is also more than double that of men of European ancestry.

2 Known Mechanisms in Prostate Cancer

Over the past several years significant efforts have been invested in understanding the molecular basis of prostate cancer. A number of mouse models (*Pten* $-/-$ [8], *Myc* hi/lo [9], *Nkx3-1* $-/-$ [10], and *Id4* $-/-$ [11] and reviewed in ref. 12) have been developed that recapitulate several aspects of human disease initiation and progression. Most if not all of the studies have pointed towards the central role of androgen receptor (AR) in prostate cancer [13, 14]. AR, a member of the nuclear receptor superfamily, is required for normal prostate function and is involved in cyto-differentiation of the prostate epithelial cells [15]. In a majority of prostate cancer cases, AR function contributes to the survival and proliferation of cancer cells in primary disease and in most cases the presence of AR

continues to be indispensable after progression to hormone- independent disease. However, the prostate epithelial specific AR knockout mouse [16, 17] also develops prostate cancer, suggesting that AR function is not absolutely required for disease development. Also unlike other cancers (most notably breast and colorectal) where a familial mutation in a single gene leads to a higher risk of developing the respective cancers, no such major risk allele has been identified for prostate cancer to date. The heterogeneity of prostate cancer [18] suggests that there could be multiple initiating events leading to inactivation of tumor suppressors and/or activation of tumor promoters/oncogenes that could at some point of disease progression cross-talk with AR [18]. These early or late events may promote the transition of androgen receptor from a tumor suppressor to an oncogene.

3 Epigenetics

The definition of epigenetics has evolved over a period of time since the term was introduced by Waddington in 1942 as “causal interactions between genes and their products, which bring the phenotype into being” [19]. In broadest terms, epigenetics is a bridge between genotype and phenotype—a phenomenon that changes the final outcome of a genetic locus without changing the underlying DNA sequence [20]. Chromatin modifications, both covalent and non-covalent, are currently considered as hallmarks of changes in an epigenetic landscape [21]. Both DNA and histones, two basic components of chromatin, undergo these modifications to influence chromatin structure and gene expression. Depending upon the type and location, these modifications alter multiple cellular processes such as gene regulation [22], DNA replication [23, 24], cellular differentiation [25], and stem cell development [26].

3.1 Epigenetics and Cancer

Multiple genetic changes must occur to promote the cancer phenotype [27]. According to the classical mechanism, genomic instability (deletions or translocations) due to inactivating mutations in tumor suppressor genes or gain of function mutations in oncogenes resulting in uncontrolled growth is considered as a tumor initiating event. More recently, loss of function (expression) through epigenetic changes due to altered pattern of DNA methylation and histone modification is gaining significant attention as an additional pathway involved in cancer [28–30]. Thus combined genetic and epigenetic changes are now considered as cancer initiating events. As discussed above, aging, environment, and ancestry are the three major risk factors in prostate cancer. All three of these risk factors are intricately linked to epigenetic alterations in genomes that can lead to global changes in gene expression patterns which are generally pro-tumor. Several epigenetic alterations, such as global hypomethylation and CpG island hypermethylation, are progressively accumulated during aging and directly contribute to cell transformation [31].

Recent advances suggest that epigenetic mechanisms mediate the effect of environmental factors on the genome by disrupting cellular processes which contribute to higher cancer risk [32]. More recently, the concept of generational threshold in prostate cancer incidence is also gaining attention. Prostate cancer risk is usually low in recently migrated populations but within three generations, the risk of prostate cancer among these migrant populations is

almost similar to or higher than the native host country population [33]. This generational threshold appears in part due to epigenetic alterations (parental/offspring adopting a more westernized lifestyle) and not due to genetic mutations in the F0/F1 generations. These epigenetic changes could predispose the inherited genome of subsequent generations to mutagenic/genotoxic alterations leading to the development of sporadic prostate cancer [33].

Differences in epigenetic changes are also observed that associate with the high risk of prostate cancer seen in African American men [34]. Specifically, increased methylation is observed for *NKX2-5* and *TIMP3*, genes known to be involved in prostate carcinogenesis, in the normal prostate of African American men when compared to that of Caucasians [35].

3.2 Histone Modifications Versus DNA Methylation and a Link Between These Two Events in Relation to Prostate Cancer

Histones are no longer considered as simple “DNA packaging” molecules as they are involved in the dynamic regulation of a large set of genes through reversible posttranslational modifications that occur on their N-terminal tails, which are rich in positively charged amino acids [36]. The histone posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization [37, 38]. Among these modifications, acetylation and methylation are well studied in prostate cancer. Evidence indicates that the accumulation of acetylation and methylation at specific histone lysine or arginine residues plays a prominent role in gene regulation [38, 39]. In general, histone acetylation is associated with gene activation whereas histone de-acetylation is correlated with gene silencing [40, 41]. Histone methylation appears to play a more complex role in gene regulation. Methylation of H3K4 and to a lesser extent H3K48 and H3K79 are associated with gene activation whereas methylation at H3K9 and H3K27 are associated with gene silencing [42]. Some promoters simultaneously carry both gene-activating H3K4me3 and -inactivating H3K27me3 marks and are known as bivalent promoters [43]. Such bivalent marks could be required for the maintenance of embryonic stem cell gene expression patterns [43]. Whether such modifications are also present in cancer stem cells is proposed but remains to be investigated [44]. In prostate cancer increasing evidence suggests that large numbers of histone modifications are associated with changes in histone-modifying enzymes such as histone methyltransferases (HMTs) and histone acetyltransferases (HATs) [29]. Histone acetylation and deacetylation catalyzed by a group of enzymes collectively termed as histone acetyltransferases (HAT) and histone de-acetylases (HDAC) respectively, have emerged as the central mechanism in regulating various events leading to prostate cancer [45]. Multiple proteins with HAT and HDAC (HDAC1-11) activities are known that also target nonhistone proteins [46]. As would be expected, certain HDACs are increased in prostate cancer (HDAC1, HDAC2, and HDAC3) (reviewed in ref. 47). Paradoxically, the expression of HAT p300 is also increased in prostate cancer. In prostate cancer, HATs such as p300/CBP may target nonhistone proteins such as AR. Acetylation of AR is a gain-of-function modification that promotes prostate cancer cell growth [48].

DNA methylation includes the addition of a methyl group to cytosine residues of CpG dinucleotides [49]. DNA methylation in mammals is established by two DNA

methyltransferase enzymes. Current evidence suggests that members of the DNMT3 family (DNMT3A and DNMT3B) are involved in de novo methylation while DNMT1 is involved in the maintenance of methylation. De novo DNA methylation of certain gene promoter associated CpG dinucleotides occurs rarely in normal prostate epithelial cells but its frequency increases in prostate cancer. This phenomenon is in part thought to be supported by an increase in the expression of DNMT1 in prostate cancer cells when compared to their normal counterparts [50]. Thus DNA methylation in the promoter region of tumor-suppressor genes is likely an important epigenetic mechanism involved prostate cancer initiation and progression [51].

Histone modifications and DNA methylation can occur independently of each other; however initial histone modifications can lead to altered DNA methylation. Significant cross talk exists between the histone modifying and DNA methylating machinery [52, 53]. An example includes the interactions between EZH2, the SET domain methyltransferase, part of the polycomb repressive complex 2 (PRC2), and DNA methyltransferases [54, 55]. For instance, it has been observed that *MYT1*, a gene which is regulated by EZH2, is also regulated by DNMTs [55]. Almost 47 % of the genes regulated by DNMT3B are also bound by the PRC1/PRC2 complexes in colon cancer [56]. However, there are other studies that show that EZH2 occupancy at a given locus is not associated with DNA methylation at that same locus. For example, a recent study showed that *CDH1* and *KLF2*, both known to be regulated by EZH2 in prostate cancer, have no alterations of DNA methylation status on their promoters upon EZH2 overexpression. This observation suggests that DNA methylation has no role in *CDH1* and *KLF2* repression, mediated through EZH2 [57].

Histone deacetylases and the DNA-binding proteins associated with them could also be attracted to regions of methylated DNA during chromatin modification. Methylated DNA-binding proteins, such as MBD2, also interact with the nucleosomal remodeling complex (NuRD) and direct the complex to methylate DNA [58]. Chromatin-immunoprecipitation (ChIP) coupled with meta-analysis also showed a link between polycomb group protein (PcG)-mediated tri-methylation on H3K27 and de novo DNA methylation in cancer [59]. Genome-wide analysis combined with methylation-predicted models revealed that CpG islands which are methylation prone have a strong association with embryonic targets of PRC2 and a subset of PRC2 targets were also more likely to be hypermethylated in cancer (reviewed in ref. 60).

Changes in HMTs and HATs can result in changes in histone modification at both the global and gene-specific levels. Global histone modifications (acetylation and methylation) are also predictive of clinical outcome in prostate cancer. Histone acetylation and di-methylation of five residues in histones H3 and H4 (acetylation: H3K9, H3K18, and H4K12 and di-methylated H4R3 and H3K4) identified two prostate cancer disease subtypes with distinct risks of tumor recurrence in patients with low-grade prostate cancer. These histone modification patterns were predictors of outcome independently of tumor stage and preoperative prostate-specific antigen levels, and were also associated with invasion [61]. In another study, H3K4me1, H3K9me2, H3K9me3, H3Ac, and H4Ac were significantly reduced in prostate cancer as compared to nonmalignant prostate tissue [62].

3.3 Gene-Specific Epigenetic Changes in Prostate Cancer

In addition to recently discovered global epigenetic changes in prostate cancer, many gene-specific epigenetic modifications are also observed in prostate cancer which are summarized in Table 1 [57, 63–70].

3.3.1 Epigenetic Inactivation of Id4 in Prostate Cancer—The inhibitor of differentiation-4 helix-loop-helix protein (ID4) is a member of the inhibitor of differentiation gene family (along with Id1, Id2, and Id3) and acts as a dominant negative transcriptional regulator of basic helix-loop-helix (bHLH) family of transcription factors. Our recent studies have shown that ID4 is highly expressed in the normal prostate but is epigenetically silenced in prostate cancer due to promoter hyper-methylation [70, 71]. Prostates from Id4^{−/−} mouse models display frequent PIN like lesions that supports its role as a tumor suppressor [11].

4 Epigenetic Regulation of AR Dependent Pathways

The AR itself as well as its downstream pathways are regulated by diverse epigenetic mechanisms in prostate cancer. As might be expected, these epigenetic modifications in the AR pathway are generally defined as pro-tumorigenic. Studies have shown that histone lysine methylation status contributes significantly to AR signaling which correlates with an increased expression of several histone demethylase proteins (LSD1, JMJD2C, GASC1, and KDMC4) in prostate cancer [72–74]. Most of the histone demethylase proteins physically interact with AR to facilitate its recruitment to androgen response element (ARE) sites and subsequent activation of the downstream target genes [73]. AREs are particularly enriched for H3K4me1 or H3K4me2, generally associated with transcriptional enhancers [75].

5 Role of Histone Methyl-Transferase in Prostate Cancer

The expression of EZH2, a key component of the PRC2 complex, which specifically trimethylates H3K27, is increased in prostate cancer [64, 76]. The consequence of high EZH2 expression together with the observations that show increased mutations in KDM6A [72, 73] which can de-methylate H3K27me3 suggests the inactivation of genes primarily associated with prostate differentiation. A surprising new role of EZH2 has also emerged in prostate cancer. Recent studies have shown that EZH2 could also act as a co-activator of AR and requires the intact methyl-transferase activity EZH2 [77].

6 Data Sources for Detection of Epigenetically Altered Genomic Loci in Prostate Cancer

Until recently, epigenetic analyses for DNA methylation and histone tail modification were carried out on *individual* genetic loci by assays that utilized traditional DNA sequencing or polymerase chain reaction (PCR) of genomic DNA that had been modified by bisulfite treatment or enriched by chromatin immunoprecipitation (ChIP) with antibodies against specific histone tail post translational modifications. However with the advent of next-generation sequencing (NGS) and high-density arrays, and global genomic profiles of both DNA methylation and histone tail modifications, generated using techniques such as methylation-sensitive restriction enzyme sequencing (MRE-SEQ), methylated DNA

immunoprecipitation sequencing (MEDIP-SEQ), reduced representation bisulfite sequencing (RRBS) [78], array hybridization, and ChIP-SEQ, are now widely available for public interrogation [79–82].

Multiple cultured cell types and tissues have been investigated and the quantitative data have been published and deposited in publicly accessible databases found on the World Wide Web where all of the data can be accessed by individuals for visualization and/or download for their own use. Experiments using cell culture models and human tissues, representing both prostate cancer epithelia as well as healthy prostate epithelia, have been profiled. Here, we present examples of two data sources that are useful for prostate cancer researchers interested in epigenetic alterations. These examples are not meant to be exhaustive and will likely have been superseded or augmented with additional data by the time the reader is performing their specific research. One example is the UCSC Genome Browser (<http://genome.ucsc.edu>) site that contains reference sequence and working draft assemblies for a large collection of genomes. In addition, this site contains data and visualization tracks for independent research results including those interrogating the underlying chromatin structure of complete genomes. One must be familiar with the basic workings of the UCSC genome browser and track visualization methods in order to take advantage of these data sets. We refer readers to the “Guide to the UCSC Genome Browser” (<http://www.nature.com/scitable/ebooks/guide-to-the-ucsc-genome-browser-16569863>) for an easily accessible introduction to its usage.

One specific model system for examining comparative DNA methylation profiles in prostate cancer can be found on the Human Feb. 2009 (GRCh37/hg19) Assembly on the UCSC Genome Browser. This assembly contains a super track under the “Regulation” section of tracks named “ENC DNA Methyl” that contains DNA methylation data that was produced using several experimental methods by the ENCODE production groups (<http://encodeproject.org>) [79]. A track named “HAIB Methyl RRBS” from within this supertrack contains Reduced Representation Bisulfite Seq data. Replicate subtracks for a representative sample of the prostate epithelial cell line, PrEC (Lonza-CC-2555), and for LNCaP (ATCC CRL-1740) are contained within this track. The user can choose to display on the UCSC Browser only the sub-tracks for these cell lines in addition to the RefSeq Gene Track and CpG island track for additional genomic annotations to aid in result interpretation. Representative images for our favorite prostate cancer-related genes, *ID4* and *ZIC2*, are represented in Fig. 1. The UCSC Genome Browser allows one to view the entire gene of interest to identify regions of differentially methylated CpG dinucleotides and their overlapping coordinates relative to the various parts of the gene of interest and any neighboring CpG islands (Fig. 1a, c). Alternatively the user can zoom in to the level of nucleotide visualization and see the actual genomic DNA sequence surrounding the individual CpG that is being interrogated (Fig. 1b, d). In this figure, individual CpGs are represented by tick marks on the tracks labeled on the left, where green represents unmethylated CpGs or 0 % methylation, yellow represents 50 % methylation and red indicates 100 % methylation. One can easily distinguish between areas that are unmethylated in PrEC and methylated in LNCaP, indicating hypermethylation has occurred in cancer in the 5′ region of *ID4* and in the 3′ region of *ZIC2*.

Another example using a modified version of the UCSC Genome Browser, known as the UCSC Cancer Genomics Browser, can be found at <https://genome-cancer.ucsc.edu>. This site contains data for copy number variation, mutation, gene expression, DNA methylation and other genomic interrogations of multiple organ specific cancer tissues from “The Cancer Genome Atlas” (TCGA) (<http://cancergenome.nih.gov>) as well as from other sources. Again we refer the reader to the User Guide under the Help section of this site (<https://genome-cancer.ucsc.edu/proj/site/help/>) for detailed instructions on viewing specific data from within the Cancer Genomics Browser. The prostate cancer data from TCGA includes data from currently over 300 prostate adenocarcinoma (PRAD) tissue samples that have been interrogated for multiple genomic assays including global DNA methylation by the HumanMethylation450 BeadChip (www.illumina.com), gene expression by RNA-Seq, and other genome-wide cancer assays. The TCGA prostate samples also include results from adjacent normal epithelia as well as epithelia from non-diseased prostate tissue. The user can load both DNA methylation and gene expression data vertically contiguous on the browser to allow for additional functional genomic interpretation and hypothesis generation about the correlated consequences of specific DNA methylation patterns and associated gene expression levels. This allows the user to compare the groups of samples (normal versus cancer) against each other from within the single assay as well as between different assays. For example, Fig. 2a shows the results of DNA methylation in the top panel and exon-level gene expression by RNA-SEQ in the lower panel for *ID4* in PRAD. The results on the Cancer Genomics Browser are depicted in heat-map format with the genomic loci representing the column(s) of the heat map and the samples represented in rows. Samples can be labeled further with clinical annotations such as normal versus cancer, Gleason score, and primary pattern. In each experimental panel (e.g., DNA methylation or RNA-SEQ) normal and cancer samples are sorted from bottom to top, respectively, and are represented by pink and blue coloring in the sample type annotation column found on the right of each individual heat map. Additional sample annotation columns, selected by the user, can also be viewed next to sample type. Samples are sorted by the annotation column that is displayed immediately to the right of the heatmap. The user has the option to sort by any of the annotation columns that are displayed by moving it to the first position adjacent to the heatmap.

It is readily observable from the normalized exon level gene expression heat map that the normal tissue expresses higher levels of *ID4* (red rows) than the cancers (green, black, and red rows). One can zoom in (Fig. 2b) to the individual levels to reveal the locations of methylated CpG dinucleotide locations in cancer (red) and the corresponding unmethylated locations in normal (blue). These data suggest that methylation of these CpG locations near to and internal to *ID4* are correlated with downregulation of the tumor-suppressor *ID4* in prostate cancer, consistent with our previously reported data [70, 71]. These results are also consistent with the methylation patterns found in PrEC and LNCaP data described above. When the same data are depicted for *ZIC2*, similar methylation patterns are seen in normal and tumor tissues as were found in PrEC and LNCaP cell lines, i.e., hypermethylation in cancer (Fig. 2c). However, the gene expression data reveal a different pattern from that of *ID4*. Even though regions of *ZIC2* are hypermethylated in cancer, there is an extreme increase in *ZIC2* gene expression in cancer tissue samples (Fig. 2d), consistent with

previously published reports [83]. Where *ID4* is methylated and downregulated in PRAD, *ZIC2* is methylated but is significantly *up-regulated* in PRAD. In the case of *ZIC2*, these data allow one the opportunity to formulate an alternative hypothesis as to why DNA methylation does not result in the downregulation of gene expression but instead results in the up-regulation of gene expression. As can be seen from the positional information, the increased DNA methylation is not in the region of the promoter or transcription start site (TSS) of *ZIC2*, as is seen in *ID4*. It is instead found downstream in the 3' region of *ZIC2*, conceivably indicating that the binding of a repressor of *ZIC2*, such as GATA3, is excluded from binding by methylated DNA in that region of *ZIC2* and can no longer down-regulate *ZIC2* transcription in cancer. These examples of comparative DNA methylation and expression analyses in prostate cancer illustrate the confirmatory and discovery powers of functional epigenomics in the genes of *ID4* and *ZIC2*, respectively. Finally, if so desired the user-processed dataset can be downloaded in tabular quantitative format from the Cancer Browser for generation of custom figures from the user's own statistical and graphical software.

7 Concluding Remarks

Epigenetic alterations have now emerged as major contributors to prostate cancer disease initiation and progression. The detailed investigation of the molecular mechanisms involved in regulating these epigenetic changes will remain the major focus of current and future research. With the advent of newer genome-wide technologies, it is now possible to investigate the global epigenome and understand the role that risk factors (e.g., age, geographic ancestry, and environment) and molecular mechanisms (e.g., AR and other tumor suppressors/oncogene regulation, cellular heterogeneity, the transition to castration-resistant prostate cancer, and individual responses to therapies) play in the clinical course of PCa. Since epigenetic changes involve alteration in the expression of key enzymes and pathways without altering the DNA sequence (e.g., somatic mutations), strategies that target these proteins in order to reverse these epigenetic changes are attractive as future preventive and therapeutic interventions.

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Fig. 1. UCSC Genome Browser-generated images displaying differentially methylated CpG dinucleotides relative to the various parts of the gene of interest and any neighboring CpG islands (**a** and **c**). Alternatively the user can zoom in to the level of nucleotide visualization and see the actual genomic DNA sequence surrounding the individual CpG that is being interrogated (**b** and **d**). In this figure, individual CpGs are represented by *tick marks* on the tracks labeled on the *left*, where *green* represents unmethylated CpGs or 0 % methylation, *yellow* represents 50 % methylation, and *red* indicates 100 % methylation

Fig. 2.

The Cancer Genomics Browser displays epigenetic data from cancer and normal tissues. Panels **a** and **c** show the results for prostate adenocarcinoma (PRAD) DNA methylation in the *top* heatmap (red and blue) and sample normalized exon-level gene expression by RNA-SEQ in the *lower* heatmap (red and green) for *ID4* and *ZIC2*, respectively. Panels **b** and **d** are zoomed to nucleotide level to clearly demarcate the difference in methylation at specific CpG dinucleotides between normal and cancer samples

Table 1

List of genes that are epigenetically silenced in prostate cancer

Target	Function	Epigenetic modification
<i>DAB 2 interacting protein (DAB2IP)</i>	Inhibition of NF- κ B/Ras pathway	Histone methylation
Adrenoceptor beta 2 (<i>ADRB2</i>)	β -adrenergic signaling	Histone methylation
E-cadherin (<i>CDH1</i>)	Cell-cell adhesion, tumor suppressor	Histone methylation
Prostate secretory protein of 94 amino acids (<i>PSP94</i>)	Inhibits MMP secretion	Histone methylation
Slit homolog 2 (<i>SLIT2</i>)	Chemo-repellent protein	Histone methylation
TIMP metalloproteinase inhibitor 2/3 (<i>TIMP2/3</i>)	ECM degradation	Histone methylation
Raf-1 kinase inhibitor protein (<i>RKIP</i>)	Inhibition of Raf and NF- κ B pathways/ metastasis suppressor	Histone methylation
Kruppel-like factor 2 (<i>KLF2</i>)	Tumor suppressor	Histone modification
Prostate cancer-associated transcript 1 (<i>PCAT-1</i>)	Transcriptional repressor lincRNA	Histone methylation
Glutathione <i>S</i> -transferase Pi (<i>GSTP1</i>)	DNA repair, intra cellular detoxification	DNA methylation
Adenomatous polyposis coli (<i>APC</i>), retinoic acid receptor beta (<i>RARβ</i>), <i>RAAF1</i> , inhibitor of DNA binding (<i>Id4</i>),	Tumor-suppressor genes	DNA methylation
Androgen receptor (<i>AR</i>), estrogen receptor 1 (<i>ESR1</i>), estrogen receptor 2 (<i>ESR2</i>)	Hormone receptor genes	DNA methylation
<i>CDH13</i> , <i>CD44</i>	Cell adhesion genes	DNA methylation
Cyclin D2 (<i>CCND2</i>), <i>CDKN1B</i> , Stratifin (<i>SFN</i>)	Cell-cycle control genes	DNA methylation
Growth arrest and DNA-damage-inducible protein GADD45 alpha (<i>GADD45a</i>), PYD And CARD Domain (<i>PYCARD</i>), <i>RPRM</i> , <i>GLIPR1</i>	Apoptotic genes	DNA methylation
<i>O</i> -6-methylguanine DNA-methyltransferase (<i>MGMT</i>)	Remove alkyl adducts from <i>O</i> ⁶ -guanine	DNA methylation