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DNA METHYLATION IN PROSTATE CANCER: POSSIBLE PROGNOSIS MARKERS AND HYPOTHESIS ON CAUSAL RELATIONSHIP BETWEEN EPIGENETICS AND TUMOR PROGRESSION

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1. INTRODUCTION

1.2 EPIGENETICS

Epigenetics (literally 'over' or 'upon' genetics), originally defined, in the early 1940s, by C.H.Waddington as ‘the causal interactions between genes and their products, which bring the phenotype into being’, was used to explain why genetic variations sometimes did not lead to phenotypic variations and how genes might interact with their environment to yield a phenotype (Rodriguez-Paredes, 2011). Waddington’s definition initially referred to the role of epigenetics in embryonic development; however, the definition of epigenetics has evolved over time as it is implicated in a wide variety of biological processes. The current definition of epigenetics is ‘the study of mitotically and/or meiotically heritable changes in gene expression that occur independent of changes in the primary DNA sequence’. Most of these heritable changes are established during differentiation and are stably maintained through multiple cycles of cell division, enabling cells to have distinct identities while containing the same genetic information. This heritability of gene expression patterns is mediated by epigenetic modifications, which include methylation of cytosine bases in DNA, posttranslational modifications of histone proteins as well as the positioning of nucleosomes along the DNA and miRNAs. The complement of these modifications, collectively referred to as the epigenome, provides a mechanism for cellular diversity by regulating what genetic information can be accessed by cellular machinery (Sharma, 2010).

1.3 DNA METHYLATION

Methylation of the 5’-position of cytosine residues is a reversible covalent modification of DNA, resulting in production of 5-methyl-cytosine and approximately 3% of cytosines in human DNA are methylated. In mammals, cytosine methylation is restricted to those located 5’ to a guanosine (commonly annotated as CpGs, where the intervening ‘p’ represents the phosphodiester bond linking cytosine- and guanosine-containing nucleotides). The modification of cytosine is catalyzed by the enzymes DNA methyltransferases (DNMTs) using S-adenosyl-L-methionine (AdoMet, SAM) as the methyl donor. The methyl group of AdoMet is bound to a sulfonium ion that thermodynamically destabilizes the molecule and makes the relatively inert methylthiol of the methionine part very reactive toward activated carbon atoms. The reaction involves DNMT DNA binding domain, flipping the target cytosine out of the double helix, with the consequent formation of a transient covalent complex with the cytosine residue. DNMT adds a cysteine thiolate to the 6- carbon of the

substrate cytosine, followed by transfer of the methyl group to the 5-carbon (Daniel, 2011). These methyl groups allow normal hydrogen bonding and project into the major groove of DNA, changing the biophysical characteristics of the DNA. They probably have two effects: they inhibit the recognition of DNA by some proteins while they facilitate the binding of other proteins to the DNA. In general, DNA methylation is associated with gene repression. As DNA methylation patterns can be maintained following DNA replication and mitosis, is also associated with inheritance of the repressed state. Moreover cells could influence specific gene expression having the ability to both methylate and demethylate DNA.

DNA methyltransferases (DNMTs) are the family of enzymes responsible for DNA methylation. Four DNMTs have been identified in mammals: DNMT1, DNMT2, DNMT3a and DNMT3b. DNMT1 maintains DNA methylation during replication by copying the methylation pattern of the parent DNA strand onto the newly synthesized strand and prefers hemimethylated templates. Consequently, it passes the epigenetic information through cell generation. DNMT3a and DNMT3b are responsible for de novo DNA methylation, targeting unmethylated CpG dinucleotides as well as working with DNMT1 to ensure propagation of methylation patterns during DNA replication. DNMT2 has reportedly only weak DNA methylation ability in vitro and appears to be involved in methylation of RNA. Relatively slow 'passive' DNA demethylation may occur if methylated CpGs fail to be propagated following DNA replication. However, more rapid 'active' demethylation also occurs, but the exact molecular mechanisms are not clear. Demethylation might be caused by the substitution of methylated cytosines through an enzymatic process in which a glycosylase plays a major role or by a deamination-induced repair process. Other proposed mechanisms like a deamination of the methylated cytosine to thymine induced by the DNA methyltransferase DNMT3B itself in the absence of the universal methyl donor S-adenosylmethionine. However, all postulated mechanisms are not very efficient indicating that the underlying mechanisms might be more complex than currently suggested.

While most of the CpG sites in the genome are methylated, the majority of CpG islands usually remain unmethylated during development and in differentiated tissues. However, some CpG island promoters become methylated during development, which results in long-term transcriptional silencing. Gene expression and DNA methylation are inversely correlated in many genes during early development, moreover many genes show promoter hypermethylation associated with gene silencing in specific tissues.

Genome wide studies characterizing the de novo hypermethylation of promoters during differentiation in mouse models identified 5% of the CpG islands as hypermethylated and, in

consequence, silenced in somatic tissues, but not in germline cells, similar result was identified in human tissues (Berdasco, 2010). Many other genes, like melanoma antigen gene (MAGE family), are unmethylated in germline cells but methylated in somatic cell types.

These results support the hypothesis that cell-type-specific patterns of cytosine methylation mediate control of cell-type-specific gene expression and cellular differentiation. Studies of profiling of CpG methylation at chromosomes 6, 20, and 22, that compare eight somatic tissues, identifie several CpG islands that are differentially methylated. Specifically, 17% of the analyzed genes (873) were differentially methylated in their 5'UTRs, but only one third of the differentially methylated 5'UTRs shows an inverse correlation with transcription. Further work is needed to test whether this absence of correlation is due to limitations of the analytical techniques or to the existence of additional methylation-independent regulatory mechanisms (Berdasco, 2010).

DNA methylation is implicated in establishing patterns of monoallelic gene expression. X-chromosome inactivation in female cells induced to equalize the imbalance of the ''extra'' X chromosome gene expression as compared to the one X chromosome in males is an example of CpG island hypermethylation during development. Some tissue-specific CpG island methylation has also been reported to occur in a variety of somatic tissues, primarily at developmentally important genes. Furthermore, genomic imprinting is another example of monoallelic expression in which epigenetic chromosomal modifications drive differential gene expression according to which parent transmitted the chromosome to the progeny (Berdasco, 2010). Expression is exclusively due either to the allele inherited from the mother or from the father. DNA methylation seems to be the main mechanism for controlling genes subjected to imprinting. Maybe the regulation of this process is based on the cluster organization of imprinted genes controlled by regulatory elements such as Differentially Methylated Regions (DMRs). These elements are organised in Imprinting Control Regions (ICRs) that regulate imprinting in many genes with different function such as growth and cellular proliferation. DNA methylation of DMRs is thought to interact with histone modifications and other chromatin proteins to regulate parental allele-specific expression of imprinted genes. Several syndromes and pathologies such as cancer are associated with deletions or aberrations in DNA methylation of ICRs.

Repetitive genomic sequences, that are globally dispersed in the human genome, are heavily hypermethylated, to prevent chromosomal instability translocations and gene disruption by silencing non-coding DNA and transposable DNA elements or endoparasitic sequences. This

mobile elements of the genome need to be silenced completely and stably to prevent them from moving around the genome (Sharma, 2010; Berdasco, 2010).

1.4 CpG ISLANDS

In human somatic cells, m5C accounts for 1% of total DNA bases and affects 70%–80% of all CpG dinucleotides in the genome (Bird, 2002).

This modified residue is distributed throughout the majority of the genome including gene bodies, endogenous repeats and transposable elements and functions to repress transcription (Illingworth, 2009).

Methylcytosine has mutagenic properties and spontaneously deaminates to thymine resulting in the under representation of CpG (21% of that expected in the human genome). The genome is globally punctuated however by non-methylated DNA sequences called CpG islands (CGIs) which have an elevated G + C content, little CpG depletion and frequent absence of DNA methylation. These conspicuous unique sequences are approximately 1 kb in length. Computational analysis of the human genome sequence predicts 25,500 CpG islands (Illingworth, 2010). CGIs are associated with the promoter regions of 60–70% of human genes and have been shown to colocalise with the promoters of all constitutively expressed genes and approximately 40% of those displaying a tissue restricted expression profile and also developmental regulator genes (Bird, 2011; Illingworth, 2009). CGI promoters encompass a class of transcription start site (TSS) of approximately 60% of human protein coding genes which can initiate from multiple positions. Consistent with promoter association, CGIs are generally characterised by a transcriptionally permissive chromatin state. These findings suggest that CGIs may provide a means to distinguish gene promoter regions from the large proportion of transcriptionally irrelevant intergenic chromatin. Study investigating the distribution of transcription factor (TF) binding sites indicates that this sites are slightly enriched in promoter proximal sequences, and also highly abundant throughout the genome (approximately 16 sites per 100 bp). It seems clear that the presence of binding sites alone are insufficient to identify promoters, which supports the idea that CGIs serve to recognition for TF.

Not all CGIs localise to annotated TSSs. The CXXC Affinity Purification (CAP) technique using to isolate clusters of unmethylated CpG island from genomic DNA, followed by high-throughput sequencing of this fraction, showed that many CGIs in the human genome are not coincident with annotated promoters, but are remote from annotated promoters and are either intergenic or within the body of coding regions (intragenic). These “orphan” CGIs co-localise

with peaks of H3K4me3, which is signature of active promoters, and evidence suggests that a large proportion recruit RNA polymerase II (RNAPII) and give rise to novel transcripts. Intragenic methylation seems to have a major role, in regulating cell specific alternative promoters in gene bodies in a tissue- and cell type-specific manner. Moreover the majority of methylated CpG islands have been shown to be in intragenic and intergenic regions, whereas less than 3% of CpG islands in promoters are methylated. (Maunakea, 2010). Illingwoth et al. found that de novo methylation during development predominantly affects orphan CGIs in both humans and mice, with few protein-coding gene promoters being methylated. For example, in colorectal tumors, cancer-specific de novo methylation affects both CGI categories equally, with a strong preference for those marked in ES (embryonic stem) cells by H3K27me3 – the chromatin modification that is associated with polycomb-mediated repression. These findings sustain the idea that all CGIs correspond with promoters and that many orphan CGIs are associated with novel transcripts that may have regulatory significance (Illingworth, 2010).

Many studies suggested that CGI promoters may often lack TATA boxes and display heterogeneous TSSs. These observations are compatible with the idea that non-methylated CGI promoters are organized in a particular chromatin structure that predisposes them in a transcriptionally permissive state within which initiation can occur at a number of locations. CGIs tend to lack core promoter elements such as the BRE (TFIIB-Recognition Element), DPE (Downstream Promoter Element) and DCE (Downstream Core Element) and display dispersed initiation patterns. Moreover there are exceptions to this generalization: the human genes for a-globin, MyoD1, and erythropoietin, for example, have CGI promoters, yet possess TATA boxes. Chomatin signature like nucleosome deficiency and instability is a feature of CGI promoters as well as histone modification that consist of high levels of histone H3 and H4 acetylation, which are characteristic of transcriptionally active chromatin, and low level of histone H1, which is regarded as antagonistic to transcription. Moreover recent studies reveal that H3K4me3 is a signature histone mark of CGI promoters, often persisting even when the associated gene is inactive (Deaton, 2011).

The mechanism by which CGIs remain hypomethylated during the period of global de novo methylation during early development remains unclear (Antequera, 2003; Illingworth, 2009).

A simple suggestion would be that CGIs are intrinsically refractory to de novo methylation by DNA methyltransferases due to their DNA sequence. This seems unlikely however, as CGIs contain a substantially elevated density of CpG sites, the preferred substrate of the DNMT enzymes.

A potential biological reason for the maintenance of unmethylated CpGs at many promoters has recently emerged from studies of proteins that interact preferentially with CGIs. Various protein factors, including CGBP (CpG-binding protein) possess a CXXC domain, which can specifically bind to non-methylated CpG sites. In mouse the protein Cfp1 (CXXC finger protein 1) contains a CXXC domain that binds, in a specific manner, to CpG only when it is unmethylated and colocalises with almost all CGIs in the genome (Thomson, 2010). Another possibility is that an equivalent recruitment mechanism could target a demethylation activity, which specifically removes in a active manner the methyl residues from the cytosine base, to CGIs (Wu and Zhang, 2010). However, no such demethylase activity has thus far been identified in somatic tissues. An attractive scenario is that CGIs are subject to sporadic de novo methylation, but are continually clean by a mechanism involving oxidation of 5-methylcytosine. Defects in this system may predispose to de novo CGI methylation. Regardless of the detailed molecular mechanism, there is evidence that the methylation-free state of CGIs is causally related to their function as promoters (Deaton, 2011). A plausible alternative is that bound transcription factors sterically preclude DNMT association at CGI sequences. Evidence for this mechanism is supported by mouse transgenic experiments in which ablation of binding sites for the ubiquitous transcription factor Sp1 (Specificity Protein 1) facilitate de novo methylation of the aprt (adenine phosphoribosyltransferase gene) promoter CGI.

It exists a close relationship between transcription in the early embryo and lack of CGI methylation, but mechanisms that relate the two events are unknown. During early development the binding of transcription factors is required for establishment of the DNA methylation-free state. Also the presence of RNAPII at CGIs is associated with resistance to DNA methylation in cancer. CGIs often colocalize with ORIs (origins of DNA replication) and it has been speculated that intermediates in the process of replication initiation lead to local exclusion of DNA methylation but a causal relationship between ORI function and CGIs has not been established yet.

Other kinds of DNA-based metabolism might be responsible for excluding DNA methylation from these regions. For example, CGI promoters are typically loaded with polymerases that create short abortive transcripts even when the associated gene is inactive. This ‘‘futile’’ transcription cycle may somehow protect CGIs from the action of DNA methyltransferases, allowing these ‘‘silent’’ promoters to exclude DNA methylation. Another possible explanation for the immunity of most CGIs to DNA methylation is that their signature chromatin mark, H3K4me3, interferes with DNA methyltransferase activity by chromatin

binding of Dnmt3L, a member of the family of the de novo DNA methyltransferases. Multiple potential mechanisms for preventing CGI methylation, including those discussed above, are not mutually exclusive, but may act in concert. Maybe chromatin modification by itself is insufficient but is required a combination of factors—including, perhaps, initiation of transcription—to exclude DNA methylation from CGIs (Deaton, 2011).

1.5 TRANSCRIPTION REGULATION

CGIs are typically in a non-methylated state, even when the corresponding gene is transcriptionally inactive. There are, however, well-known examples of CGIs that become hypermethylated during normal development, leading to stable silencing of the associated promoter (Deaton, 2011). Microarrays probed with DNA enriched for methyl-CpGs identified 3–4% of hypermethylated CGI-promoters in a panel of somatic tissues. Promoters with relatively reduced CpG content were more often hypermethylated. These global studies indicate that sites of CGI methylation frequently localise to genomic regions distal to promoters. Consistent with this observation, bisulfite analysis identified 2.1% of promoter-associated CGIs as hypermethylated (>80% of CpGs) relative to more than 9% of the complete CGI complement. However, despite this observation the exact proportion of hypermethylated CGIs varies widely between these microarrays studies (9–25%). It seems that CGI methylation is not the primary inactivating signal in gene silencing, but acts to lock in the silent state (Illingwoth, 2009; Antequerra, 2003).

In contrast to the rarity of methylated CGIs at the promoters of annotated genes, orphan CGIs are methylated much more frequently. About 17% of orphan CGIs have been found in a methylated state, compared with 3% of CGIs at annotated gene promoters. By further separating orphan CGIs into intragenic and intergenic categories, it becomes apparent that intragenic CGIs are especially prone to methylation (20%–34%). Accordingly, CGIs located within gene bodies show the greatest number of DNA methylation differences between different somatic cells and tissues (Maunakea, 2010; Illingworth, 2010). Functionally, it can be speculated that some of the transcripts initiating from gene body CGIs are regulatory ncRNAs which presence or absence affects expression of the associated protein-coding gene or a nearby gene. Another possibility is that these sites of unusual chromatin and transcription affect alternative splicing of the gene in which they are located in a manner that differs with methylation status. It is also possible that a methylated CGI within a gene body down-

regulates transcriptional elongation, as reported in a transgenic cell model. Further studies are required to elucidate the consequences of methylation at these sites (Illingworth, 2009, 2010).

DNA methylation inhibits transcription by interfering with its initiation. Because 5-methylcytosine is in the major groove of the DNA helix, it is possible that this modified cytosine interferes directly with the binding of transcription factors. Many factors are known to bind CpG-containing sequences, and some of these fail to bind when the CpG is methylated. However, it is unlikely to be a widespread mechanism for transcriptional silencing because most transcription factors do not have CpG dinucleotides within their DNA binding sites. DNA methylation is suggested to lead to transcriptional silencing by either preventing or promoting the recruitment of regulatory proteins to DNA. One mechanism involves DNMTs interacting with transcription factors, resulting in site-specific methylation at promoter regions. This site-specific methylation is responsible for the assembly at these locations of proteins that recognise methylated DNA. These assemblies then directly influence further action of the transcriptional machinery or cause alterations in chromatin structure, which in turn affect normal gene expression mechanisms. Some studies provided the preliminary evidence of this mechanism showing that DNMT3a was recruited to the RARb2 (Retinoic acid receptor B2) promoter by the oncogenic transcription factor, PML–RAR, leading to promoter methylation and silencing of the RARb2 gene. Similar gene-specific transcriptional silencing, with the observation of suppression of P21 expression via Myc targeted methylation of the P21 promoter. Within this pathway, p53 appears to recruit DNMT1, stimulating DNMT1-mediated DNA methylation and resulting in the repression of p21 expression. Seems that transcription factors interact directly with various DNMTs acting as potential DNA ‘anchors’ for the DNMTs, thus aiding in the site-specific methylation of promoter regions. Thus, the dual ability of some transcription factors to bind to DNA via specific recognition sequences, and also to interact with DNMTs, may promote widespread site specific DNA methylation at promoter regions. Once such site-specific methylation occurs, recruitment of methyl-binding proteins, as outlined below, may result in further effects on transcriptional activity and chromatin structure.

Various DNA methyl-binding proteins (MBPs) exist, and are grouped into similar ‘families’ according to their structural similarity. One family shares a related DNA binding domain (methylated DNA-binding domain, MBD) and the MBD family includes the proteins MBD1, MBD2, MBD3, MBD4 and MECP2. MBD1-3 proteins are transcriptional repressors that act through various mechanisms, resulting in the recruitment of corepressors and histone deacetylases. MBD4 is a thymidine glycosylase repair enzyme. It is not associated with

transcriptional inactivation and is likely to have a role in limiting the mutagenicitiy of methylcytosine. MECP2 binds methylated CpG via its MBD domain and exerts repressive effects on transcription over distances of several hundred base pairs via its second functional domain, a transcriptional repression domain that recruits the co-repressor, Sin3 complex, which contains histone deacetylase 1 and 2, or other co-repressor complexes. Alternatively MECP2 can alter chromatin compaction by binding to linker DNA and nucleosomes, resulting in a physical barrier to the transcriptional machinery. The second family of MBPs contains a common zinc finger domain and consists of the proteins Kaiso, ZBTB4 and ZBTB38. The nucleo/cytoplasmic distribution of this family of proteins is variable and is said to respond to intracellular signalling, including the Wnt pathway. Recent evidences suggest that Kaiso can regulate transcriptional activity via modulating histone deacetylase 1 (HDAC1) and b catenin complex formation, and interacting with transcriptional factors. The third family of methyl DNA-binding proteins contains UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) and UHRF2, which recognize and bind semi-methylated DNA through their SET- and RING finger-associated domains (SRA proteins). Binding of SRA proteins to methylated DNA directs DNMT1 to these sites, resulting in further alteration of DNA methylation and additional recruitment of other MBPs and their associated activities. Thus, methyl-binding proteins react to the methylation status of DNA at specific sites, often associated with promoters of genes. These methyl-binding proteins appear to exert their effect by recruiting additional enzymes, such as histone deacetylases, which, as described in the following sections, also have important roles in epigenetic control of gene expression (Gibney, 2010; Sharma, 2010; Daniel, 2011).

Recent studies have shown that DNA methylation is also important for the regulation of non-CpG island promoters. For example, tissue-specific expression of MASPIN (Mammary Serine Protease Inhibitor), which does not contain a CpG island within its promoter, is regulated by DNA methylation. Similarly, methylation of the non-CpG island Oct-4 promoter, strongly influences its expression level. Since CpG islands occupy only 60% of human gene promoters, it is essential to elucidate the role of non-CpG island methylation in order to fully understand the global role of DNA methylation in normal tissue (Sharma, 2010).

However many genes display a relatively poor correlation between CGI hypermethylation and the transcriptional status of associated genes. There are several potential explanations for this lack of correlation. Hypermethylation of the single promoter associated CGI would lead to stable transcriptional silencing. The majority of methylated CGIs are located within intragenic

regions where the effect on transcription is less clear. Many genes can generate multiple transcripts by utilising alternative transcription starts sites.

Alternative promoters could be inactivated by CGI methylation. Where intragenic islands do not associate with a known TSS, it is possible that their methylation could prevent spurious gene body transcription which could otherwise interfere with the correct expression of the parent gene. There is evidence that intragenic CGIs can localise to sites of antisense non-coding RNA (ncRNA) trans, CGI methylation results in the derepression of genes silenced by ncRNAs. Many hypermethylated CGIs are located in intergenic DNA outside coding sequences and therefore have no obvious regulatory role in gene transcription. Transcription initiation which negatively regulate the expression of the sense transcript.

Several germ line and embryonic specific genes associate with methylated CGI promoters and can be reactivated by depletion of DNA methylation levels. Several studies have identified differential CGI.

Methylation between somatic tissues associated with constitutively repressed genes. This suggests that methylation is stochastically accumulated in different cell types in the absence of transcription. This fits with the observation that CGI methylation is a relatively late event during X-inactivation following gene repression. Absence of TFs at silenced promoters could facilitate transient de novo methylation. This possibility would align with the notion that methylation may be regarded as the basal state of the genome and is excluded from specific regions by the presence of bound factors. Alternatively DNMT recruitment could be mediated by initial repressive events to target DNA methylation and irrevocably silence transcription of the associated gene (Illingworth, 2009; Deaton, 2011).

1.6 METHYLATION in CANCER

The epigenetic pattern present in normal cells undergoes extensive distortion in cancer. These epimutations, along with widespread genetic alterations, play an important role in cancer initiation and progression. The cancer epigenome is characterized by global changes in DNA methylation and histone modification patterns as well as altered expression profiles of chromatin-modifying enzymes. These epigenetic changes result in global dysregulation of gene expression profiles leading to the development and progression of disease states. Epimutations can induce silencing of tumor suppressor genes independently and also in conjunction with deleterious genetic mutations or deletions; representig the second hit required for cancer initiation according to the ‘two-hit’ model proposed by Knudson. Epimutations can also promote tumorigenesis by activating oncogenes and inactivating tumor

suppressors genes. The events that lead to initiation of these epigenetic abnormalities are still not fully understood. Nevertheless, since epigenetic alterations, like genetic mutations, are mitotically heritable, they are selected for in a rapidly growing cancer cell population and confer a growth advantage to tumor cells resulting in their uncontrolled growth (Sharma, 2010). A cancer epigenome is characterised by genome-wide hypomethylation and site-specific CpG island promoter hypermethylation. The mechanisms that initiate these global changes are still under investigation, but recent studies indicate that some changes occur very early in cancer development and may contribute to cancer initiation.

Global DNA hypomethylation plays a significant role in tumorigenesis and occurs at various genomic sequences including repetitive elements, retrotransposons, pericentromeric regions, CpG poor promoters, introns and gene deserts. However, the range of affected loci also includes growth regulatory genes, imprinted genes, developmentally critical genes, and tissue-specific genes. DNA hypomethylation at repeat sequences leads to increased genomic instability by promoting chromosomal rearrangements. Hypomethylation of retrotransposons can result in their activation and translocation to other genomic regions, thus increasing genomic instability. Many human cancers show similar loss of DNA methylation and genomic instability. In addition, DNA hypomethylation can lead to the activation of growth-promoting genes, such as R-Ras and MAPSIN in gastric cancer, S-100 in colon cancer and MAGE in melanoma, and a loss of imprinting (LOI) in tumors. In Wilms' tumor, hypomethylation-induced LOI of IGF2, an important autocrine growth factor, results in its pathological biallelic expression. LOI of IGF2 has also been linked with an increased risk of colorectal cancer. DNA hypomethylation leads to aberrant activation of genes and non-coding regions through a variety of mechanisms that contribute to cancer development and progression. In contrast to hypomethylation, which increases genomic instability and activates proto-oncogenes, aberrations in DNA methylation patterns of the CpG islands in the promoter regions of tumor suppressor genes are accepted as being a common feature of human cancer. Site-specific hypermethylation contributes to tumorigenesis by silencing tumor suppressor genes. Since the initial discovery of CpG island hypermethylation of the Rb promoter (a tumor suppressor gene associated with retinoblastoma), various other tumor suppressor genes, including VHL (associated with von Hippel-Lindau disease), CDKN2A (Cyclin-dependent kinase inhibitor 2A), hMLH1 (a homolog of Escherichia coli MutL), and BRCA1 (breast-cancer susceptibility gene 1) also shown tumor-specific silencing by hypermethylation. These genes are involved in cellular processes, which are integral to cancer development and progression, including DNA repair, cell cycle, cell adhesion, apoptosis and angiogenesis.

Epigenetic silencing of such tumor suppressor genes can also lead to tumor initiation by serving as the second hit in the Knudson's two-hit model. In addition to direct inactivation of tumor suppressor genes, DNA hypermethylation can also indirectly silence additional classes of genes by silencing transcription factors and DNA repair genes. Promoter hypermethylation-induced silencing of transcription factors, such as RUNX3 (runt-related transcription factor 3) in esophageal and gastric cancer and transcription factors GATA-4 and GATA-5 in colorectal and gastric cancers leads to inactivation of their downstream targets. Silencing of DNA repair genes (e.g. MLH1, BRCA1 etc.) enables cells to accumulate further genetic lesions leading to the rapid progression of cancer. A CpG island hypermethylation profile of human primary tumors shows that hypermethylation of tumor-suppressor genes are specific to the cancer type. Each tumor type can be assigned a specific, defining DNA ''hypermethylome,'' rather like a physiological or cytogenetic marker. These marks of epigenetic inactivation occur not only in sporadic tumors but also in inherited cancer syndromes, in which hypermethylation may be the second lesion in Knudson's two-hit model of cancer development. These CpG hypermethylation ''maps'' have been identified first in cancer cell lines then in primary tumors, including acute myeloid leukemia, glioblastomas, astrocytomas, and ovarian epithelial carcinoma.

While the ability of DNA hypermethylation to silence tumor suppressor genes in cancer is well established, how genes are targeted for this aberrant DNA methylation is still unclear. One possibility is that silencing specific genes by hypermethylation provides a growth advantage to cells resulting in their clonal selection and proliferation. Tumor-specific CpG island methylation can occur through a sequence-specific instructive mechanism that directs DNMTs to specific genes by their association with oncogenic transcription factors. Large stretches of DNA can become abnormally methylated in cancer causing some CpG islands to be hypermethylated as a result of their location inside such genomic regions that have undergone large-scale epigenetic reprogramming. Another interesting mechanism proposes a role of histone marks in the tumor-specific targeting of de novo methylation and will be discussed in detail in the next section. Interestingly, regions that are hypermethylated in cancer are often premarked with H3K27me3 polycomb mark in ES cells suggesting a link between the regulation of development and tumorigenesis. This observation also partially explains the theory of 'CpG island methylator phenotype' (CIMP) that hypothesizes that there is coordinated methylation of a subset of CpG islands in tumors since many of these CIMP loci are known polycomb targets. Further understanding of how specific genomic regions are

targeted for DNA hypermethylation in cancer will potentially lead to additional therapeutic targets (Sharma, 2010).

The regulatory mechanisms underlying the differential expression patterns of housekeeping genes compared with tissue-specific genes are also poorly characterized. For a long time, it was accepted that all known housekeeping genes and half of the tissue-specific genes have associated CpG islands. Normally, housekeeping genes have a nonmethylated CpG island tightly associated with their promoter and not to be regulated by DNA methylation. Several genes that are considered as constitutively expressed in all cells are known to be inactivated by CpG promoter hypermethylation in cancer cells, affecting a wide range of cellular pathways, such as cell cycle (Rb, p16 INK4a , p15 INK4b), DNA repair (BRCA1, MGMT, MLH1), transcription factors (GATA-4, GATA-5, VHL), epigenetic enzymes [NSD1 (nuclear receptor binding SET domain protein 1), RIZ1 (retinoblastoma protein-interacting zinc finger gene)], receptors [CRBP1 (Cellular retinol-binding protein-1), ESR1 (EStrogen Receptor 1), TSHR (thyroid stimulating hormone receptor)], signal transduction [APC (Adenomatous polyposis coli), RASSF1A ((Ras association domain family 1), WIF1 (Wnt inhibitory factor 1)], toxic catabolism and drug resistance (GSTP1: Glutathione S-transferase P), metastasis and cell invasion [CDH1 (Cadherin-1), TIMP3 (Metalloproteinase inhibitor 3)], apoptosis [DAPK (Death-associated protein kinase), TMS1 (target of methylation-induced silencing 1), CASP8 (Caspase 8)], and angiogenesis (THBS1: Thrombospondin 1).

Finally CpG methylation constitutes a mechanism of epigenetic control of differentiation genes, allowing the expression in a time- and tissue-dependent manner. These same genes can also be deregulated in cancer by aberrant CpG promoter hypermethylation. For example, the tissues-specific expression of maspin, which encodes the mammary serine protease inhibitor protein and is expressed only in cells of epithelial origin, is epigenetically regulated by DNA methylation. Additionally, the aberrant CpG hypermethylation of maspin leads to gene silencing in cancers, such as breast, thyroid, skin, and colon.

1.7 METHYLATION as BIOMARKER

In cancer research and detection, a biomarker refers to a substance or process that is indicative of the presence of cancer in the body. It might be either a molecule secreted by a malignancy itself, or it can be a specific response of the body to the presence of cancer (Laird, 2003).

Tumour biomarkers are potentially useful in the identification of individuals at increased risk of developing cancer, in screening for early malignancies and in aiding cancer diagnoses. Following a diagnosis of cancer, biomarkers may be used for determining prognosis,

predicting therapeutic response, surveillance following curative surgery for cancer and monitoring therapy (Duffy, 2009).

Biomarkers capable of distinguishing diseased or malignant cells from normal ones must be specific, sensitive, and detectable in specimens obtained through minimally invasive procedures to be clinically applicable moreover are very important sample extraction, storage, and handling to ensure intra- and inter-laboratory reproducibility. In routine clinical practice, most tumour diagnostics is carried out by biochemical assays determining the presence and/or quantity of enzymes, receptors, growth factors, or hormones, that are measured in either serum or plasma or in tumour tissue. Compared with other molecular structures such as mRNA, miRNA and certain proteins, the use of DNA for the measurement of tumour marker, can be more easily transferred from a research laboratory setting into routine diagnostics. The advantage of using DNA as a biomarker are numerous: is amplifiable, unlike proteins, thus allowing measurements on small amounts of test sample by PCR and related techniques and is stable and can keep for long periods of time. Most importantly, relatively intact DNA can be isolated from formalin-fixed, paraffin-embedded tissue and other archived material.

Methyl groups on cytosines are part of the covalent structure of the DNA. Once methylation is acquired, it is in most cases chemically and biologically stable over time.

A large number of promoters become hypermethylated during carcinogenesis in both early and advanced cancers. If the methylation pattern is specific for a certain tumour type and/or correlates with clinically important parameters, DNA methylation might still be a useful biomarker for tumour diagnosis or risk assessment (Tost, 2010; Duffy 2009).

DNA methylation can be analyzed with a growing number of methods that are high throughput and quantitative assays eliminating the need for normalization.

As most methods determine the ratio between methylated and unmethylated CpGs, DNA methylation analysis is independent of the total amount of starting material. It provides a binary and positive signal that can be detected independent of expression levels. It is therefore easier to detect than negative signals.

Analysis of methylation in homogeneous samples can be relatively easy, heterogeneity of clinical specimens pose a major difficulties to data analysis. Clinical specimens contain a mixture of components, bring different types of cells with its own methylation peculiarities. The composition of samples also changes with time, with different proportions of the same fragment methylated in different samples. These variability impose another level of complexity that has to be considered; even the adjacent sections of the same tumor will have slightly different composition and thus quantitatively different methylation patterns.

Moreover, the natural history of cancer can bring together cells with different degrees of neoplastic transformation that will have different levels of methylation. (Levenson, 2010).

The analysis of DNA methylation patterns is also complicated by the fact that some changes are due to exposure to environmental influences as well as accumulation of DNA methylation at some promoters during aging; a significant increase in DNA promoter methylation with age was found for several genes. To be useful as biomarker, age-associated DNA methylation changes have therefore to be distinguished from cancer predisposing alterations (Tost, 2010) studies investigating a potential diagnostic utility for methylated genes should as a minimum include age-matched controls. Certain benign diseases, especially benign tumours, may also exhibit altered gene methylation.

The high cancer specificity of methylated genes present in blood or in other biological fluids is highly desirable if these markers are to be used in screening for early malignancy. Highly specific DNA methylation markers might thus be combined with highly sensitive tests in order to screen for cancer, panels of tests may be more useful in screening than single tests.

Methylation can be detected in cell-free circulating DNA (cfcDNA) in blood. Potential for cancer detection based on abnormal methylation in cfcDNA has been demonstrated for different cancers, including prostate, breast, gastric, testicular and bladder cancers, and melanoma. Detection is based on the analysis of the same set of promoters for different diseases with results most frequently expressed as a ratio of hypermethylation relative to healthy controls. Non malignant diseases produced specific methylation patterns that were very different from patterns of malignant diseases. Importantly, methylation patterns were unique to the analyzed disease, raising hopes that cfcDNA can be used to identify the site and the nature of the disease. Thus, benign, inflammatory and malignant diseases could be differentially identified, suggesting that molecular diagnosis based on methylation analysis of cfcDNA is possible (Levenson, 2010). DNA methylation patterns might be able to help with the identification of metastatic tumours of unknown primary origin.

A further advantage is the potential reversal of epigenetic changes by treatment with pharmacological agents, while genetic changes are irreversible.

If the core region of a CpG island in a promoter that is controlling transcriptional activity is defined, the stable DNA-based analyte can be used as a proxy to monitor the (re-)activation of gene expression during treatment. Drug-specific changes in methylation profiles are detectable soon after drug application. Similarly, drug specific profile changes have been identified in cfcDNA of patients treated with different drugs, suggesting that an active compound that alters gene expression may induce changes in cfcDNA methylation. These

observations expand potential application of the methylation profiling from detection and diagnosis to treatment monitoring through detection of drug-specific changes in patients' cfcDNA. At the same time, they may open the possibility of early discovery of resistance, which may manifest either as a reversal of drug-induced changes or as induction of another layer of changes, this time specific for resistance.

For optimum management of patients with cancer, accurate prognostic and predictive factors are necessary. Such factors are particularly important in cancer types that have widely varying outcomes and for which systemic adjuvant therapy may be beneficial. predictive biomarkers are factors that are associated with either response or resistance to therapy.64 Predictive markers are necessary in oncology as cancers of similar histological types may vary widely in their response to a specific therapy. For many of the published investigations that related methylated genes to patient outcome, it was not clear whether a prognostic effect, a predictive effect or a combination of both was assessed. The use of methylated genes for aiding prognosis and therapy prediction has been most extensively investigated in breast and brain cancers.

The sensitive and specific detection of tumor-specific DNA methylation patterns at distal sites makes DNA methylation a biomarker of choice for the clinical management of cancer patients.

A major unresolved analytical issue is the optimum system/ platform to be used for determining gene methylation in the clinical setting. According to the recent National Cancer Institute workshop on the clinical application of methylated DNA sequences as cancer biomarkers, bisulphite sequencing is optimal for the detection of CpG island methylation of new genes, pyrosequencing is best for quantitation of individual CpG sites while quantitative methylation-specific PCR is best for sensitive detection of methylated alleles. Following selection of a specific analytical platform, assays for clinically important methylated genes must be optimised, standardised and must undergo technical validation including evaluation in external quality assurance programmes. all the published studies so far have been small scale and retrospective in design. In order for methylated genes to enter routine clinical use, these preliminary findings must be confirmed in high-level evidence studies such as prospective trials or meta/pooled analysis of individual data from smaller-scale studies. Furthermore, the measurement of methylated genes must be able to provide additional information to that available from existing diagnostic, prognostic and predictive modalities. Most importantly, the additional information must be clinically relevant, i.e. provide

information that improves patient outcome, enhances quality of life or leads to reduced health care costs.

1.8 PROSTATE CANCER

Prostate cancer is one of the commonest tumour among men in several Western populations, being the most frequent cancer in the US and Western Europe, and the second most common cancer in Italy (Ferlay, 2010). There are large international disparities in incidence with a North-South gradient, which is also evident within Europe. The introduction of prostate-specific antigen (PSA) testing in the late 1980s has dramatically affected incidence of prostate cancer worldwide. Already in 1990, 60% of all newly diagnosed prostate carcinomas in the USA were detected by PSA testing (Jones, 1995). In Italy, prostate cancer incidence doubled from the early to the late 1990s (www.registri-tumori.it).

This prostate cancer epidemic may be explained by two factors. First, the ability of PSA screening to identify pre-clinical and indolent prostate cancers which would have been clinically detected after some years if the PSA was not carried out resulting in significant overdiagnosis (Draisma, 2003). Second latent prostate cancer of clinical irrelevance is rather frequent, with an estimated prevalence up to 35% in autopsy studies (Yatani, 1988).

Results from recent randomized trials in the US and Europe comparing the effect of PSA-screening vs. no screening on overall mortality and mortality from prostate cancer are somewhat inconclusive (Djulbegovic, 2010). Guidelines recently prepared by the European Association of Urology conclude that current evidence is insufficient to warrant widespread population-based screening by PSA (Heidenreich, 2011).

Therefore, the debate on whether PSA testing should be offered to the population and whether PSA-screening is able to decrease mortality is still ongoing (Ilic, 2006). PSA testing has low sensitivity and positive predictive value, implying high proportions of both false-positive and false-negative tests (Thompson, 2005). Overdiagnosis leads to overtreatment, with the resultant costs, side effects and long term complications. Indeed, a great increase in radical prostatectomies has paralleled the increased use of PSA testing. Furthermore, there are no clear clinical guidelines for men found with a high PSA and a negative biopsy. These people often undergo many biopsies over the ensuing years, resulting in patient anxiety and an increased likelihood that an indolent cancer is eventually found. In a recent Italian study for example, out of 1700 men with a PSA level of at least 4.0 ng/ml, 53% were re-tested, 24% received a biopsy and 9.5% were diagnosed with prostate cancer over a 24 month period (Leoni, 2008).

The Gleason score for tumor-cell differentiation, the main prognostic factor in prostate cancer (Egevad, 2002), shows strong predictive ability, but most of PSA detected patients share the same Gleason score (6 or 7) and cannot be further separated with respect to prognosis.
It is also important to emphasize that the natural history of the carcinoma of the prostate is, at the moment, known only in superficial way and it is hard to establish which tumours may kill the patient if left untreated and it is not known if diagnosis and treatment of early tumours improve survival.
The PSA debate, and the current inability to distinguish between indolent and aggressive prostate cancers, necessitates investigations into new diagnostic and prognostic markers.
Apart from PSA, several proteins which may be used as biomarkers for prostate cancer diagnosis and progression have been suggested in the last few years and new others are being investigated such as Cromogranina A, PSMA (Prostate-specific membrane antigen) e PCA3 (Prostate cancer antigen 3). In particular iperexpression of PCA3 is associated with malignant transformation of prostate cells and it is possible to find its mRNA in the urine of the patients. It shows low sensibility and high specificity.

1.9 RATIONALE OF THE RESEARCH

A number of recent studies indicate that new markers can be found by understanding the role of epigenetic changes in cancer development and progression (Esteller, 2007; Jones, 2007).
It has been suggested that detection of aberrant DNA methylation in tumour suppressor and DNA repair genes may be used as a biomarker for early diagnosis of cancer and for tumour progression, in terms of invasive and metastasis potential, aggressiveness, and recurrence (Garinis, 2002; Esteller, 2002).
Detection of de-novo DNA hypermethylation in some specific genes has been reported to distinguish between normal and prostate cancer tissue with high sensitivity and specificity (Perry, 2006). Robust data indicate that methylation in the GSTP1, the most widely investigated gene in prostate cancer epigenetics, is methylated in at least 80% of prostate cancers (Bastian 2005; Perry 2006). Some studies assessed multiple genes simultaneously identifying values of sensitivity and specificity for diagnosis of prostate cancer well above 90% (Yegnasubramanian 2004, Bastian 2006, Jeronimo 2004). Furthermore, methylation in single genes and methylation indices have been found to be associated with clinicopathologic indicators of poor prognosis, although there is inconsistency between studies (Perry, 2006; Bastian, 2005; Yegnasubramanian, 2004; Jeronimo, 2004). Most of these studies, however, had small sample size and short follow-up. Moreover the association between promoter

hypermethylation in prostate cancer and clinical outcome or mortality is poorly understood and has been less investigated (Rosenmbaum, 2005; Henrique, 2007). Further evaluations are needed for the prognostic value of epigenetic alterations in prostate cancer cells.

Several studies analized the methylation status of selected genes in non neoplastic tissue adjacent to tumor, which remains after surgical resection of the tumor.

The purpose of this studies is mainly to assess the state of methylation of certain genes in the tissue without features of malignancy, and evaluate if it is comparable to the epigenetic events of primary tumor (Martone, 2007) and whether it is able to predict the risk of recurrence of malignant disease (Tan, 2008).

A recent study analyzed the methylation status of the promoter of some genes (GSTP1, RARβ2, and APC) in prostate tumor tissue and in the non-neoplastic (histological evidence of malignancy) tissue adjacent to tumor. The author found that in non-neoplastic tissue promoter methylation of APC and RARβ2 was increased and suggest that hypermethylation in non neoplastic tissue may be a risk factor for prostate cancer. (Steiner, 2010).

DNA methylation is mediated by enzymes of the family of the DNA methyltransferases (DNMTs). Documented over-expression of DNMT3b in the tumour tissue of several cancer sites, as well as DNMT3b inhibition obtained by de-methylant agents, indicate a direct relation between DNMT3b activity and the DNA methylation status. Single nucleotide polymorphisms (SNPs) in the DNMT3b gene have been reported to influence the intensity of the enzyme function and to play a role in cancer aetiology and progression. There is therefore interest in understanding how much of the early changes in DNA methylation are due to the activity of DNMT enzymes. Accordingly, a number of cancer studies have investigated variants in DNMT genes as potential markers for disease development and progression (Singal, 2005; de Vogel, 2009; Cebrian, 2006; Shen, 2002).

1.9.1 SELECTED GENES

Candidate genes for the study of the prognostic value of epigenetic events should be selected on the basis of their function, their involvement in prostate cancer genesis, and the available evidence from previous studies on prostate cancer progression (Bastian, 2005; Yegnasubramanian, 2004). Several genes have been implicated in the tumour genesis of prostate cancer including GSTP1 (Glutathione S-transferase), APC (Adenomatous Polyposis Coli) and RUNX3 (Runt-related transcription factor 3)

These genes are involved in cell-cycle signal transduction (APC), DNA protection (GSTP1) and apoptosis (RUNX3).

- GSTP1: It represents an enzyme family that can detoxify reactive chemical species by catalysing their conjugation to reduced glutathione. GSTP1 is a "caretaker" gene, defending cells against genomic damage mediated by carcinogens or oxidants. A number of studies revealed that methylation in GSTP1 in the tumour tissue or the serum may be used as a diagnostic and possibly prognostic marker for prostate cancer (Enokida, 2005; Kang, 2004; Bastian, 2004).
- APC: It is tumour suppressor gene, initially identified in colorectal cancer. It interacts with beta-catenin, a protein involved in cellular adhesion and motility. Aberrant methylation of the promoter region of APC gene and loss of its specific transcript is present in prostate tumor tissue and may be associated with clinical parameters (Enokida, 2005; Kang, 2004).
- RUNX3: It is one of the genes with a RUNT domain, a transcription factor controlling genes activation. It is reported to be an important tumour suppressor gene in gastric cancer. In prostate cancer, the detection of methylation in RUNX3 has been associated with both PSA levels and the Gleason score (Kang, 2004).

2. AIMS of the STUDIES

This research project is divided in three studies on prostate tumor tissue and non neoplastic tissue adjacent to tumor tissue.

In general in these studies we evaluate the importance of methylation status to investigate the development and progression of prostate cancer and possible regulation of mechanism which leads to methylation status through the efficiency of DNMT3b enzyme.

The relevance of methylation status could suggest a possible role of hypermethylation of selected genes as a prognostic marker.

2.1 STUDY 1: DNA METHYLATION IN PROSTATE TUMOR

Aim of the study was to investigate the prognostic value of promoter methylation in selected genes (GSTP1, APC, RUNX3) using a very long follow-up period, evaluating prostate cancer-specific mortality and testing the results in two independent cohorts selected before and after the introduction of the PSA testing.

Specific aims were:

1. To estimate the proportion of cancers with methylation in the selected genes in patients diagnosed before and after the introduction of PSA testing
2. To evaluate whether methylation in the selected genes correlates with tumour characteristics
3. To follow-up the prostate cancer patients for long-term mortality to understand whether the detection of methylation correlates with the risk of death, and is therefore a prognostic marker
4. To test the prognostic value of the selected markers in two independent series of patients

2.2 STUDY 2: DNA METHYLATION IN NON NEOPLASTIC TISSUE ADJACENT TO PROSTATE TUMOUR

We studied in non-neoplastic prostate tissue adjacent to tumour of prostate cancer patients promoter hypermethylation in selected genes (APC, GSTP1) to understand whether hypermethylation in non neoplastic tissue is an early marker of prostate cancer development and progression. Patients are members of the cohort recruited in study 1 selected because their tumour specimens contain also a well recognizable normal tissue.

Specific aims were:

1. To estimate the prevalence of hypermethylation of selected genes in non neoplastic tumor adjacent to tumour tissue

2. To understand if, within the same patient, the methylation pattern in the non-neoplastic tissue correlates with that of the tumour tissue

3. To evaluate whether the methylation in the non-neoplastic tissue correlates with the risk of death and is, therefore, a marker of prognosis

2.3 STUDY 3: DNMT3b GENOTYPING

In this study we conducted analyses to assess and estimate the relationships between a DNMT3b variant (rs406193 C>T SNP), DNA methylation in the tumour tissue, tumour aggressiveness (as measured by the Gleason score) and long-term prostate cancer mortality. In particular, we considered the possible causal relationships between the studied variables, assuming that: i)DNMT activity affects tumour tissue methylation, ii) methylation status affects tumour morphology, and thus the Gleason score, iii) DNA methylation affects mortality both directly and indirectly via Gleason score.

3. METHODS

3.1 STUDY 1

3.1.1 STUDY DESIGN

We identified two independent cohorts of prostate cancer patients. The first cohort (cohort-1980s) included 298 eligible patients diagnosed in 1982-1988, and therefore before the introduction of PSA testing, whereas the second cohort (cohort-1990s) consists of 280 eligible patients diagnosed in 1993-1996. The second cohort was used to validate the findings in the first cohort and to study any possible changes in the methylation patterns between the 1980s, before the introduction of PSA testing, and the 1990s, during the PSA era. For each patient we obtained slices of paraffin embedded tumour tissue (PET) and tested DNA for detection of promoter methylation in the three selected genes. We studied the prostate cancer survival in association with promoter methylation in GSTP1, APC, and RUNX3. Because these genes are involved in signalling and transcription pathways, their inhibition by promoter methylation may plausibly have a role in prostate cancer progression. The study was approved by the local ethical committee.

3.1.2 STUDY POPULATION

The two cohorts include 459 consecutive patients of any age, diagnosed with prostate cancer at the Pathology Ward of the San Giovanni Battista Hospital in Turin. They received a biopsy or radical prostatectomy (RP) or a transurethral resection of the prostate (TURP) at the same hospital. Patients were resident in Turin or in the hinterland.

3.1.3 PATIENT INFORMATION

DNA extraction from the PETs was successful in 77% (228) of the patients in the 1980s cohort and 90% (253) of patients in the 1990s cohort. Patients with successful extraction remained for further analysis. For each patient we obtained from the pathology report the name and surname, the date of birth and diagnosis, residence, a description of the tumour histology, tumour grade and, limited to members of the cohort-1990s, the Gleason score. No information on PSA variables was available. Three patients in the 1980s cohort and two patients in the 1990s cohort with incorrect demographic information were excluded from the study. Diagnostic slides for patients in the 1980s cohort were traced and re-evaluated by a pathologist (L.D.), who assigned the Gleason score. We could not trace the slides of eight tumors. In those cases, we used the information on tumor grade that was available in the

pathology report; well-differentiated tumors were translated to a Gleason score of 6 or less, moderately differentiated tumors corresponded to a score of 7, and poorly differentiated tumors had a score of 8+. All this information was extracted and a dataset created.

3.1.4 FOLLOW-UP

We observed the patients from the date of the pathology report to February 13, 2006 for the 1980s cohort and to January 15, 2007 for the 1990s cohort. Dead patients were censored on their date of death. Information on vital status and copies of the death certificates came from the demographic offices of Turin and the towns of the hinterland, and we ascertained migration at the Migration Office. Follow-up information was 95% complete (nine patients lost) for the 1980s cohort and 96% complete (eight patients lost) for the 1990s cohort. Patients with no follow-up information were excluded from the study. The death certificates for eight patients in the 1980s cohort were not retrievable. These patients were excluded from the analyses focusing on prostate cancer mortality.

3.1.5 MOLECULAR METHODS

3.1.5.1 DNA EXTRACTION

Genomic DNA was extracted from 3-5 sequential paraffin sections of primary tumour tissues, cut by using every time a new disposable microtome blade to prevent tissue carries over. The tissue specimens were dewaxed twice in xylene and washed twice in 100% ethanol. After removal of the ethanol, an over night digestion with 1:10 Proteinase K (20 mg/ml) at 55°C followed by DNA purification with phenol and precipitation with isopropanol according to the standard protocols was performed (Sambrok, 2001). In order to check the DNA adequacy beta-globin gene fragments of 152 base pairs (bp) were amplified by PCR using a set of primers chosen according to published sequences (Van Duin, 2002). These molecular techniques allow the analysis of the DNA methylation status, even in archival paraffin-embedded and micro dissected tissues. It represent the most versatile, rapid and cost-effective molecular tool presently available (Gillio-Tos, 2007). A successful extraction from tissue samples may depend on state of conservation of PETs that influences fragmentation and denaturation of DNA. Amplicons were analysed on 2% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination. If a patient had multiple blocks of PET, a block embedding tissue with tumor cells was chosen after histologic review of the

corresponding slide. If a patient had both biopsy and prostatectomy blocks, we analyzed the biopsy.

3.1.5.3 BISULPHITE MODIFICATION

Bisulfite modification was performed based on principle that bisulfite treatment of DNA converts unmethylated cytosines to uracil, whereas methylated cytosines remain unaltered. Thus, methylated and unmethylated DNA become distinguishable after bisulfite conversion by sequence-specific primers Genomic DNA samples underwent bisulphite modification using CpGenomeTM DNA modification Kit (Intergen Co., New York, NY) following the manufacturer's recommendations. Two microliters of a carrier DNA included in the kit (DNA Modification Reagent IV) were added to all samples. Positive controls for methylated [CpGenomeTM universal methylated DNA (Intergen Co.)] and unmethylated status [normal human lymphocyte DNA] were included in each modification set (Esteller, 1999). The bisulphite-modified genomic DNA was used immediately for methylation-specific PCR or stored at -80°C.

3.1.5.3 MSP-PCR

Methylated and unmethylated DNA sequences thus become distinguishable after bisulphite conversion by sequence-specific primers. The modified DNA were used as a template for PCR amplification using primers specific for either the methylated or the modified unmethylated DNA. The specific primers sets and their relative annealing temperature for methylated and unmethylated form of the selected genes promoters (GSTP1, APC, RUNX3) were chosen according to published sequences (Kang, 2004; Jeronimo, 2003) (Table 1). For PCR amplification, 4 µl of bisulfite-modified DNA were added in a final volume of 25 µl PCR mix. containing 1X PCR buffer (15 mmol/L Tris, pH 8.0; 50 mmol/L KCl; and 6.7 mmol/L MgCl2), deoxynucleotide triphosphates (2 mmol/L each), primers (0.4µmol/L each per reaction), and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 10 minutes at 95°C, 30 seconds at 95°C, 1 minute at primer-specific annealing temperature (Table 1), 1 minute at 72°C for 45 cycles, and 7 minutes at 72°C (Tsuchiya, 2000). All the PCR amplifications were performed in a Gene® Amp PCR System 9700 Thermal Cycler (Applera). Bisulfite-modified CpGenome^{TM} universal methylated DNA (Intergen Co.) were used as a positive control for methylated alleles, and bisulfite-modified DNA from normal human lymphocytes were used as a positive control for unmethylated alleles. Negative PCR controls without DNA were included in every

PCR run. Ten microliters of each PCR amplification were directly loaded on 2% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination. The sensitivity of MSP is 1:1000, detecting one methylated genome in 1000 unmethylated genome (Esteller, 2002).

Table 1. Primers and MSP PCR annealing temperature

Gene	**Primer sense (5'→ 3')**	**Primer antisense (5'→ 3')**	**bp**	**AT °C**
GSTP1 M	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	91	59
GSTP1 U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	97	59
APC M	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	98	55
APC U	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	108	59
RUNX3 M	TTCGTTTATTTTGTCGTCGT	CGCTATTATACGTATTCCCG	100	55
RUNX3 U	TTTGGGTTTATGGGAATATG	TTCTCACAACAACAACAACC	120	55

3.1.6 STATISTICAL ANALYSES

Cohort-1980s and cohorts-1990s were analysed separately.

We compared clinical and pathologic characteristics of the patients in the 1980s cohort and the 1990s cohort using univariate χ^2 and t tests (Armitage, 2002). Variables were classified as reported in Table 3. No information on PSA variables, tumor stage, and number of positive and negative biopsies was available in the pathology reports.

For each gene, we used logistical regression to estimate, separately for the 1980s and 1990s cohorts, the prevalence odds ratios (POR) of methylation in association with pathologic and demographic variables (Breslow, 1980). Patients with missing methylation status in one gene were excluded from the corresponding analyses.

For the older cohort, we ended follow-up after 14 years to apply the same maximum follow-up for both cohorts. The effect of methylation in each of the three genes on overall survival was investigated using Kaplan-Meier method (Kaplan, 1958) and differences in survival between patients with methylated and unmethylated cancers were evaluated with the log-rank test. Duration of follow-up was used as the time-scale. In presence of competing risks Kaplan-Meier estimates for cause-specific survival are biased because the assumption that all events are independent is violated (Haesook, 2007). Therefore the effect of methylation status in each of the three genes on cumulative mortality from prostate cancer was investigated taking

into account competing risks, (Kalbfleisch, 1980) and differences in overall prostate cancer mortality were evaluated with the Gray's test (Gray, 1988). Gray's test compare the cumulative incidence curves of a particular type of failure among different groups in presence of competing risk. Differences with methylation status in each of the three genes were tested using Gray's test to compare equality of cumulative incidence curve for different failure's type across methylation status.

Using age as the temporal axis, we used Cox proportional hazards regression models to estimate hazard ratios (HRs) with 95% CIs of prostate cancer mortality by methylation status and Gleason score (two categories:<8 and ≥8). Patients were censored at death from causes other than prostate cancer. Both a graphical check and formal tests based on Schoenfeld residuals, to exclude a nonlinear relationship between the residuals and the function of time, ($P > .15$) indicated that the proportional hazard assumption was met. We included the following covariates in the models: source of tumor tissue, Gleason score, and follow-up duration (time-dependent variable in three categories:<1 year, between 1 and 3 years, and 3+years). Inclusion of place of residence in the models did not affect HR estimates. HRs specific for categories of follow-up duration were estimated introducing terms for the interaction between this variable and methylation status. We also investigated the interaction between Gleason score and methylation in APC and RUNX3. In exploratory analyses, we further studied the association between survival and number of methylated genes. Patients with missing methylation status in at least one of the genes were excluded from this analysis (n=53, 12% of the patients). To understand whether a lack of cancer cells in some tissue slices biased our estimates, we provisionally restricted survival analyses to patients positive for methylation in GSTP1, for whom tumor cells were most likely sufficiently represented, and patients who underwent biopsy, where all available tissue was paraffin embedded in one single block.

Software packages used for general analysis were Stata and R. The R "cmprsk" package was used to perform Gray test. We used also "Design" library developed by Frank E. Harrell Jr. to plot Kaplan-Meier curves.

3.2 STUDY 2

3.2 1 STUDY DESIGN

We studied a sub-cohort of prostate cancer patients from the two cohorts (1980s' and 1990s'). For each patients we obtained PETs and the corresponding slides. The slides were re-evaluated by a pathologist to identify portion of non neoplastic tissue adjacent to tumour

tissue. We studied, in non neoplastic tissue, the promoter methylation of APC and of GSTP1 to evaluate the association with mortality. The study was approved by the local ethical committee.

3.2.2 STUDY POPULATION

The subpopulation selected from the cohort of study 1 includes patients of any age, diagnosed with prostate cancer at the Pathology Ward of the San Giovanni Battista Hospital in Turin. They received a biopsy or radical prostatectomy (RP) or a transurethral resection of the prostate (TURP) at the same hospital. We selected 157 patients from the cohort of study 1 for whom it is possible identified non neoplastic tissue adjacent to tumor tissue.

3.2.3 FOLLOW UP

We observed the patients from the date of the pathology report to August 31, 2011. Dead patients were censored on their date of death. Information on vital status and copies of the death certificates came from the demographic offices of Turin and the towns of the hinterland, and we ascertained migration at the Migration Office.

3.2.4 SLIDES COLLECTION

For each patient included in the study, PETs were available as well as the corresponding histological slides stained with hematoxylin-eosin (originally these were the diagnostical slides) at the Pathology Ward of the San Giovanni Battista Hospital in Turin.

The slides corresponding to the PETs of the cohort 1980s appeared bleached, no interpretable, because the staining tends to fade. We cut PETs slice with 3-4 μm thick sections and prepared fresh hematoxylin-eosin slides. The PET and the slides of each patients were identified reporting the same numbers. For each patient included in the study, each slide uniquely correspond to a PET with the same number.

3.2.5 NON NEOPLASTIC TISSUE SELECTION

All slides were analyzed by a pathologist to identify portions of non neoplastic tissue within the prostate tumour. If present, these portions were highlighted on the slide. Based on the information provided by the pathologist for each patient was selected a PET containing non neoplastic cells. Those patients for whom it has not been possible to identify any area of non-neoplastic tissue, were excluded from the study. For some patients blocks containing normal

tissue were more than one. For this case the choice of suitable PET were done according to the following criteria:

1) The pathologist suggested a priority based on histological features.
2) If the pathologist had not been given any priority, the choice was made ontwo criteria: the distance from the non neoplastic tissue marked on the slide from the surrounding tumor tissue (primary criterion) and the extension of non-neoplastic (secondary criterion) zone.

The selected PETs were cut with a microtome and depending on the extention of the non neplastic zone were cut a different number of slices: 5 slices for biopsies and small pieces, 4 slices for larger pieces. The tissue slices (thickness of 10 μm) were spread on a slide and allowed to dry. The slices overlapped exactly with the corresponding hematoxylin-eosin slide. Using this guide, the slices were subjected to macrodissection removing portion of non neoplastic tissue with a disposable scalpels with a fine tip to avoid contamination by surrounding tumour tissue. The fragments of tissue removed from the same patient were placed in a single tube.

3.2.6 MOLECULAR METHODS

3.2.6.1 DNA EXTRACTION

The tissue specimens were de waxed three times in xylene and washed three times in 100% ethanol. For the extraction and purification of DNA was used the commercial kit QIAamp ® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany): after removing ethanol the tissue was dried at room temperature (or thermal block at 37 ° C to shorten the time) and was incubated with 180 μl of buffer ATL (tissue lysis buffer) and 20 μl of proteinase K for 1 h at 56 ° C, and then for 1 h at 90 ° C. The tissue cells in the sample were lysed by adding 200 μl of Buffer AL, and the lysate obtained was washed with 200 μl of absolute ethanol, and then transferred on to the column and purified through the silica membrane of resin contained in the column. The resin holds, linking them, the DNA molecules. The membrane was washed twice with washing buffers AW1 and AW2 and finally DNA was eluted in a volume variable ATE (42 μl of DNA extracted from biopsies, 72 μl of DNA extracted from TURP operators and pieces). The extracted DNA was then stored at -80 ° C.

The concentration of each sample was assessed on a volume of 1.5 μl of DNA by UV-visible spectrophotometer (NanoDrop).

3.2.6.2 BISULPHITE MODIFICATION

DNA samples were extracted from PETs and underwent sodium bisulfite conversion, using the commercial kit EpiTect bisulfite kit (Qiagen, Hilden, Germany). For each sample 1 mg of DNA was modified. The volume of DNA modified by bisulfite conversion depend on the concentration of the DNA and the maximum volume of genomic DNA was 40 µl as recommended by the manufacturer's protocol. DNA was added to 85 µl of bisulfite Mix, 15 µl or 35 µl of the DNA Protect Buffer and the final total volume of 140 µl was reached with distilled H2O. The samples were placed in a thermal cycler and the reaction of modification, keep 5 hours with thermal profile as follows: 5 minutes at 95°C, 25 minutes at 60°C, 5 minutes at 95°C, 85 minutes at 60°C, 5 minutes at 95°C, 175 minutes at 60°C and final hold at 20°C.

The samples were then added to 310 µl of Buffer BL +10 µg of carrier RNA and 250 µl of absolute ethanol. The samples were then passed on a spin column and the washed with 500 µl of BW (wash buffer). Desulfonation was performed by adding 500 µl (BD Desulfonation Buffer) and incubating for 15 minutes at room temperature. The spin column was washed twice with 500 µl of BW and allowed to dry, the elution of the modified DNA was performed in two steps, using 21 µl of EB (elution buffer) resulting in a total volume of 42 µl, then stored at -80 ° C.

3.2.6.3 REAL TIME PCR with SPECIFIC PROBE

To determine the methylation pattern of APC and GSTP1 promoters was set up a Real Time PCR assay with specific probe. Each PCR reaction contains: 1x PCR buffer, 5.5 µM MgCl 2, 200 µM dNTPs, 600 nM of both primers, 200 nM and 80nM of specific probe for the methylation of the promoter of APC and GSTP1 respectively, 5U of Taq, 2 µl of modified DNA with sodium bisulfite and distilled H2O to obtain a final volume of 25 µl The reaction was carried out in a thermocycler (iCycler, BioRad). PCR conditions were as follows: 1,30 minutes at 95°C, 15 seconds at 95°C, 1 minute at primer-specific annealing temperature (60°C) for 50 cycles, and 10 minutes at 72°C. It was used a positive control (universal methylated DNA CpGenome, Chemicon). Negative controls were included to exclude the presence of contamination.

Primers and probe used were initially selected from those published in the literature (Eads, 2001; Woodson, 2004), then modified: their sequence is shown in Table 2. Primers amplified a 73 bp region of the APC promoter (GenBank Accession number: U02509). For GSTP1

primers amplified a 72 bp fragment. Specific probe identifies methylation status of four CpG islands for each gene.

We performed the Real Time PCR on tumour samples corresponding to selected non neoplastic tissues to compare results obtained with the same technique with high sensitivity.

Table 2. Primers and probes for Real Time PCR

	GSTP1	**APC**
PRIMER F	5'- GATTTGGGAAAGAGGGAAAGGT -3'	5'-GGATTAGGGCGTTTTTTAT-3'
PRIMER R	5'- CAAAAAAACGCCCTAAAATC -3'	5'-GTGTGGGCGTACGTGATCGATATGTG-3'
PROBE	Fam- TGCGCGGCGATTTCGGG -tamra	Fam-TTCGTCGGGAGTTCGTCGATTG-tamra

3.2.7 STATISTICAL ANALYSES

We calculated frequencies of DNA methylation in non-neoplastic tissue and studied methylation in the tumor tissue and Gleason score as determinant of methylation using logistic regression. To estimate the association between the Gleason score and the methylation in non-neoplastic tissue adjacent to the tumor, was used an ordered logistic regression model. We tested for the proportional-odds assumption by an approximate likelihood-ratio test. This estimate was checked for the following potential confounding factors: the cohort of origin, age at diagnosis (65-70, 70-75,> 75 versus <65), calendar year at diagnosis, and the type of tumor tissue (prostatectomy and TURP versus biopsy). In order to model methylation in margins we used a standard logistic regression. Because of competing nature of events (prostate cancer mortality vs mortality from other causes), for the survival analysis was applied the model of Fine & Gray (Fine and Gray, 1999), an extension of the Cox proportional hazards model. This model estimates the hazard corresponding to the cause-specific cumulative incidence function, i.e. the probability of event of interest occurring before other competing events, in the presence of randomly right censored data. In this context, for example, death from other causes of death to preclude the occurrence of prostate cancer. We refer to this as the sub-hazard ratio (sub-HR) (for simplicity we in tables HR). We checked and tested whether the proportionality assumption by Schoenfeld's residuals. This analysis was controlled for the following potential confounding factors: age at diagnosis (65-70, 70- 75,> 75 versus <65), calendar year at diagnosis, the type of tumor tissue (prostatectomy and TURP vs. biopsy), methylation of the tumor, and tumor grade (Gleason score = 7 and> 7 vs <7). All statistical analysis were carried out for both APC and GSTP1

genes. The level of significance was set at 5%. The statistical analysis were performed using the program STATA 11.1 (www.stata.com).

3.3 STUDY 3

3.3.1 STUDY DESIGN

We studied prostate cancer patients from the two cohorts (1980s' and 1990s'). We genotyped each patient for the rs406193 C>T SNP of the DNMT3b gene. We evaluate the association between variant selected of DNMT3b gene and mortality for prostate cancer.

The study was approved by the local Ethical Committee.

3.3.2 STUDY POPULATION

This study is based on 451 members of two cohorts of consecutive prostate cancer patients included in study 1.

Genotyping was not successful for 13 patients, because of poor DNA quality. These patients were excluded from the study leaving 438 patients for further analyses.

3.3.3 DNMT3b GENOTYPING

We targeted the single-nucleotide polymorphism rs406193 located in the 3' flanking region of the DNMT3b DNA sequence; C is the wild type and T the variant allele. We carried out SNP genotyping using a validated commercially available TaqMan® SNP Genotyping Assay kit (Applied Biosystems, Foster City, CA, USA). The assay was performed according to the manufacturer instructions on the iCycler iQ™ Real Time PCR Detection System (Bio-Rad®, Hercules, CA, USA). Specifically, 20 ng of DNA sample were added to a reaction solution of 25 µL containing 2X iQ™ SuperMix (Bio-Rad) and a 20X working stock solution of SNP Genotyping Assay (Applied Biosystems) including two specific primers and two MGB (Minor Groove Binder)-Taqman probes. Reaction conditions were as follows: 10 min at 95°C; 40 cycles of 15 s at 92°C, 90 s at 60°C. Fluorescence was measured in the iCycler iQ™ Real Time PCR Detection System with an automatic software for data analysis (Bio-Rad). All of the samples were tested in duplicate.

3.3.4 STATISTICAL ANALYSIS

We conducted statistical analyses on the basis of the assumed causal relationships between the main variables involved in the study (DNMT3b variant, DNA-methylation, Gleason score and prostate cancer mortality). Specifically, we assumed that: i) DNMT3b activity affects the methylation status of the three assessed genes as well as of several other genes, ii) methylation status affects the tumour morphology and thus the Gleason score and not vice versa, iii) DNA methylation may affect mortality.

In all analyses, due to low frequency of homozygous for TT, we compared CC carriers with carriers of at least one T. Age at diagnosis in 5-year classes, period of diagnosis (1980s or 1990s) and source of tumour tissue (biopsy, TURP, prostatectomy) were included as potential confounders in all analyses.

We estimated the associations among the variables shown in Figure 3: the effects of DNMT variant rs406193 on the number of methylated genes, Gleason score and prostate cancer mortality. We used ordinal logistic regression to estimate the odds ratio (OR), and corresponding confidence intervals (CIs), for the variant rs406193 on the number of methylated genes. We used three levels for the dependent variable (0-1, 2 or 3 methylated genes out of APC, GSTP1, and RUNX3) and the odds ratios estimated in this model should be interpreted as the effect of the rs406193 variant on each increase in number of methylated genes. We used logistic regression to estimate the OR of having a high Gleason score (8+). In the latter analysis, the number of methylated genes was not included in the model as it was assumed to be an intermediate variable in the path between DNMT3b activity and Gleason score (Figure 1).

We evaluated the effect of rs406193 on prostate cancer mortality by using a Cox regression model to estimate hazard ratios (HRs) of prostate cancer mortality and corresponding 95% CIs, with age as the time-scale. Subjects were censored at death for causes other than prostate cancer. The proportional hazard assumption was checked by the formal test based on Schoenfeld's residuals. Since in a competing risks framework there is no direct correspondence between the effects of the exposure on the hazard function and its effects on the cumulative incidence function, we also used the Fine and Gray model to estimate the hazard corresponding to the cause-specific cumulative incidence function (subdistribution hazard ratios) (Fine, 1999). The results from the two models were very similar and, for simplicity, we report in the paper only the estimates obtained from the Cox model.

We conducted further analyses to assess to what extent the effect of rs406193 variant (exposure) on cause-specific mortality (outcome) is mediated through the Gleason score

(mediator). Specifically we estimated the effect of rs406193 variant on prostate cancer mortality without and with adjusting for the Gleason score, assuming (i) no residual confounding between the mediator and the outcome, (ii) no residual confounding between the exposure and the mediator and (iii) no interactions between the exposure and the mediator as well as between the mediator and variables affected by the exposure (Petersen, 2006).

4. RESULTS

4.1 STUDY 1

We identified 298 patients for the cohort-1980s and 280 patients for the cohort-1990s. The proportion of successful DNA extraction from PETs was 77% (n=228) in the cohort-1980s and 90% (n=253) in the cohort-1990s.

However, due to lack or incorrect demographical information 12 (5.2%) patients of cohort-1980s and 10 (3.9%) patients of cohort-1990s were excluded, leaving 216 and 243 patients for the analysis. Vital status was obtained for all patients. Causes of death were missing for 8 and 0 patients for cohort-1980s and cohort-1990s, respectively.

Patients of the cohort-1990s were younger than those of the cohort-1980s. They had a median survival time of 6.3 years, which was twice that observed in the cohort-1980s (3.1 years). In the 1990s cohort, tumor tissue was obtained from transurethral resections of the prostate and radical prostatectomies more often than in the 1980s cohort (Table 3).

The prevalence of methylation in APC was lower (P = .047) in the 1980s cohort, whereas methylation in GSTP1 (P = .002) and RUNX3 (P<.001) was higher in the cohort-1980s (76% and 84.6%) than in the cohort-1990s (62.8% and 48%) (Table 3). This difference remained after adjusting for Gleason score, age, source of the tumor tissue and year of collection of the tumour tissues.

Methylation in GSTP1 and APC was positively associated with Gleason score only in the 1990s cohort (Table 4). In both cohorts, pair-wise comparisons revealed that methylation in each gene was independent from methylation in the other two genes (P>.25).

Results of analysis on overall survival for methylation in the three genes and tumour grade are reported in Figure 1. In cohorts-1980s the methylation status in APC was associated with survival (p-value<0.05). An association was found also in the cohort-1990s between methylation in RUNX3 and survival (p-value=0.0161).

Figure 2 shows the cumulative mortality for prostate cancer. Patients with methylation in APC had a higher prostate cancer mortality than patients with an unmethylated cancer (Figure 2A). This association was statistically significant only in the 1990s cohort (P = .02). Methylation in RUNX3 was associated with survival in the 1990s cohort (P=.05) but not in the 1980s cohort (Figure 2B). Methylation in GSTP1 was not associated with survival.

When we evaluated prostate cancer mortality in association with methylation in APC we found an HR of 1.42 (95% CI, 0.98 to 2.07) in the cohort-1980s and 1.57 (95% CI, 0.95 to 2.62) in the 1990s cohort (HR = 1.49; 95% CI, 1.11 to 2.00 for the two cohorts combined;

Table 5). In the 1990s cohort, the adjusted HR estimate was lower than the crude one, mainly because of the introduction of Gleason score into the model. In the 1980s cohort, the association between methylation in APC and mortality was stronger and statistically significant in the first year of follow-up (adjusted HR of 2.66 95% CI: 1.12-6.31), whereas in the 1990s cohort, the HR estimate increased with duration of follow-up: after 3 years of follow-up we found an HR=1.86 (95% CI: 0.93-3.72) (Table 5).

Results were not consistent between the two cohorts for methylation in RUNX3 which was associated with prostate cancer mortality in the 1990s cohort (HR=1.56; 95% CI, 0.95 to 2.56) but not in the 1980s cohort (Table 5). Methylation in GSTP1 was not associated with prostate cancer mortality.

Results of the analyses on prostate cancer mortality restricted to patients who underwent biopsy or were positive for GSTP1 methylation did not substantially change increased HR estimates for methylation in APC and RUNX3. The effect of methylation in APC or RUNX3 at different levels of Gleason score is summarized in Table 6. In the 1980s cohort, Gleason score had a small impact on the HR estimates, whereas in the 1990s cohort, we found a doubled HR from prostate cancer mortality among patients with a Gleason score less than 8 both for methylation in APC (HR = 2.09; 95% CI, 1.02 to 4.28) and in RUNX3 (HR = 2.40; 95% CI, 1.18 to 4.91). In the two cohorts combined, the HR of prostate cancer mortality increased with increasing number of methylated genes (P = .002 for linear trend; Table 7).

4.1.1 TABLES AND FIGURES

Table 3. Characteristics of the two cohorts of prostate cancer patients after 14 years of Follow-Up

Characteristic	1980s Cohort (1982-1988) No. of Patients	%	1990s Cohort (1993-1996) No. of Patients	%	P*
No. of patients	216		243		
No. of person-years	1,040		1,591		
Mortality					
Overall	195		177		
As a result of prostate cancer	121		76		
As a result of other causes	74		101		
Missing cause of death	8		0		
Survival time, years					
Median	3.1		6.3		
Range	0-14		0-14		
Age, years					.003
Mean	72.3		70.0		
Standard deviation	7.5		8.7		
Residence					.692
Turin	153	70.8	168	69.1	
Turin hinterland	63	29.2	75	30.9	
Source of tumor tissue					< .001
Biopsy	182	84.3	164	67.5	
TURP	11	5.1	45	18.5	
Radical prostatectomy	23	10.6	34	14.0	
Gleason score					< .001
< 7	32	14.8	136	56.0	
7	85	39.4	34	14.0	
8+	99	45.8	73	30.0	
Methylation in *GSTP1*					.002
Yes	159	76.1	150	62.8	
No	50	23.9	89	37.2	
Missing	7	—	4	—	
Methylation in *APC*					.047
Yes	76	35.9	106	45.1	
No	136	64.1	129	54.9	
Missing	4	—	8	—	
Methylation in *RUNX3*					< .001
Yes	170	84.6	103	48.1	
No	31	15.4	111	51.9	
Missing	15	—	29	—	

Abbreviation: TURP, transurethral resection of the prostate.
*P value for difference between 1980s cohort and 1990s cohort.

(Richiardi, Fiano et al., JCO, 2009)

Table 4. Association between Gleason score and prevalence of methylation in GSTP1, APC, and RUNX3 in 1980s and 1990s cohorts

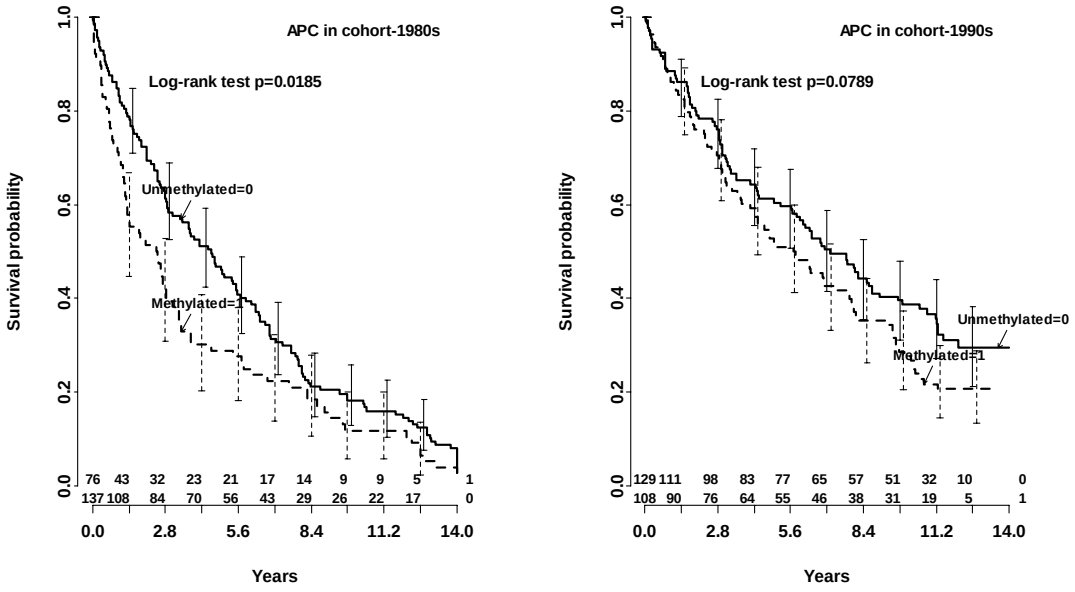
	GSTP1			*APC*			*RUNX3*		
Gleason Score	Prevalence of Methylation (%)	POR*	95% CI	Prevalence of Methylation (%)	POR*	95% CI	Prevalence of Methylation (%)	POR*	95% CI
1980s cohort									
< 7	65.5	1	—	25.8	1	—	89.3	1	—
7	82.1	2.27	0.81 to 6.34	40.5	1.66	0.64 to 4.31	81.0	0.40	0.10 to 1.70
8+	74.0	1.18	0.45 to 3.13	35.1	1.25	0.48 to 3.25	86.2	0.58	0.13 to 2.53
1990s cohort									
< 7	57.0	1	—	38.5	1	—	44.7	1	—
7	62.5	1.21	0.53 to 2.77	48.5	1.39	0.63 to 3.08	48.4	1.21	0.53 to 2.76
8+	73.6	2.02	1.06 to 3.84	55.6	2.07	1.13 to 3.81	53.6	1.36	0.73 to 2.52

Abbreviation: POR, prevalence odds ratio.
*POR adjusted for age, source of tumor tissue, year of tissue collection, and residence.

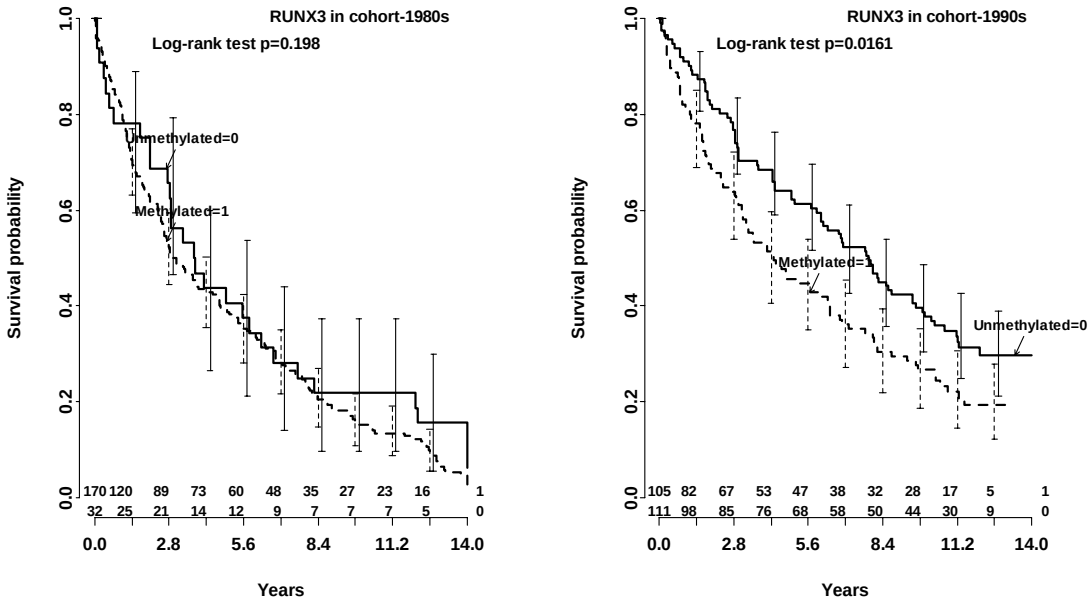
(Richiardi, Fiano et al., JCO, 2009)

Figure 1. Kaplan-Meier overall survival probability by methylation in APC (A), RUNX3 (B) and GSTP1 (C)

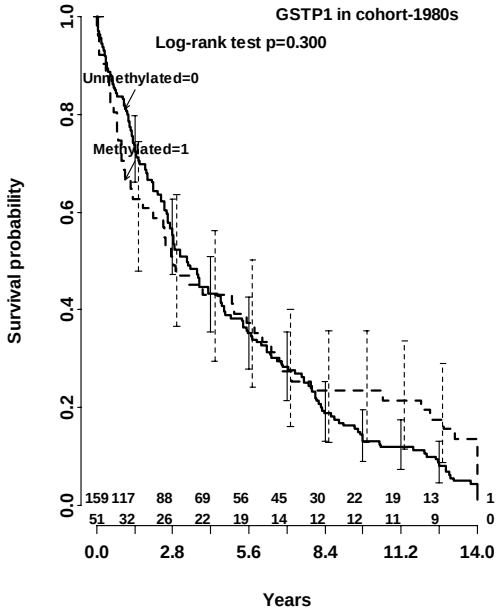
A



B



C


GSTP1 in cohort-1980s
Log-rank test p=0.300
Unmethylated=0
Methylated=1
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
159 117 88 69 56 45 30 22 19 13 1
51 32 26 22 19 14 12 12 11 9 0
0.0 2.8 5.6 8.4 11.2 14.0
Years

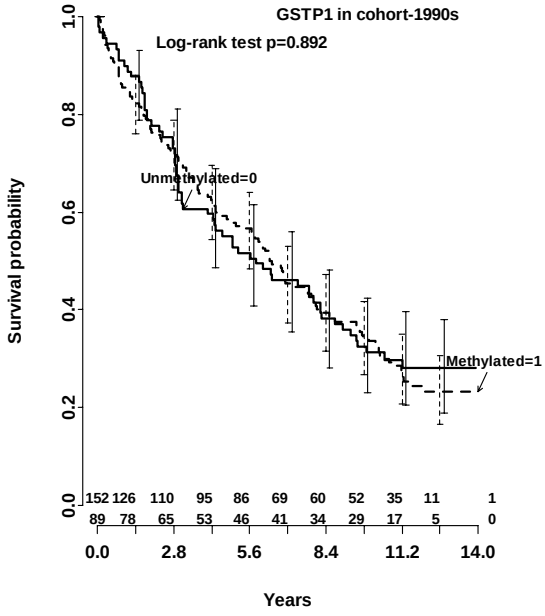
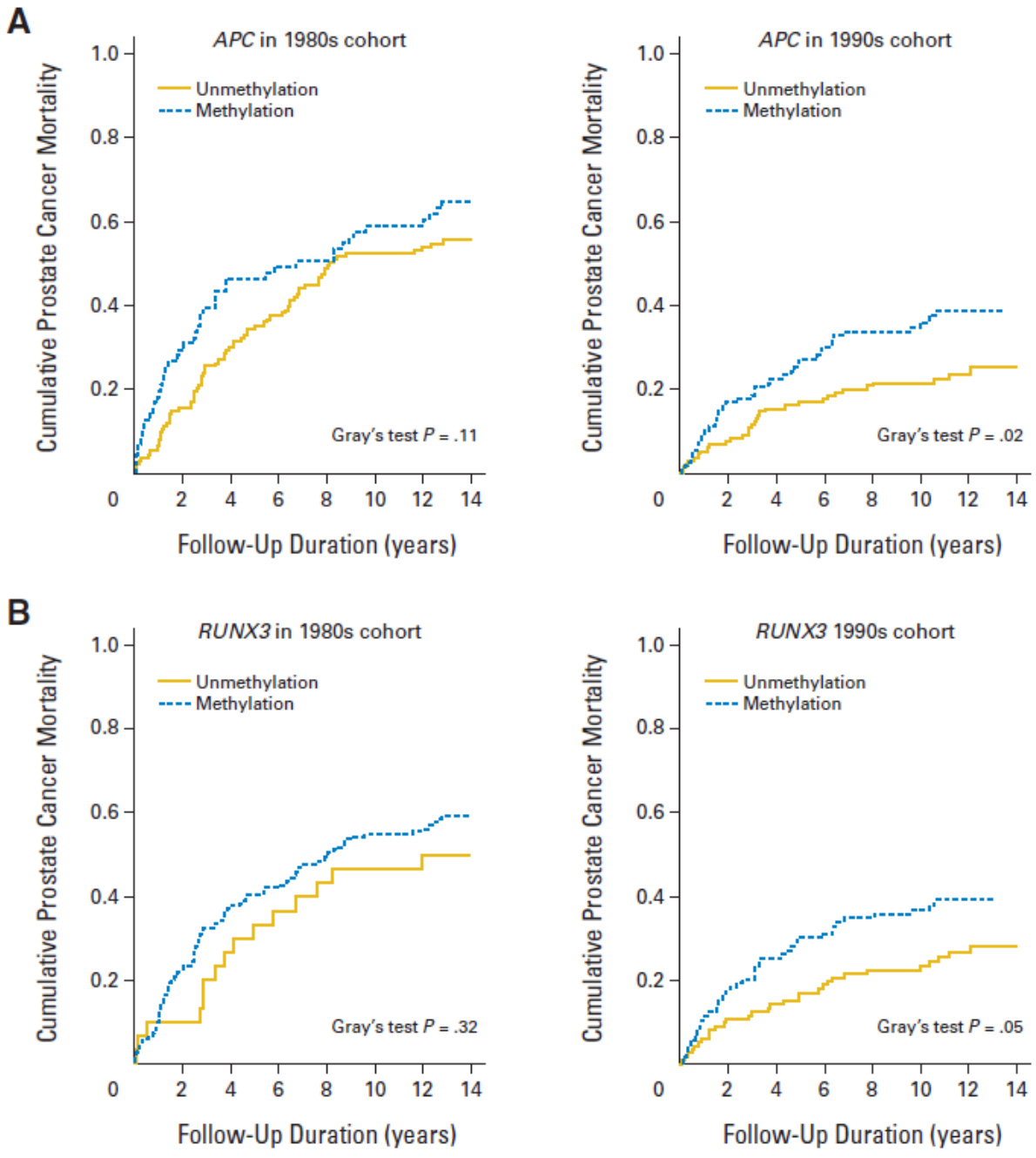

GSTP1 in cohort-1990s
Log-rank test p=0.892
Unmethylated=0
Methylated=1
Survival probability
1.0
0.8
0.6
0.4
0.2
0.0
152 126 110 95 86 69 60 52 35 11 1
89 78 65 53 46 41 34 29 17 5 0
0.0 2.8 5.6 8.4 11.2 14.0
Years

Figure 2. Cumulative prostate cancer mortality by methylation status in (A) APC and (B) RUNX3 in the 1980s cohort and 1990s cohort



(Richiardi, Fiano et al., JCO, 2009)

Table 5. Prostate cancer mortality for methylation in GSTP1, APC, and RUNX3 by duration of Follow-Up in 1980s and 1990s cohorts

Methylation and Gleason Score	No. of Deaths	Overall 14-Year Follow-Up			Follow-Up Period					
					< 1 Year		1-3 Years		3+ Years	
		Crude HR	HR*	95% CI	HR*	95% CI	HR*	95% CI	HR*	95% CI
1980s cohort										
Methylation in *GSTP1*										
No	26	1	1		1		1		1	
Yes	89	1.08	1.00	0.64 to 1.58	0.42	0.17 to 1.03	0.94	0.41 to 2.12	1.64	0.79 to 3.41
Methylation in *APC*										
No	74	1	1		1		1		1	
Yes	46	1.46†	1.42	0.98 to 2.07	2.66	1.12 to 6.31	1.41	0.72 to 2.75	1.10	0.63 to 1.93
Methylation in *RUNX3*										
No	15	1	1		1		1		1	
Yes	97	1.32	1.22	0.70 to 2.14	0.97	0.27 to 3.43	2.39	0.72 to 7.89	0.93	0.45 to 1.94
Gleason score										
< 8	50	1	1		1		1		1	
8+	71	2.53†	2.17	1.48 to 3.18	3.39	1.29 to 8.91	1.87	0.97 to 3.60	2.07	1.21 to 3.52
1990s cohort										
Methylation in *GSTP1*										
No	17	1	1		1		1		1	
Yes	58	2.02†	1.44	0.82 to 2.54	1.62	0.52 to 5.04	0.94	0.34 to 2.62	1.74	0.75 to 4.04
Methylation in *APC*										
No	31	1	1		1		1		1	
Yes	41	1.99†	1.57	0.95 to 2.62	1.48	0.55 to 3.96	1.15	0.42 to 3.16	1.86	0.93 to 3.72
Methylation in *RUNX3*										
No	30	1	1		1		1		1	
Yes	40	1.74	1.56	0.95 to 2.56	1.58	0.60 to 4.14	1.33	0.48 to 3.68	1.66	0.85 to 3.27
Gleason score										
< 8	36	1	1		1		1		1	
8+	36	3.50†	3.27	2.00 to 5.37	6.50	2.08 to 20.3	3.61	1.31 to 9.94	2.34	1.16 to 4.73

Abbreviation: HR, hazard ratio.
*HR was adjusted for follow-up duration, Gleason score, and source of tumor tissue; Gleason score was also adjusted for methylation in *APC*.
†$P < .05$.

(Richiardi, Fiano et al., JCO, 2009)

Table 6. Prostate cancer mortality for Gleason score strata and methylation in APC and RUNX3 in 1980s and 1990s cohorts

	Gleason < 8			Gleason 8+		
Methylation	No. of Deaths	HR*	95% CI	No. of Deaths	HR*	95% CI
1980s cohort						
Methylation in *APC*						
No	30	1		44	1	
Yes	20	1.52	0.85 to 2.73	26	1.36	0.81 to 2.26
Methylation in *RUNX3*						
No	6	1		9	1	
Yes	38	1.14	0.45 to 2.88	59	1.09	0.52 to 2.29
1990s cohort						
Methylation in *APC*						
No	16	1		15	1	
Yes	20	2.09	1.02 to 4.28	21	1.07	0.51 to 2.26
Methylation in *RUNX3*						
No	14	1		16	1	
Yes	21	2.40	1.18 to 4.91	19	0.84	0.38 to 1.85

Abbreviation: HR, hazard ratio.
*HR was adjusted for follow-up duration and source of tumor tissue.

(Richiardi, Fiano et al., JCO, 2009)

Table 7. Prostate cancer mortality for number of methylated genes in 1980s and 1990s cohorts combined

No. of Methylated Genes	No. of Deaths	HR*	95% CI
0	5	0.80	0.31 to 2.08
1	32	1.00	
2	94	1.53	1.02 to 2.30
3	44	1.97	1.24 to 3.15

Abbreviation: HR, hazard ratio.
*HR was adjusted for follow-up duration, Gleason score, source of tumor tissue, and cohort. $P = .002$ for trend.

(Richiardi, Fiano et al., JCO, 2009)

4.2 STUDY 2

We identified 157 patients, 28 for the cohort 1980 and 129 for the cohort 1990. Vital status was missing for one patient. The tumour tissues were obtained from biopsies, transurethral resections of the prostate and radical prostatectomies in similar proportion. Prevalence of methylation in non neoplastic tissue was 58% for GSTP1 and 56% for APC. Prevalence of methylation in tumour corresponding to non neoplastic tissue was 84% and 82% for GSTP1 and APC respectively (Table 8).

Methylation was found at the same time in tumour and non neoplastic tissue in 48.8% of patients for APC and in 45.4% for GSTP1. Methylation was found only in non neoplastic in 21.4% and 24% of patients for APC and GSTP1 respectively (Table 9).

When we considered the methylation of APC in non neoplastic tissue we found an association with prevalence of APC promoter methylation in tumour for the same gene (OR=3.27, 95% CI, 1.12 to 9.53), but the association is lower we consider the methylation of GSTP1 (OR=1.31, 95% CI, 0.47-3.65) (Table 10 A).

Similar results were obtained for methylation of GSTP1 in non neoplastic tissue in association with the methylation of the same gene (OR=2.66) or different gene (APC OR=1.07) in tumour (Table 10 B).

In table 11 we report mortality for prostate cancer and for other causes in relationship with the methylation status of the gene analysed in tumour and non neoplastic tissue adjacent to tumor.

Patients with methylation in APC and GSTP1 in non neoplastic tissue adjacent to tumour had a higher risk of mortality than those with unmethylated promoter. HR for prostate cancer mortality for APC methylation in non neoplastic tissue was 2.91 (95% CI, 1.49-5.67) (Table 12 A) for GSTP1 was 3.48 (95% CI, 1.67-7.27) (Table 12 B). When we adjusted for Gleason score and methylation in tumor tissue the HR decreased for the two genes, HR=1.80 and HR=1.61 for APC and GSTP1 respectively but the results were not statistically significant (Table 13). We performed the analysis of mortality with a five-years follow up and the association with methylation of non neoplastic tissue was higher HR=4.08 for APC and HR=4.79 for GSTP1 (Table 14). When we adjusted for Gleason score and methylation in tumor tissue the association decreased (data not shown).

We found a strong association between number of methylated genes in non neoplastic tissue and mortality also after adjustment for Gleason score and methylation in tumor tissue (Table 15, Table 16).

4.2.1 TABLES

Table 8. Characteristics of patients

Characteristics of Patients	N (%)
Number	157
Cohort	
1980s	28 (17.8)
1990s	129 (82.2)
Age at diagnosis	
40-64	26 (16.6)
65-69	35 (22.3)
70-74	40 (25.5)
>=75	56 (35.7)
Life status	
Death from prostate cancer	43 (27.4)
Death from other causes	85 (54.1)
Censoring	28 (17.8)
Missing	1 (0.6)
GSTP1 Methylation in Tumor	
No	28 (17.8)
Yes	129 (82.2)
APC Methylation in Tumor	
No	25 (15.9)
Yes	132 (84.1)
GSTP1 Methylation in NNTAT	
No	91 (58.0)
Yes	66 (42.0)
APC Methylation in NNTAT	
No	88 (56.1)
Yes	69 (43.9)
Gleason score	
<7	58 (36.9)
7	41 (26.1)
8+	55 (35.0)
Missing	3 (1.9)
Source of tumor tissue	
Biopsy	54 (34.4)
TURP	54 (34.4)
Radical prostectomy	49 (31.2)

NNTAT: non neoplastic tissue adjacent to tumor

Table 9. Frequency of methylation of APC and GSTP1 in non neoplastic tissue adjacent to tumor (NNTAT) and tumor

	APC Methylation in Tumor	
APC Methylation in NNTAT	No	Yes
No	22(78.6)	66(51.2)
Yes	6(21.4)	63(48.8)
	GSTP1 Methylation in Tumor	
GSTP1 Methylation in NNTAT	No	Yes
No	19(76.0)	72(54.5)
Yes	6(24.0)	60(45.4)

Table 10. Association between gene specific [APC (A) and GSTP1 (B)] methylation in tumor and risk of methylation in non neoplastic tissue adjacent to tumor (NNTAT)

A

	APC Methylation in NNTAT	
Methylation in Tumor	**OR**	**95% CI**
APC		
No	1	-
Yes	3.27	1.12-9.53
GSTP1		
No	1	-
Yes	1.31	0.47-3.65
Age at diagnosis		
40-64	1	-
65-69	1.54	0.51-4.64
70-74	2.14	0.73-6.29
>=75	1.98	0.71-5.49
Cohort		
1980s	1	-
1990s	1.67	0.11-24.08
Source of tumor tissue		
Biopsy	1	-
TURP	0.85	0.37-1.97
Radical prostectomy	0.64	0.26-1.60

B

	GSTP1 Methylation in NNTAT	
Methylation in Tumor	**OR**	**95% CI**
APC		
No	1	-
Yes	1.07	0.40-2.85
GSTP1		
No	1	-
Yes	2.66	0.86-8.27
Age at diagnosis		
40-64	1	-
65-69	0.51	0.16-1.58
70-74	0.40	0.13-1.23
>=75	0.57	0.20-1.61
Cohort		
1980s	1	-
1990s	0.11	0.01-1.41
Source of tumor tissue		
Biopsy	1	-
TURP	0.20	0.08-0.49
Radical prostectomy	0.28	0.11-0.70

OR adjusted for age at diagnosis, cohort and source of tumor tissue

Table 11. Life status associated to methylation status for APC and GSTP1 in tumor and in non neoplastic tissue adjacent to tumor (NNTAT), and to Gleason score

		Censoring (N=28)	Death from prostate cancer (N=43)	Death from other causes (N=85)
APC Methylation	**Tumor**			
	No	4 (14.8)	3 (11.1)	20 (74.1)
	Yes	24 (18.6)	40 (31.0)	65 (50.4)
	NNTAT			
	No	19 (21.6)	17 (19.3)	52 (59.1)
	Yes	9 (13.2)	26 (38.2)	33 (48.5)
GSTP1 Methylation	**Tumor**			
	No	15 (16.5)	19 (20.9)	57 (62.6)
	Yes	13 (20.0)	24 (36.9)	28 (43.1)
	NNTAT			
	No	15 (16.5)	19 (20.9)	57 (62.6)
	Yes	13 (20.0)	24 (36.9)	28 (43.1)
Gleason score	**Tumor**			
	<7	14 (24.1)	3 (5.2)	41 (70.7)
	7	8 (20.0)	11 (26.8)	21 (51.2)
	8+	6 (10.9)	28 (50.9)	21 (38.2)
	Missing	-	18 (33.3)	2 (66.7)

Table 12. Association between methylation status for APC (A) and GSTP1 (B) in non neoplastic tissue adjacent to tumor (NNTAT) and mortality for prostate cancer

A

	HR	95% CI
APC Methylation in NNTAT		
No	1	-
Yes	2.91	1.49-5.67
Age at diagnosis		
40-64	1	-
65-69	3.38	0.89-12.82
70-74	3.23	0.42-24.89
>=75	10.80	1.52-76.57
Cohort		
1980s	1	-
1990s	3.69	0.39-34.87
Source of tumor tissue		
Biopsy	1	-
Radical prostectomy	0.25	0.07-0.80
TURP	1.26	0.61-2.58

B

	HR	95% CI
GSTP1 Methylation in NNTAT		
No	1	-
Yes	3.48	1.67-7.27
Age at diagnosis		
40-64	1	-
65-69	3.70	0.96-14.30
70-74	3.58	0.49-25.88
>=75	12.41	1.84-83.44
Cohort		
1980s	1	-
1990s	7.60	0.53-109.72
Source of tumor tissue		
Biopsy	1	-
Radical prostectomy	0.33	0.11-1.00
TURP	2.14	0.98-4.64

HR adjusted for age at diagnosis, cohort and source of tumor tissue

Table 13. Association between methylation status for APC (A) and GSTP1 (B) in non neoplastic tissue adjacent to tumor (NNTAT) and mortality for prostate cancer adjusted for methylation in tumor and Gleason score

	HR	95% CI
APC Methylation in Tumor		
No	1	-
Yes	1.25	0.43-3.64
GSTP1 Methylation in Tumor		
No	1	-
Yes	2.23	0.40-12.50
APC Methylation in NNTAT		
No	1	-
Yes	1.80	0.81-4.03
GSTP1 Methylation in NNTAT		
No	1	-
Yes	1.61	0.62-4.15
Gleason score		
<7	1	-
7	4.54	1.13-18.29
8+	9.08	2.59-31.85
Age at diagnosis		
40-64	1	-
65-69	3.08	0.63-9.24
70-74	3.67	0.37-25.10
>=75	10.63	1.23-64.11
Cohort		
1980s	1	-
1990s	5.76	0.20-167.54
Source of tumor issue		
Biopsy	1	-
Radical prostectomy	0.44	0.14-1.37
TURP	1.03	0.40-2.67

HR adjusted for methylation status in tumor, Gleason score, age at diagnosis, cohort and source of tumor tissue

Table 14. Association between methylation status for APC (A) and GSTP1 (B) in non neoplastic tissue adjacent to tumor (NNTAT) and mortality for prostate cancer at 5 years of follow up

A

	HR	95% CI
GSTP1 Methylation in NNTAT		
No	1	-
Yes	4.79	1.95-11.79
Age at diagnosis		
40-64	1	-
65-69	8.06	1.61-40.35
70-74	46.48	4.45-485.39
>=75	290.02	21.27-3953.50
Cohort		
1980s	1	-
1990s	8.10	0.29-222.44
Source of tumor tissue		
Biopsy	1	-
Radical prostectomy	0.31	0.08-1.17
TURP	2.01	0.85-4.76

B

	HR	95% CI
APC Methylation in NNTAT		
No	1	-
Yes	4.08	1.58-10.58
Age at diagnosis		
40-64	1	-
65-69	5.67	0.87-37.10
70-74	46.28	3.95-541.84
>=75	276.38	18.95-4029.91
Cohort		
1980s	1	-
1990s	4.10	0.19-90.70
Source of tumor tissue		
Biopsy	1	-
Radical prostectomy	0.24	0.05-1.06
TURP	1.20	0.50-2.90

HR adjusted for age at diagnosis, cohort and source of tumor tissue

Table 15. Association between number of genes methylated in non neoplastic tissue adjacent to tumor (NNTAT) and mortality for prostate cancer

	HR	95% CI
N of Methylated genes NNTAT		
0	0.65	0.26-1.66
1	1	-
2	3.28	1.48-7.26
Age at diagnosis		
40-64	1	-
65-69	3.12	0.80-12.19
70-74	3.14	0.39-25.25
>=75	9.91	1.36-71.94
Cohort		
1980s	1	-
1990s	4.70	0.35.62.78
Source of tumor issue		
Biopsy	1	-
Radical prostectomy	0.30	0.09-0.96
TURP	1.97	0.95-4.09

HR adjusted for age at diagnosis, cohort and source of tumor tissue

Table 16. Association between number of genes methylated in non neoplastic tissue adjacent to tumor (NNTAT) and mortality for prostate cancer adjusted for methylation in tumor tissue and Gleason score

	HR	95% CI
N of Methylated genes in NNTAT		
0	0.85	0.31-2.31
1	1	-
2	2.56	0.94-6.98
N of Methylated genes in tumor		
0	1.96^{-06}	4.33^{-07}-8.86^{-06}
1	1	-
2	1.04	0.30-3.56
Gleason score		
<7	1	-
7	5.27	1.27-21.97
8+	8.65	2.61-28.70
Age at diagnosis		
40-64	1	-
65-69	2.07	0.54-7.99
70-74	2.85	0.34-24.10
>=75	8.03	1.08-59.60
Cohort		
1980s	1	-
1990s	4.29	0.19-99.26
Source of tumor issue		
Biopsy	1	-
Radical prostectomy	0.39	0.11-1.39
TURP	1.12	0.48-2.61

HR adjusted for methylation status in tumor, Gleason score, age at diagnosis, cohort and source of tumor tissue

4.3 STUDY 3

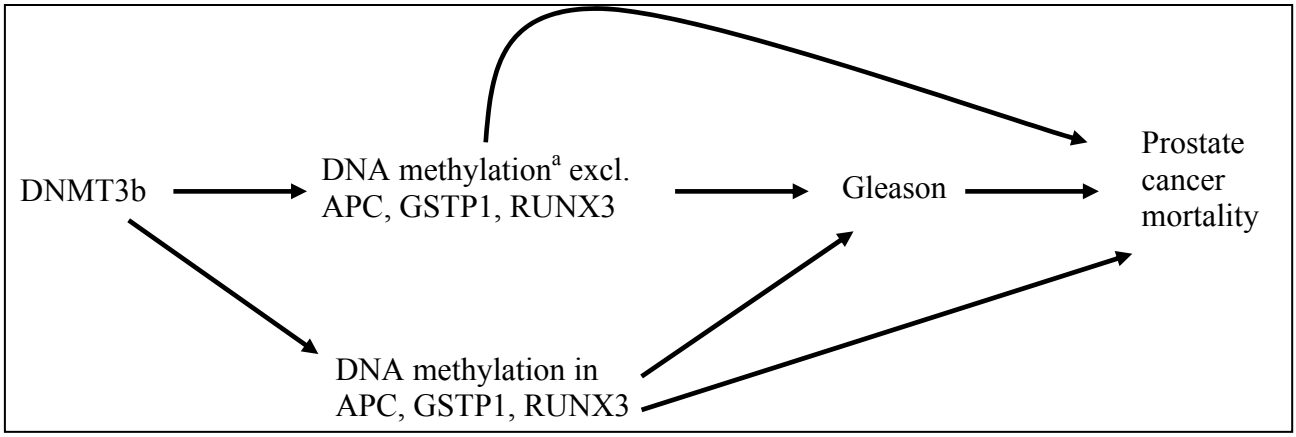
The characteristics of the 438 patients included in the study are summarised in Table 17. Allelic frequencies were: CT = 42.3%, TT = 2.7% and CC = 55%; 189 patients died from prostate cancer. With regard to the three main variables considered in our analysis (Figure 3), 188 of the patients had a Gleason score of at least 8, 80 had promoter methylation in all the three evaluated genes (APC, GSTP1 and RUNX3), and 199 were T carriers in rs406193.

As reported in Table 18, there was little or no association between carriers of the rs406193 T allele and the number of methylated genes (OR per each increase in one methylated gene = 0.84, 95% CI: 0.57-1.23). Conversely, the rs406193 T allele had an OR of 0.57 (95% CI: 0.39-0.85) of having a Gleason score of at least 8 (Table 19).

The findings summarized in Table 20 suggest that carriers of the rs406193 T allele had a decreased risk of dying for prostate cancer (HR: 0.81, 95% CI: 0.61-1.09), although the confidence intervals were wide and the effect was modest. After controlling for the Gleason score, the HR increased to 0.93 (95% CI: 0.69.1.26), indicating that the effect of rs406193 variant on decreasing the risk of death was partly explained by its effect on the Gleason score.

4.3.1 TABLES AND FIGURE

Figure 3. The assumed causal relationships between DNMT3b, tumour tissue DNA methylation, Gleason score and prostate cancer mortality



a Unmeasured variable

DNMT3b genotype was the exposure variable evaluated in association with prostate cancer mortality. DNA methylation, both in genes we previously studied (APC,GSTP1, RUNX3) and in other unmeasured genes, was considered as intermediate variable. Gleason score was included as further intermediate variable.

The graph shows that the effect of DNMT3b T variant on the outcome could be partially captured by the Gleason score.

In our study, when we did not adjust for Gleason score the protective effect of DNMT3b T variant on the outcome resulted in a HR of 0.81 (Table 4). This is the total effect on prostate cancer mortality, which includes both the direct effect (not mediated by Gleason) and the indirect effect (mediated by Gleason).

When we adjusted for Gleason score, the protective effect of DNMT3b T variant on the outcome resulted in lower estimate (HR 0.93, Table 4), that is only the direct effect (not mediated by Gleason) of DNMT3b genotype on prostate cancer mortality.

Table 17. Characteristics of patients

Characteristics of patients	N (%)
Number	438
Mortality	
Overall	368 (84%)
Due to prostate cancer	189 (43%)
Due to other causes	179 (41%)
Mean age at diagnosis	72
Source of tumour tissue	
Biopsy	325 (74%)
TURP	56 (13%)
Radical prostectomy	57 (13%)
Period of diagnosis	
1982-1986	197 (44%)
1993-1996	241 (55%)
N of Methylated genes	
0-1	136 (31%)
2	177 (40%)
3	80 (18%)
Missing	45
DNMT3b genotype	
CC	239 (55%)
CT+TT [a]	199 (45%)
Gleason score	
<8	250 (57%)
8+	188 (43%)

[a] Allelic frequency: CT = 42.3%; TT = 2.7%

Table 18. Association between genotype and the number of methylated genes

DNMT3b rs409361	N Methylated genes				
	0-1	2	3	OR	95% CI
CC	74 (54%)	96 (54%)	46 (58%)	1	-
CT+TT	62 (46%)	81 (46%)	34 (42%)	0.84	0.57-1.23

OR adjusted for age at diagnosis, period of diagnosis and source of tumour tissue;

Table 19. Association between genotype and Gleason score

DNMT3b rs409361	Gleason			
	<8	8+	OR	95% CI
CC	125 (50%)	114 (83%)	1	-
CT+TT	125 (50%)	74 (17%)	0.57	0.39-0.85

OR adjusted for age at diagnosis, period of diagnosis and source of tumour tissue

Table 20**.** Association between genotype and the mortality for prostate cancer

DNMT3b rs406193	N of Deaths	HR1	95% CI	HR2	95% CI
CC	104	1	-	1	-
CT+TT	85	0.81	0.61-1.09	0.93	0.69-1.29

HR1 adjusted for age at diagnosis, period of diagnosis and source of tumour tissue
HR2 adjusted as HR1 and for Gleason score

5. DISCUSSION

5.1 STUDY 1

We found that methylation in APC is associated with prostate cancer mortality, particularly among those with a highly to moderately differentiated tumor. A similar association was found for methylation in RUNX3 in the 1990s cohort, whereas methylation in GSTP1 was not associated with risk. The results also indicated a shift in the methylation patterns from the 1980s to the 1990s.

Gene-specific prevalences of methylation were different between the two cohorts. These differences are unlikely a result of selection of patients or laboratory heterogeneities for the following reasons: the same methodologies for molecular analyses were used in the two cohorts; within each cohort, the lack of association of the year of tissue collection with prevalence of methylation suggests that tissue preservation did not affect the results; both cohorts included an unselected series of patients, and the catchment area of the hospital did not change over time; and in the 1990s cohort, a higher number of patients received radical prostatectomy, but the differences between the two cohorts remained in the comparison restricted to patients who underwent biopsy. The decrease in age at diagnosis and increase in survival that we observed in the 1990s cohort is consistent with an effect of opportunistic PSA screening (Draisma, 2003). It has been estimated that, each year, more than 10% of men older than 50 years received a PSA test at the end of the 1990s in Northern Italy (Russo, 2002; D'Ambrosio, 2004). Therefore, one plausible explanation for the observed difference in methylation prevalences between the 1980s cohort and the 1990s cohort could be that PSA-detected prostate cancers are characterized by a different methylation pattern. This could be explained either by a larger proportion of early tumors or a greater proportion of indolent cancers in the 1990s cohort. Distinguishing between these two mechanisms cannot be done with our data, but it would help in the understanding of the biology of prostate cancer and possibly in the identification of new prognostic markers. The methylation prevalences that we found are in accordance with previous data. Prevalence of GSTP1 methylation has been estimated to be greater than 60%, with large heterogeneities between studies (Perry, 2006; Bastian, 2004; Meiers, 2007). The two largest studies conducted so far found prevalences of 73% in 179 patients 22 and 66% in 291 patients. Most of the studies that investigated methylation in APC in prostate cancer found a prevalence of greater than 50% (Bastian, 2005; Yegnasubramanian, 2004; Jeronimo, 2004; Kang, 2004; Cho, 2007; Rosenbaum, 2005; Henrique, 2007; Bastian, 2004; Bastian, 2007 Tokumaru, 2004; Maruyama, 2002, Enokida,

2005). Little is known about the prevalence of methylation in RUNX3 from previous studies (Cho, 2007). Our data suggest that methylation in APC may be involved in prostate cancer progression. Even if we lacked information on some important variables, such as PSA and TNM stage, residual confounding is unlikely to be a major limitation. First, we adjusted for Gleason score, which is a strong prognostic variable. Second, studies evaluating the correlation between methylation in APC and clinicopathologic variables found heterogeneous results, with most of the association estimates being weak (Bastian, 2005; Jeronimo, 2004; Maruyama, 2002). The confounding potential of these variables after adjustment for Gleason score should thus be limited. To date, little is known about the role of epigenetics and promoter methylation in prostate cancer progression. Our results on APC are predated by similar findings from two recent smaller studies. In a cohort of 74 prostate cancer patients who underwent prostatectomy, approximately 70% of the patients experienced PSA reoccurrence, metastasis, or death, with an HR for promoter methylation in APC of 3.0 (95% CI, 1.4 to 6.3) (Rosenbaum, 2005). A three-fold statistically significantly increased HR has also been reported by Henrique et al, who investigated 83 prostate cancer patients, of whom 15 died from prostate cancer during follow-up. The APC complex is known from studies of colorectal cancer cells to function as a gatekeeper in the cell, preventing the transcription of gene products that promote cell proliferation and survival rather than differentiation and apoptosis (Baylin, 2006). Hypermethylation of APC implies silencing of this gatekeeper, making the cell vulnerable to further epigenetic and genetic changes and, thus, progression toward the development of invasive cancer. This is consistent with our finding of a decreasing survival with number of methylated genes, which further supports a role of gene hypermethylation in cancer progression. We also found that hypermethylation of APC is associated with survival among patients with tumors of high to moderate differentiation but not among those with a poorly differentiated tumor. This finding may suggest that changes in APC might occur early in the tumorigenesis in the prostate, making the cell vulnerable to further changes, but in tumors with poor differentiation, changes in APC add little to the malignant potential. In this large survival analysis of two independent series of unselected prostate cancer patients, we found that hypermethylation in the promoter of the APC gene is involved in prostate cancer progression. The possibility of using this as a prognostic marker will have to be addressed in future independent cohorts with more detailed clinical information available. When comparing patients from the 1980s, before the introduction of PSA testing, with those from the 1990s, after the introduction of PSA, we found a

considerable change in the methylation pattern, possibly indicating different biologic behavior of PSA-detected and other prostate cancers.

5.2 STUDY 2

We found a positive association between hypermethylation of APC and GSTP1 promoter in non neoplastic tumor adjacent to tumor and mortality for prostate cancer. Evidence suggest that hypermethylation is an early event in the process of carcinogenesis, the identification of hypermethylation in non-neoplastic tissue adjacent to the tumor may have a high prognostic value: it is indicative of a methylation pattern already altered, even in the absence traces of histological features of malignancy.

The prevalence of methylation in non neoplastic tissue for the two genes was high. We excluded problems of contamination from adjacent tumour tissue because we analysed non neoplastic fragments near but isolated from the tumour. Patients that showed methylation in non neoplastic tissues died for prostate cancer more frequently.

The prevalence of promoter methylation in APC and GSTP1 in tumour, corresponding to non neoplastic tissue selected for this study was higher than in the original study (study 1). It depends on technique used to analyse methylation pattern: real time PCR with specific probe is a technique more sensitive (1:10.000 CpG methylated) than end-point PCR used in the cohort study (1:1.000 CpG methylated).

Some cases (N = 6 for APC, N=6 for GSTP1) show a discrepancy in the state of methylation, specifically APC or GSTP1 promoters were methylated in non-neoplastic tissue and the corresponding tumor is not methylated.

A plausible explanation for these results could derive from the peculiar characteristic of prostate cancer to be a multifocal tumor. Tumor foci can in turn progress at different rates depending on the nature of the genetic alterations present that in part differing degrees of biologic aggressiveness (Andreoiu, 2010). Therefore it should be consider that, and it might be the case, discrepancies in methylation status are explained by different origin between the two foci.

Another explanation could consider that methylation pattern can be reversed in the tumour. It is described the possibility of a sort of reversal of hypermethylation in advanced stages of cancer. A study in ovarian cancer cells, for example, shows that tumor of advanced stage have lower rate of methylation (Watts et al., 2008). Since the process of hypermethylation is an important epigenetic dysregulation in the early stages of tumor progression, it is possible that,

while not detectable in tumor tissue, in the tissue around it is detectable because of a possible development of new tumor derived by infiltrating cells.

Taken together, our data suggest that the pattern of promoter hypermethylation of certain genes present in the tumor can also be detected in adjacent non-neoplastic tissue. Patients who have the APC promoter methylation in the tumor than those without this alteration, are 3 fold more likely to have this same alteration in the adjacent non-neoplastic tissue, those with promoter GSTP1 methylation have approximately the same probability equal. This probability decrease when we consider the methylation status in a different gene respect those methylated in tumour. These results of the study could further confirm that the dysregulation of this gene in tumor cells is an early event compared with histopathological signs of carcinogenesis, as already detectable in non-neoplastic tissue adjacent to tumor foci themselves. In non neoplastic tissue adjacent to tumour could be present yet pre tumoral cells derived from tumour that show pattern of methylation similar to the neoplastic population of origin. A second hypothesis could involve a possible exposure that alter methylation pattern of specific gene in different cells, but some author suggest that, even though the methylation seems a mechanism well regulated, the hypermethylation of genes is a nonspecific event (Illingworth, 2009).

The importance of assessing the pattern of hypermethylation in non-neoplastic tissue with the tumor becomes more significant when one takes into account mortality. Our results indicate that the combination of APC promoter hypermethylation in tumor tissue and adjacent non-neoplastic tissue leads to a significant increase in risk of death from prostate cancer. The promoter hypermethylation of APC in non-neoplastic tissue could play a role, therefore, of great interest as a prognostic index.

Even the promoter of GSTP1 hypermethylation in non-neoplastic tissue increases the risk of death for prostate cancer. This further suggest that the detection of hypermethylation in non-neoplastic tissue is an indicator of increased aggressiveness of the tumor regardless of the gene considered. The hypermethylation of multiple genes increases the aggressiveness of the tumor further indicating the importance of methylation in the process of cancerogenesis.

When considering hypermethylation in the non neoplastic adjacent tissue as a potential prognostic marker adjustment for Geason score is required. We found that the risks of mortality decrease if we consider Gleason score that represent a strong confounder.

5.3 STUDY 3

We found that a DNMT3b variant affects the diagnostic Gleason score in prostate cancer patients, and weekly, via this effect, the prostate cancer mortality. We assumed that its effect would be mediated by tumour tissue DNA methylation in several genes. The role of DNA methylation status and of DNMTs function in cancer development has been widely studied (Kulis, 2010). The association between DNMT and cancer survival has been studied to a lesser extent, although high levels of expression of DNMT1 and DNMT3b have been recently linked to poor lung cancer prognosis (Kim, 2006; Xing, 2008), suggesting a direct role of these enzymes in cancer progression. DNMT variants have been associated also with cancer incidence, as reported for lung (Shen, 2002; Lee, 2005), breast (Montgomery, 2004) and head and neck cancers (Liu, 2008; Wang, 2004), although the results were not always consistent (Fan, 2008; Wu, 2007; Aung, 2005).

Little is presently known about the relationship between DNMT3b variants and prostate cancer survival.

We studied two cohorts of consecutive prostate cancer patients with a long and complete follow-up, enabling the use of mortality as the outcome for the study, although we lacked information on clinical data, including PSA levels and information on treatment. We found a low frequency of the TT homozygous, that is in line with the frequencies previously reported for the same SNP in colon cancer (de Vogel, 2009).Therefore all the analyses were computed by comparison of CC carriers with carriers of at least one T.

We focused the present investigation on the single DNMT3b rs406193 variant on the basis of previous evidence of its association with a reduced risk of cancer in wide size studies (Cebrian, 2006; de Vogel, 2009), of the availability of a validated reliable commercial kit for molecular analyses, and of the probably functional effect of this SNP. As suggested by a silico tool (TESS program), the sequence motif in the SNP site is recognized by a transcription factor. When the T-allele is present, the motif is missing at the 3' flanking region of the DNMT3b gene, and the recognition by the transcription factor is prevented (Cebrian, 2006), affecting the DNMT3b levels of expression.

Our study did not aim to investigate systematically the prognostic role of DNMT3b variants or, even more broadly, of DNMT variants. Rather we were interested in discussing a model for causal relationships between the DNMT genotype, DNA methylation status, Gleason score and mortality. Under our specified assumptions (Figure 3), the model not adjusted for Gleason score (which was considered as a mediator of the effect of methylation on mortality) estimates the total effect of rs406193 variant on prostate cancer mortality. The

total effect includes both the direct effect, not mediated by Gleason, and a indirect effect mediated by Gleason. Thus, the model adjusted for the Gleason score provides an estimate of the direct effect (not mediated by Gleason) of rs406193 variant, assuming that the potential confounding introduced by adjustment for the Gleason score is small. On the hazard-ratio scale it is not possible to decompose the total effect of the exposure on the outcome into direct and indirect effects (Kaufman, 2004). Hence, in our study the causal estimate of the indirect effects of rs406193 cannot be estimated by comparing the Gleason-adjusted and the Gleason-unadjusted hazard ratios .

Our assumed causal diagram shown in Figure 3, and our findings, indicate that studies aiming to evaluate the association of DNMT variants with prostate cancer mortality should not include the Gleason score and/or DNA methylation status in the main analyses. Analogously, studies on the role of tumour tissue DNA methylation on prostate cancer mortality should not include Gleason score in the main analyses, although this is often done. Incorrect adjustment for the Gleason score probably leads to an underestimate of the effect of DNA methylation or DNMT variants. Furthermore, even if we attempted to estimate the controlled direct effect of rs406193 on prostate cancer mortality, it should be noted that, according to our proposed causal model (Figure 3), complete information on the tumour tissue methylation status would be needed to obtain a more valid estimate.

The very weak association between being a rs406193 T carrier and tumour hypermethylation in the three previously investigated genes, compared to the much stronger association found with Gleason score, suggests that the aberrant methylation probably involves a much larger number of cancer-related genes. This is consistent with findings of recent studies that observed several genes to be hypermethylated in patients with more aggressive prostate cancers (Phè, 2010). Likely, tumour tissue DNA methylation in a number of genes is associated with more aggressive tumours, and this tumour aggressiveness is partly captured by a higher Gleason score.

In conclusion, this study provides clues on the involvement of a DNMT3b variant in prostate cancer progression. It also suggests that the causal relationships between the involved variables should be taken into account in order to validly estimate the association between tumour tissue methylation and cancer mortality.

6. CONCLUSIONS

We found that methylation in APC is associated with prostate cancer mortality, particularly when a highly to moderately differentiated tumor is present. Analogous association was found for methylation in RUNX3 in the 1990s cohort. Methylation in GSTP1 was not associated with risk of prostate cancer mortality. These results suggest that hypermethylation of APC could be considered as a biomarker of prognosis in prostate cancer.

We found the hypermethylation state of the promoter of APC and GSTP1 genes is also associated in non-neoplastic tissue adjacent to tumor tissue (NNTAT) with mortality. Therefore in this tissue was detectable an early dysregulation of methylation status when no morphological signs of malignacy were present.

For the first time it has been demonstrated a significant correlation between the state of hypermethylation in non-neoplastic tissue adjacent to cancer and mortality from prostate cancer. This result suggests that the hypermethylation in non-neoplastic tissue, particularly of the APC gene, may be used as a biomarker for tumor aggressiveness of prostate cancer.

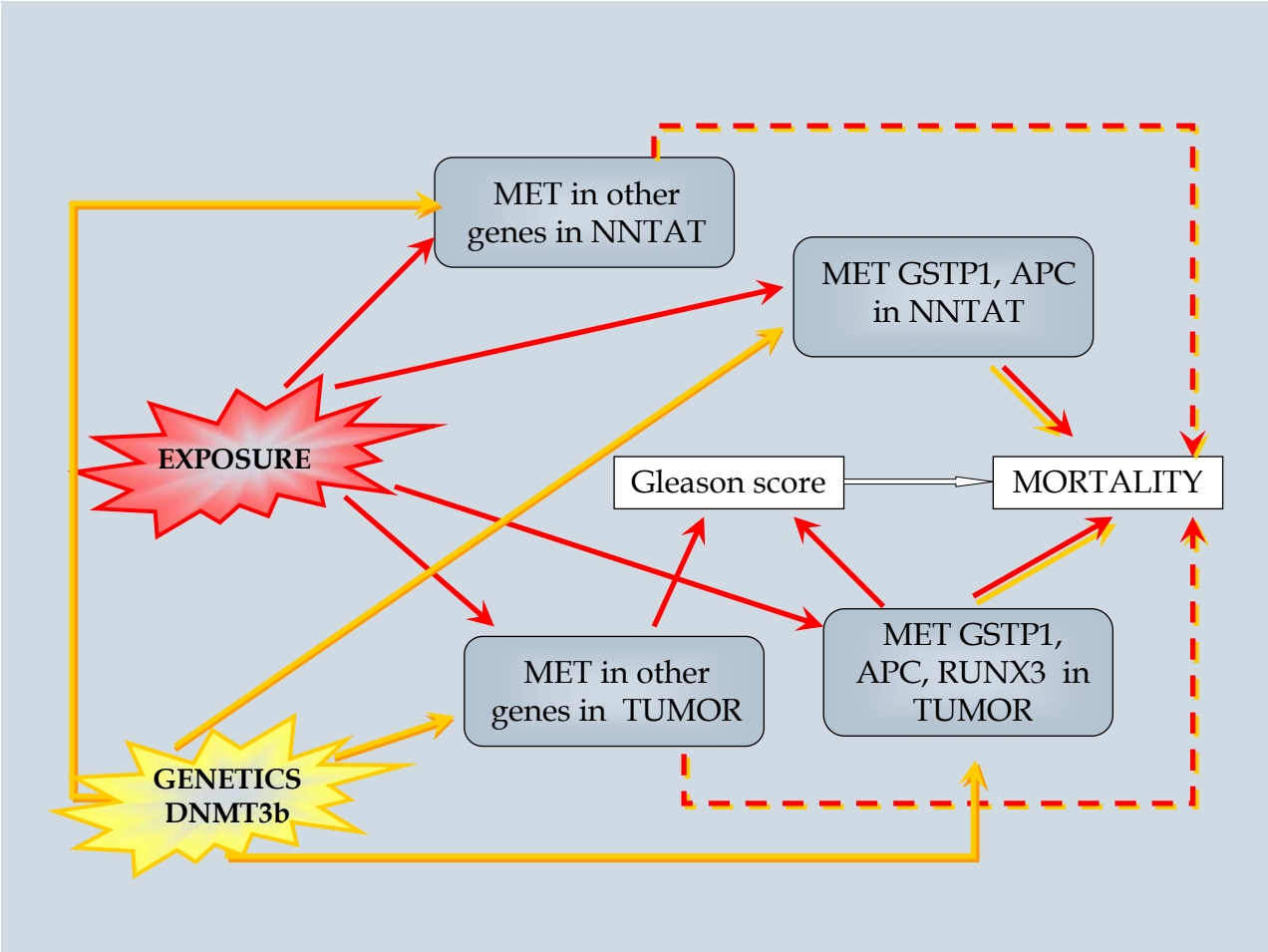
We found a weak association between variant of DNMT3b and number of genes methylated and mortality.

Pattern of methylation in prostate cancer can contribute to understand the biology of tumor and mechanisms that lead to malignancy (Figure 4). It could further represent a possible marker of prognosis. There is currently no agreement on how to treat patients with prostate cancer of low or intermediate grade, neither on how to discriminate indolent from aggressive tumors. There is therefore need for prostate cancer patients of new markers that can be measured at the time of the diagnosy to guide the patient management.

Only by understanding the challenges introduced by disease heterogeneity, a prolonged natural history, and an evolving biology and by adopting practices that take these characteristics of prostate cancer into account will we be able to develop robust molecular biomarkers and significantly impact the care of men diagnosed with prostate cancer.

(Febbo, 2009)

Figure 4. Hypothesized causal relationship among variables analized in this research



Red filled arrows indicate hypotetical causal relationships among currently unknown environmental exposures and the gene methylation status. In the studies 1 and 2 we were able to evaluate the relationship among the hypermethylation of selected genes in tumor and non neoplastic tumor adjacent to tumor (NNTAT), tumor aggressiveness (Gleason score) and prostate cancer mortality. Dashed red arrows indicate that other possible unmeasured variables (e.g. methylation in other genes not evaluated in this research) can contribute to explain the association we found with aggressiveness and mortality.

Yellow arrows (filled and dashed) indicate possible implication of the variant of the DNA-methyltransferase-3b gene in the methylation activity of the enzyme, and its possible relationship with aggressiveness and prostate cancer mortality.

In this scenario, the Gleason score represents a mediator of the effect which captures the effects of the other variables. If we would exclude the Gleason score from the scheme, all the effects of the analysed variable would be enphatized.

7. FUTURE PROSPECTS

We will set up a global analysis of methylation of tumor tissue and adjacent non-neoplastic evaluating the status of LINE-1 hypomethylation that represent a surrogate of the hypomethylation of genome in cancer. In addition, to investigate the relevance of hypermethylation of promoter of APC as biomarker of malignancy will set up an early case-control study with patients in whom prostate cancer was verified histologically, who received repeated biopsies of the prostate. We will include patients for whom a normal prostate tissue sample was taken at least 3 months before the diagnosis of cancer. For each of the patients a matched control that received two samples of prostate tissue, both negative for tumor will be randomly sampled.

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