

METHYLATION AND PROSTATE CANCER MARKERS

We use a recent result on methylated genes in examining initial prostate biopsies for carcinogenic potential. We examine each marker and why it has potential and then address a set of key questions that should be considered.

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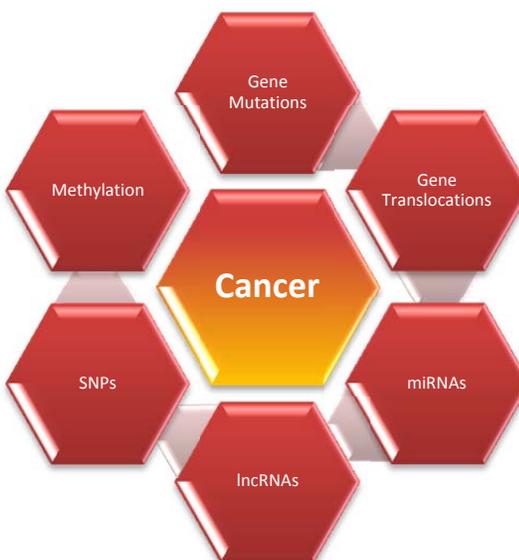
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1 INTRODUCTION

There seems to be a continuous flow of genes, miRNAs, epigenetic factors including methylation, SNPs and the like all both diagnostic and prognostic for various cancers. A decade ago one looked for a gene, some gene that somehow got broken, changed, deleted, or the like. The paradigm was the Philadelphia chromosome of a cut and paste example. With the understanding we now have of methylation we see the same occur here, and methylation can be acquired and/or genetically inherited (see imprinting examples). However methylation is still somewhat poorly understood; what causes it, why does it work positively in some cases and negatively in others?

Methylation is but one of the many facets of what we now see as causes of Cancer. We depict a short summary below.



We examine the work of Wojno et al which has received recent interest. They examine the impacts of methylation upon 3 genes and see their presence as prognostic of potential aggressive prostate cancer. Specifically they conclude:

The diagnosis of prostate cancer is dependent on histologic confirmation in biopsy core tissues. The biopsy procedure is invasive, puts the patient at risk for complications, and is subject to significant sampling errors.

An epigenetic test that uses methylation-specific polymerase chain reaction to determine the epigenetic status of the prostate cancer-associated genes GSTP1, APC, and RASSF1 has been clinically validated and is used in clinical practice to increase the negative predictive value in men with no history of prostate cancer compared with standard histopathology. Such information can help to avoid unnecessary repeat biopsies.

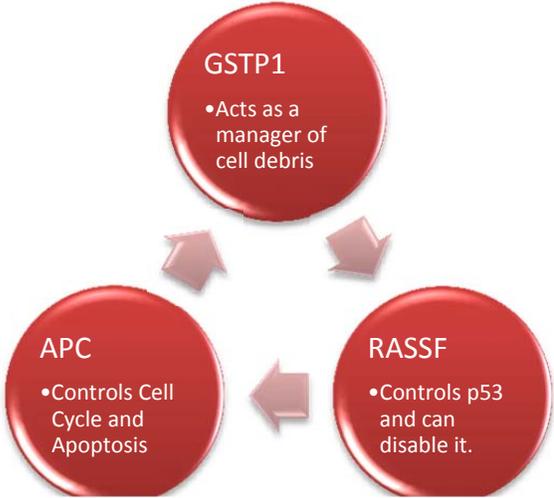
The repeat biopsy rate may provide preliminary clinical utility evidence in relation to this assay's potential impact on the number of unnecessary repeat prostate biopsies performed in US urology practices.

DNA methylation normally can result in the silencing of genes by interrupting the normal process of promoters. CpG islands are often hypermethylated and thus the gene which may regulate cell proliferation is silenced. This may result in uncontrolled cell growth. For example genes controlling MYC are not produced and MYC may then result in excess cell cycle proliferation. Methylation is hypermethylated in the regions of intergenic regions and in repetitive elements and this hypermethylation silences these regions and facilitates normal cell DNA transcription of the gene. Disruption of DNA, namely hypomethylation, in the intergenic and repetitive regions may result in possible loss of imprinting. This hypomethylation is also related to the production of lncRNAs which may in turn interfere with normal gene transcription.

Decitabine is a DNMT inhibitor. Namely, it inhibits the DNA methyltransferases that facilitate methylation (such as DNMT3 which are de novo and DNMT1 which is maintenance). Decitabine thus has then tendency on the specific hematologic cell lines in MDS to remove methylations which have caused the aberrant cell line proliferations and allow for the return of homeostasis. MDS is a quasi-malignant condition originating in the bone marrow which may in many cases result in Acute Myelogenous Leukemia. With the use of decitabine or a similar DNMTI azacitidine, demethylation of these rapidly reproducing cells may be achieved and possible a normal state of homeostasis achieved.

The use of pharmaceuticals that alter the methylation patterns of DNA can have lasting effects because those patterns may last through subsequent mitotic changes. On the one hand that may be beneficial as is the case with MDS but such broad demethylation may also alter other segments of the DNA altering essential control elements and pathways. In cell development there are two sensitive periods; germ cell development and early embryonic development. It is during these periods that methylation is cleared and reset and that a drug-like a DNMTI would pose a serious risk to the proper resetting of the marks and could result in substantial DNA expression damage.

In summary we will examine the three gene methylation proposition with this test. We summarize this below:



2 THE PROPOSITION

Let us examine what the study presents in a bit more detail. Basically it does the following:

1. It examines three gene products; *GSTP1*, *APC*, and *RASSF1*
2. It determines if the genes are methylated so that the gene products are suppressed.
3. If that is the case after a first biopsy which is deemed normal, then a second biopsy is mandated due to the high incidence of a positive result on the second biopsy for PCa.

Specifically from the paper by Partin et al on the same topic the authors' state:

The DOCUMENT multicenter trial in the United States validated the performance of an epigenetic test as an independent predictor of prostate cancer risk to guide decision making for repeat biopsy. Confirming an increased negative predictive value could help avoid unnecessary repeat biopsies. We evaluated the archived, cancer negative prostate biopsy core tissue samples of 350 subjects from a total of 5 urological centers in the United States. All subjects underwent repeat biopsy within 24 months with a negative (controls) or positive (cases) histopathological result. Centralized blinded pathology evaluation of the 2 biopsy series was performed in all available subjects from each site.

*Biopsies were epigenetically profiled for *GSTP1*, *APC* and *RASSF1* relative to the *ACTB* reference gene using quantitative methylation specific polymerase chain reaction. Predetermined analytical marker cutoffs were used to determine assay performance. Multivariate logistic regression was used to evaluate all risk factors.*

The epigenetic assay resulted in a negative predictive value of 88% (95% CI 85-91). In multivariate models correcting for age, prostate specific antigen, digital rectal examination, first biopsy histopathological characteristics and race the test proved to be the most significant independent predictor of patient outcome.

The DOCUMENT study validated that the epigenetic assay was a significant, independent predictor of prostate cancer detection in a repeat biopsy collected an average of 13 months after an initial negative result. Due to its 88% negative predictive value adding this epigenetic assay to other known risk factors may help decrease unnecessary repeat prostate biopsies.

Recall that the *negative predictive value* or NPV is defined as:

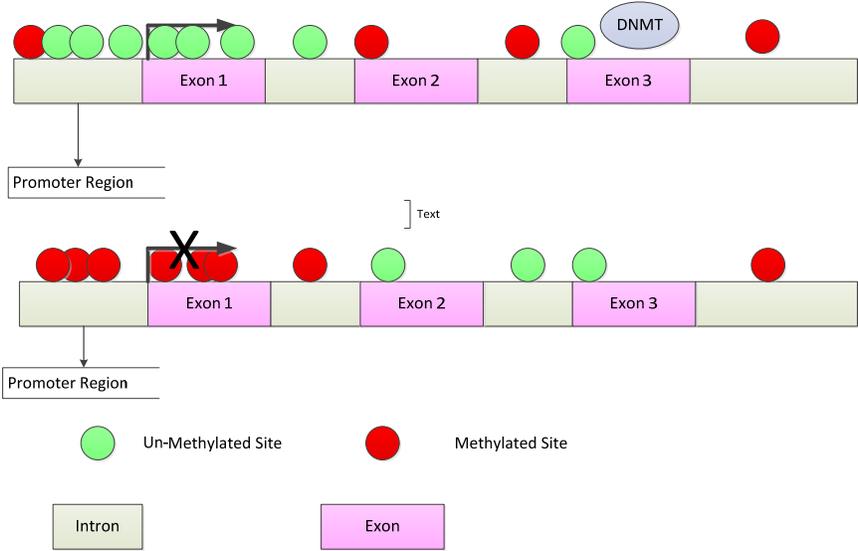
$$NPV = \frac{\text{Number True Negatives}}{\text{Number True Negatives} + \text{Number False Negatives}}$$

Thus an NPV of 88% for the sample size used implies that it is fairly high in predicting True Negatives a priori but may still miss a percentage. There of course is the issue of the pathologist

missing the PCa as well. This could be done by a sampling deficiency or confusion on a reading. It is not clear if for example a HGPIN is read.

Thus focusing on methylated genes, specifically just 3 of them, GSTP1, APC and RASSF1, they were able in a small sample to ascertain that there would be no need of a second biopsy if they were found to be unmethylated in the first.

Recall the effects of methylation as we show below:



From an article in Medical Express¹ as well as from an article in Eureka² as well as from an article in Science Codex³ we have the following summary:

More than one million prostate biopsies are performed each year in the U.S. alone, including many repeat biopsies for fear of cancer missed. Therefore there is a need to develop diagnostic tests that will help avoid unnecessary repeat biopsies. Two independent trials have now validated the performance of an epigenetic test that could provide physicians with a better tool to help eliminate unnecessary repeat prostate biopsies, report investigators in The Journal of Urology.

In the previously reported independent MATLOC (Methylation Analysis To Locate Occult Cancer) trial, a multiplex epigenetic assay (ConfirmMDx for Prostate Cancer) profiling the APC, GSTP1 and RASSF1 genes demonstrated a negative predictive value of 90%. GSTP1

¹ <http://medicalxpress.com/news/2014-07-accurate-epigenetic-unnecessary-biopsies-prostate.html>

² http://www.eurekalert.org/pub_releases/2014-07/ehs-nae072114.php

³ http://www.sciencecodex.com/new_accurate_epigenetic_test_could_eliminate_unnecessary_repeat_biopsies_for_prostate_cancer-137932

methylation is a specific biomarker for (prostate) cancer and this gene is methylated in up to 90% of prostate cancer cases. Additionally, APC and RASSF1 are important field effect markers and increase the diagnostic sensitivity of the assay.

A second multicenter study, DOCUMENT (Detection Of Cancer Using Methylated Events in Negative Tissue), has validated the performance of the epigenetic assay used in the MATLOC trial as an independent predictor of prostate cancer risk to guide decision making for repeat biopsy. In the DOCUMENT study patients with a negative biopsy were evaluated to identify those at low risk for harboring cancer missed, through biopsy sampling error, who could forego an unnecessary repeat biopsy. The validation study resulted in a negative predictive value of 88%.

"This epigenetic assay is a significant, independent predictor and has been shown to be the most valuable diagnostic aid of all evaluated risk factors in two independent trials," comments Alan W. Partin, MD, PhD, of the James Buchanan Brady Urological Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland. "Negative findings of this assay could be used to reduce concern over unsampled cancer and effectively avoid unnecessary repeat biopsies."

A total of 350 patients were enrolled in the DOCUMENT trial from five geographically dispersed medical centers: Cleveland Clinic, Eastern Virginia Medical School, Lahey Hospital & Medical Center, Johns Hopkins University, and University of California Los Angeles. Patients were grouped into those with two consecutive negative biopsies (controls) and those with a negative biopsy followed by a positive biopsy within 24 months. The initial archived, negative for cancer, prostate biopsy core tissue samples were evaluated. All of the men underwent a repeat biopsy on average one year after the initial biopsy.

Only biopsies with a minimum of eight cores per biopsy, collected no earlier than 2007, were included in the study, while initial biopsies with atypical cells suspicious for cancer, i.e. atypical small acinar proliferation by the sites' pathologists, were excluded, since this would have triggered a repeat biopsy based upon histopathology alone.

After correcting for age, prostate specific antigen (PSA), digital rectal exam, histopathological characteristics of the first biopsy, and race, this epigenetic test proved to be the most significant, independent, and strongest predictor of patient outcome with an odds ratio of 2.69 as well as the most valuable diagnostic aid of all evaluated risk factors. The slightly decreased sensitivity of the DOCUMENT trial compared to the MATLOC trial is most likely associated with a higher PSA screening prevalence in the DOCUMENT cohort.

It is important to note the following:

1. The genes selected have been studied for over two decades and especially as regards to their hypermethylation status.
2. The test is an early prognostic test which when combined with prostate biopsy data, especially a benign reading on the prostate biopsy.

3. The test has reasonable statistics given its small sample size but as we shall see there is substantial variability in these tests.

3 GSTP1

We start with a general cellular household maintenance gene, GSTP1. GSTP1 is a gene whose protein is involved in general housekeeping efforts in a cell. As Laborde states:

Glutathione transferases (GSTs) are enzymes that catalyze the conjugation of glutathione (GSH) to a variety of electrophilic substances. Their best known role is as cell housekeepers engaged in the detoxification of xenobiotics. Recently, GSTs have also been shown to act as modulators of signal transduction pathways that control cell proliferation and cell death. Their involvement in cancer cell growth and differentiation, and in the development of resistance to anticancer agents, has made them attractive drug targets. This review is focused on the inhibition of GSTs, in particular GSTP1-1, as a potential therapeutic approach for the treatment of cancer and other diseases associated with aberrant cell proliferation.

GSTP1 seems to have a beneficial capability in cells and thus if it is no longer functioning there is an accumulation of toxic elements in the cells which can thus result in cell degradation.

From NCBI we have the following descriptive⁴:

Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Based on their biochemical, immunologic, and structural properties, the soluble GSTs are categorized into 4 main classes: alpha, mu, pi, and theta. This GST family member is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases.

The above is a reasonable duplication of what we generally find in the literature. Specifics as to what and how it functions are contained in the referenced literature, Now from Townsend et al we find the following⁵:

Glutathione S-transferase Pi 1 (GSTP1) belongs to a family of phase II detoxification enzymes that catalyze the conjugation of glutathione (GSH) and electrophilic compounds, resulting in the detoxification of electrophiles. A multitude of studies in vitro and in vivo have shown that GSTP1 is important in xenobiotic detoxification, and its overexpression contributes to the drug-resistant phenotype. GSTP1/GSTP2-null mice have an increased susceptibility to skin tumorigenesis induced by chemical carcinogen. GSTP1 has been shown to form protein-protein interactions with several proteins and to act as an endogenous negative regulator. Among the ligand-binding partners identified so far are c-Jun N-terminal kinase (JNK), tumor necrosis factor (TNF)-receptor-associated factor 2 (TRAF2) and peroxiredoxin-1.

⁴ <http://www.ncbi.nlm.nih.gov/gene/2950>

⁵ <http://www.signaling-gateway.org/molecule/query?afcsid=A002956>

JNK is a mitogen-activated protein (MAP) kinase that has a pivotal role in cell survival and death pathways. Dissociation of GSTP from JNK1 in vitro and in GSTP-deficient mice shows activation of JNK activity. GSTP also has a pivotal regulatory role in the TNF- α -induced signaling cascade through protein–protein interactions with TRAF2. Dissociation of GSTP from TRAF2 leads to activation of the apoptosis signal-regulating kinase (ASK1) pathway. The protein–protein interactions with peroxiredoxin-1 mediate the S-glutathionylation of its active site cysteine, leading to enzyme activation.

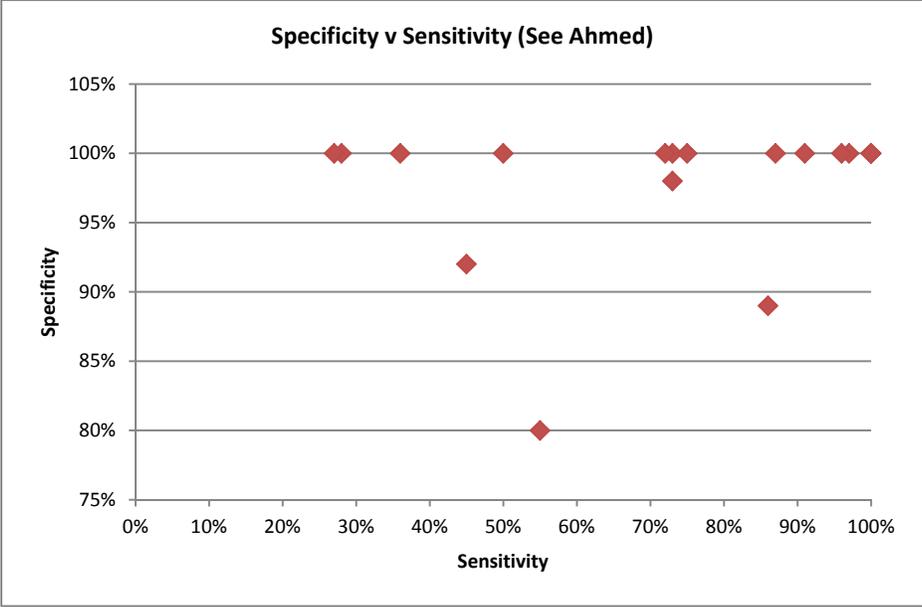
Aberrant expression of GSTP has been linked with tumor development and resistance to cancer drugs. The recent understanding of the dual functionality of GSTs has shed light on the initial confusion arising from the fact that not all drugs used to select for resistance were substrates for thioether bond formation.

This yields some detail on its functioning. It does not appear to have significant pathway functioning.

In a review by Ahmed in 2010 the author details a considerable amount of research regarding methylation and several of the genes discussed in the initial work. Specifically he presents an interesting set of tables on GTSP1. This result follows as has been modified:

<i>Gene/Gene cohort</i>	<i>Specimen</i>	<i>Sensitivity %</i>	<i>Specificity %</i>
<i>GSTP1</i>	Biopsy	91	100
<i>GSTP1</i>	Biopsy	73	100
<i>GSTP1</i>	Biopsy	75	100
<i>GSTP1, RARβ2, APC, TIG1</i>	Biopsy	97	100
<i>GSTP1</i>	Biopsy washing	100	100
<i>GSTP1</i>	ejaculate	44	NA
<i>GSTP1</i>	ejaculate	50	100
<i>GSTP1</i>	Serum	72	100
<i>GSTP1, PTGS2, TIG1</i>	Serum	45	92
<i>GSTP1, RASSF1, RARβ2</i>	Serum	28	100
<i>GSTP1</i>	Urine	27	100
<i>GSTP1</i>	Urine post massage	36	100
<i>GSTP1</i>	Urine post massage	73	98
<i>GSTP1</i>	Urine post biopsy	39	NA
<i>GSTP1, APC, EDNRB</i>	Urine post biopsy	71	NA
<i>GSTP1, INK4α, ARF, MGMT</i>	Urine	87	100
<i>GSTP1, INK4α, ARF, MGMT, RARβ2, TIMP3, CDH1, RASSF1A, APC</i>	Urine	100	100
<i>GSTP1, RARβ2, APC, RASSF1A</i>	Urine post massage	86	89
<i>GSTP1, RASSF1A, ECDH1, APC, DAPK, MGMT, p14, p16, RARβ2, TIMP3</i>	Urine post massage	93	NA
<i>GSTP1, RARβ2, APC</i>	Urine	55	80
<i>GSTP1, gal3</i>	Biopsy	96	100
<i>GSTP1, gal3</i>	Serum	100	100
<i>GSTP1, gal3</i>	Urine	100	ND

We summarize this in the Figure below:



Thus there is a rich body of literature on these genes and PCa. In fact the papers by Brooks et al in 1998 and by Wang et al in 2001 are already focusing on GSTP1 and its methylation as an association with PCa. Therefore this provides a strong basis for using this gene and its logic as a cell maintenance product also has substantial merit.

4 RASSF1

RASSF1 is an effector gene and it drives other genes that control cell growth, proliferation and apoptosis. Richter et al have recently presented an excellent summary of RASSF and their involvement in many cancers. As noted above, the Ahmed summary on GTPS1 did include RASSF1 as well. Thus RASSF1 has been recognized as a major player in many cancers. Now methylation of that gene can result in lack of its expression and it is this suppression and its sequelae which are of import.

As Richter et al state:

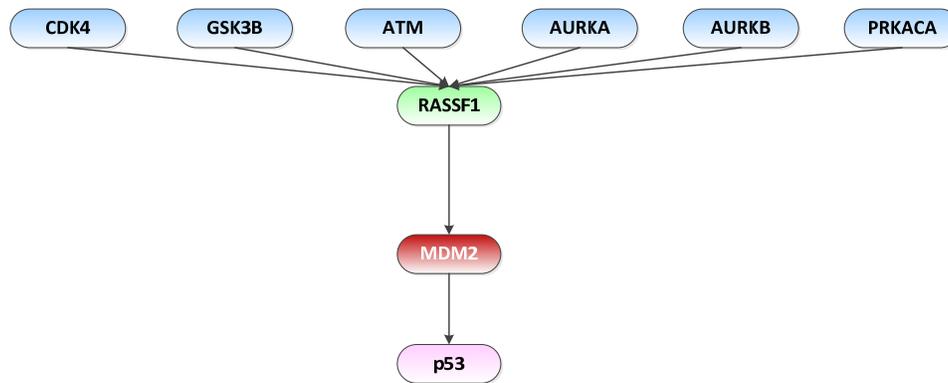
Since methylation of the RASSF1A promoter is described as an early and frequent event in tumorigenesis, RASSF1A could serve as a useful diagnostic marker in cancer screens. RASSFs are implicated in various cellular mechanisms including apoptosis, cell cycle control and microtubule stabilization, though little is known about the underlying mechanisms. Tumor suppressing functions were reported for several members. Here we review the current literature on RASSF members focusing on structural, functional and epigenetic aspects. Characterizing the cellular mechanisms that regulate the signaling pathways RASSFs are involved in, could lead to a deeper understanding of tumor development and, furthermore, to new strategies in cancer treatment.

From NCBI⁶:

This gene encodes a protein similar to the RAS effector proteins. Loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, which suggests the tumor suppressor function of this gene. The inactivation of this gene was found to be correlated with the hypermethylation of its CpG-island promoter region. The encoded protein was found to interact with DNA repair protein XPA. The protein was also shown to inhibit the accumulation of cyclin D1, and thus induce cell cycle arrest. Several alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.

Now RASSF1 is activated by a wide collection of genes but what is important is that it activates MDM2. MDM2 is an analog of MDM4 but it also activates p53.

⁶ <http://www.ncbi.nlm.nih.gov/gene/11186>



Richter et al also state:

The RASSF1 gene, which is located on the small arm of chromosome 3 (locus 3p21.3) codes for eight exons (1 α , 1 β , 2 $\alpha\beta$, 2 γ , 3, 4, 5 and 6). There are seven different RASSF1 isoforms (RASSF1A to RASSF1G) that are generated by differential usage of two promoters (distance 3.5 kb) and through alternative splicing. So far however, the biological relevance of only two isoforms, RASSF1A and RASSF1C, was demonstrated. Regarding the transcripts RASSF1B and RASSF1E there is currently not enough evidence to support a biological role, as well as for the candidates RASSF1F and RASSF1G that possibly enter the nonsense-mediated mRNA decay. The two main variants are RASSF1A and RASSF1C containing a RA domain, SARAH domain and ATM domain, whereas the C1 domain can only be found in RASSF1A.

The isoform A is being transcribed from the upstream promoter and isoform C from the downstream promoter and both promoters are located within CpG-islands. However only the upstream promoter is often hypermethylated in various tumor entities.

We briefly examine the sequellae to RASSF1 in a cell; MDM2 and p53.

4.1 MDM2

Now as Mancini et al state:

MDM4, formerly named MDMX, is an inhibitor of p53 with in vitro and in vivo oncogenic potential. The relevance of MDM4 regulation of p53 has been established by the Mdm4 knockout (KO) mice.

These animals show embryonic lethality, but have a normal development when simultaneously deleted for Trp53 gene.

Different models have been proposed to explain the activity of MDM4 towards p53, particularly to distinguish MDM4 from its analogue MDM2, the best characterized negative regulator of p53.

As the most evident phenotype of Mdm4-KO mice is a generalized cell cycle arrest, MDM4 has been considered as a negative regulator of p53 growth arresting function. Conversely, the

control of p53-apoptotic function has been attributed to MDM2 because of the presence of early embryonic cell death in Mdm2-KO mice. This model, therefore, attributes the control of distinct activities of p53 to different proteins.

In contrast to this, a second model is based on the evidence that MDM4 and MDM2 efficiently associate and regulate each other's function. It has been proposed that the interdependence of the two MDM proteins is the basis for the negative non-overlapping regulation of p53. The presence of apoptosis in Mdm4-KO mice in neuronal progenitors, an increased transcription of some p53 targets genes, have raised a third hypothesis: MDM4 controls the transcriptional function of p53, whereas MDM2 controls its protein levels. All these models apply mainly to the regulation of p53 in unstressed conditions and/or during the mouse development, although some data also extend them to stressing situations.

MDM4, also called Mdm4 p53 binding protein homolog, is located at 1q32. From NCI we have⁷:

This gene encodes a nuclear protein that contains a p53 binding domain at the N-terminus and a RING finger domain at the C-terminus, and shows structural similarity to p53-binding protein MDM2. Both proteins bind the p53 tumor suppressor protein and inhibit its activity, and have been shown to be overexpressed in a variety of human cancers. However, unlike MDM2 which degrades p53, this protein inhibits p53 by binding its transcriptional activation domain. This protein also interacts with MDM2 protein via the RING finger domain, and inhibits the latter's degradation. So this protein can reverse MDM2-targeted degradation of p53, while maintaining suppression of p53 transactivation and apoptotic functions.

p53 data is extensive⁸. The p53 pathway can be developed based upon the detailed NCI data⁹ or from the KEGG genome database we have shown below in a modified form¹⁰:

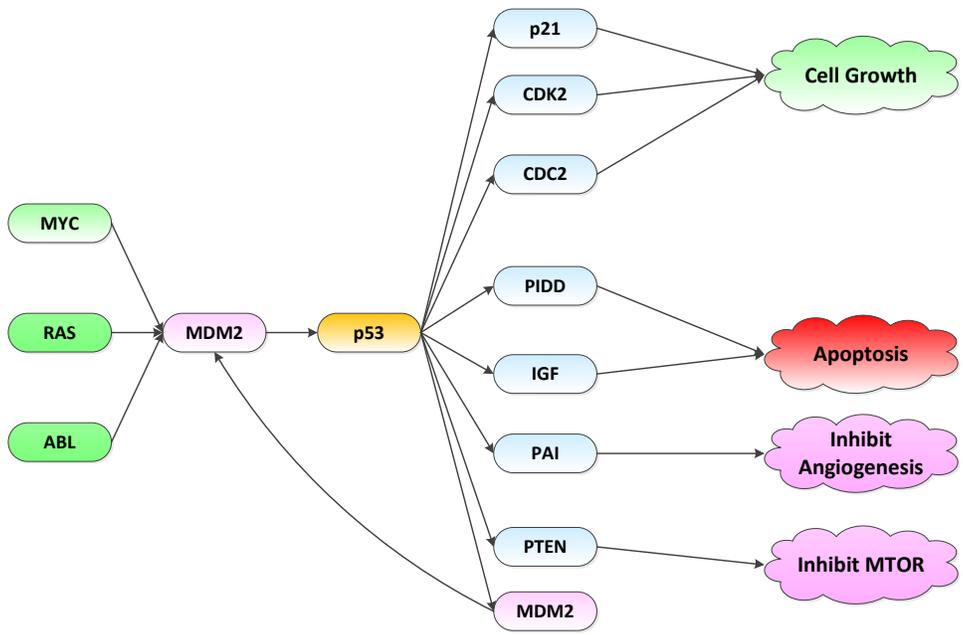
⁷ <http://www.ncbi.nlm.nih.gov/gene/4194>

⁸ <http://www.mmmp.org/MMMP/public/biomap/searchBiomap.mmmp>

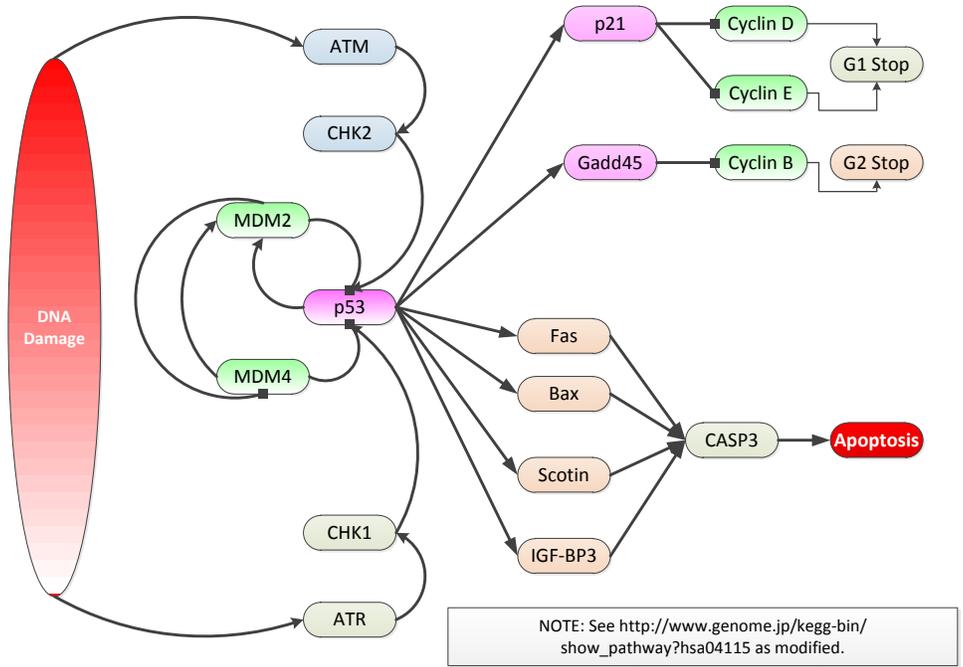
⁹

http://pid.nci.nih.gov/search/pathway_landing.shtml?pathway_id=200207&source=NATURE&genes_a=4194&genes_b=&what=graphic&jpg=on&ppage=1

¹⁰ http://www.genome.jp/kegg-bin/show_pathway?hsa04115



We demonstrate the interactions below in a slightly different manner focusing on MDM2. Note MDM2 and MDM4 interact and it is through this that we have control over p53. Thus any blockage of p53 is a loss of apoptotic capability as well as loss of G1 and G2 stoppage.



NOTE: See http://www.genome.jp/kegg-bin/show_pathway?hsa04115 as modified.

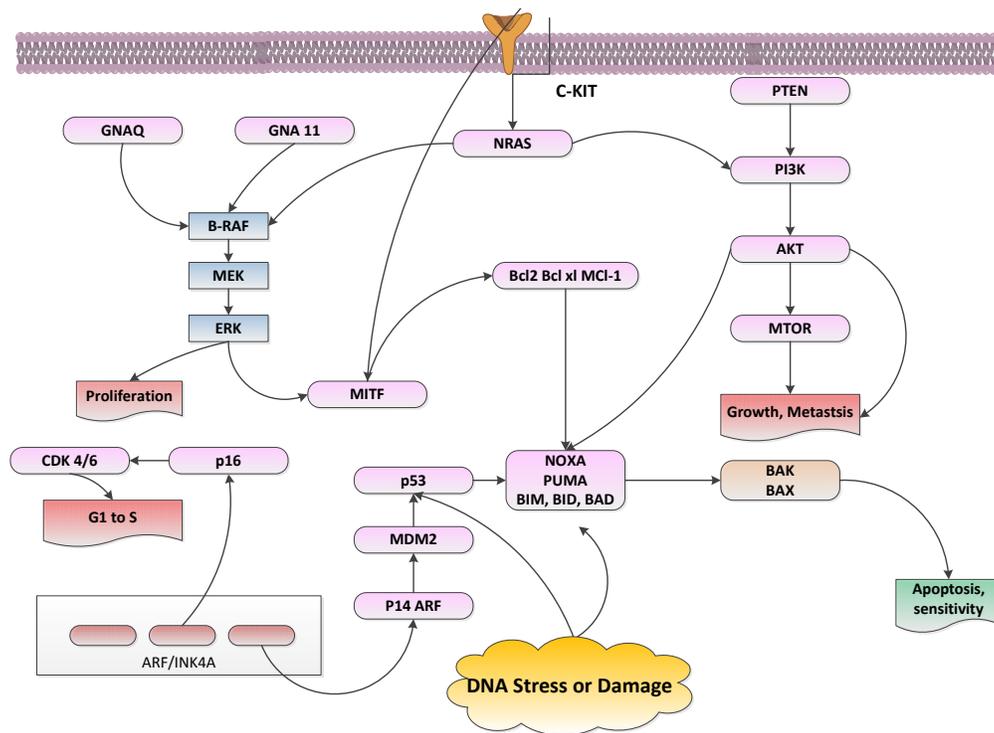
4.2 p53

p53 is a classic cancer related gene. Originally thought of an oncogene it was later understood to be just the opposite. It is often seen turned off in many cancers.

As Muller et al state:

In about half of all human cancers, the tumor suppressor p53 protein is either lost or mutated, frequently resulting in the expression of a transcriptionally inactive mutant p53 protein. Loss of p53 function is well known to influence cell cycle checkpoint controls and apoptosis. But it is now clear that p53 regulates other key stages of metastatic progression, such as cell migration and invasion. Moreover, recent data suggests that expression of mutant p53 is not the equivalent of p53 loss, and that mutant p53s can acquire new functions to drive cell migration, invasion, and metastasis, in part by interfering with p63 function.

The relationships between MDM2 and p53 are shown below:



Thus, turning p53 off, results in conditions favorable for metastatic growth.

5 APC

APC is the adenomatous polyposis coli gene associated with a form of colon cancer. It was one of the key genes discussed by Vogelstein in his model for multiple hits in colon cancer. Like many genes related to cancers it is key in controlling cell cycle progression and apoptosis. It is reasonable to see such a gene in PCa being methylated and suppressed.

As Wantanabe et al noted two decades ago APC was involved in American PCa but surprisingly not in Japanese men. They stated:

APC is a tumor-suppressor gene and its mutation is widely involved in the early stages of tumor development in the digestive tract, including the stomach, colorectum and pancreas, where K-ras and p53 gene mutations are frequently found. The APC gene is known to be expressed in the human prostate. Frequent allele loss at the APC locus, 5q21, has been found in the advanced stages of primary prostate cancer in Americans ... we examined 31 human primary prostate cancers (three cases at stage A, 10 at stage B, five at stage C and 13 at stage D) and four cases of lymph node metastasis from the stage D cases, for mutations in the APC gene.

A mutation was detected in only one of the 35 samples (3%). This mutation, present in a primary stage B cancer, had a T to C transition in exon 15 at the first position of codon 956, resulting in substitution of histidine for tyrosine. This study clarified that APC gene mutations are not largely involved in the development of clinical prostate cancer.

As Jaiswal and Naryan state:

The APC (adenomatous polyposis coli) gene product is involved in cell cycle arrest and in apoptosis. The loss of APC function is associated with the development of colorectal carcinogenesis. In previous studies, we have shown that the APC gene is inducible and that the DNA damage-induced level of APC mRNA requires p53. In the present study, we examined the role of p53 in the transcriptional regulation of APC promoter and characterized two p53-binding sites on the cloned APC promoter (pAPCP).

They continue:

In a simple model, Wntless/Wnt signaling regulates the assembly of a complex consisting of Axin (and its homolog Axil and conductin), APC, β -catenin, and glycogen synthase-3b kinase (GSK3b). Axin (Axil/conductin) binds to APC, β -catenin, and GSK3b and thereby promotes β -catenin phosphorylation and subsequent ubiquitination and degradation in the proteasome. GSK3b regulates this process by phosphorylating components of the complex. Activation of the Wntless/Wnt signaling pathway inhibits GSK3b and stabilizes β -catenin. Stabilizing mutations in β -catenin or truncation in APC also occur both in colon cancer and melanoma cells and increase the stability of β -catenin.

The stabilized pool of β -catenin associates with members of the Tcf-Lef family of transcription factors and regulates transcriptional expression of proto-oncogene and cell cycle regulator c-myc, the G1/S-regulating cyclin D1, the gene encoding the matrix-degrading metalloproteinase,

matrysin, the AP-1 transcription factors c-jun and fra-1, and the urokinase type plasminogen activator receptor. Thus, the regulation by APC of the level of b-catenin plays a role in β -catenin/Tcf- Lef-mediated transcriptional regulation of genes. However, the mechanisms by which these transcriptional changes contribute to early stage colorectal carcinogenesis are still unclear.

As Morin and Weeraratna state:

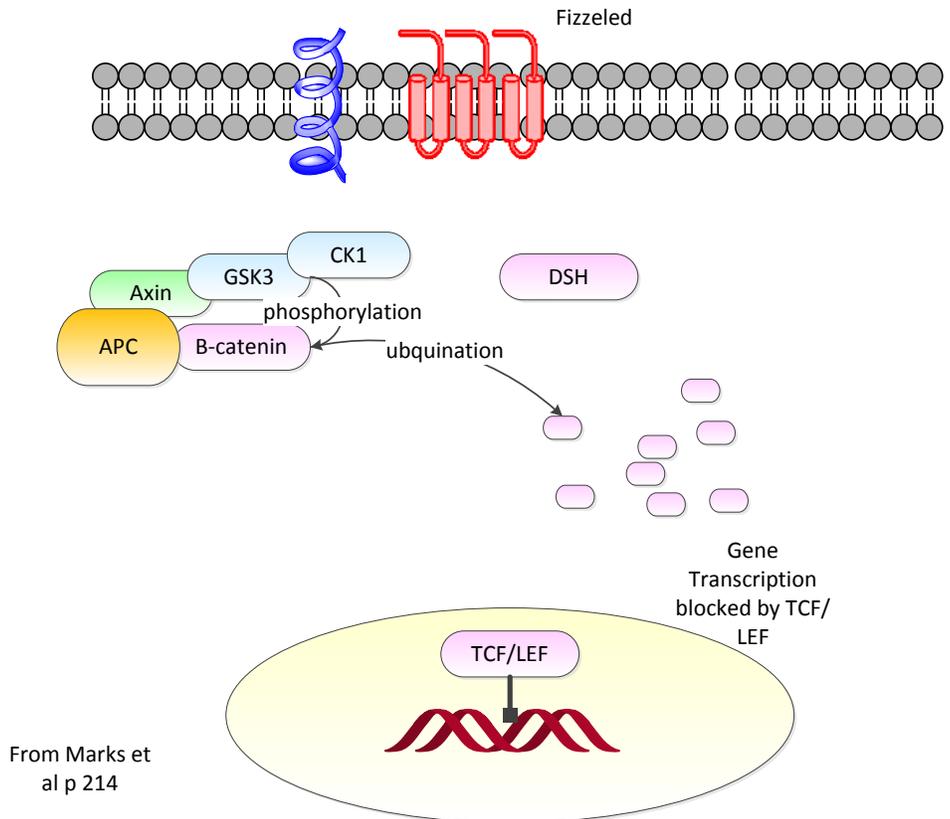
APC plays important roles in cell cycle, motility, adhesion, and signaling. It is often referred to as a multitasking protein, and the disruptions of its interactions may lead to the inability of APC to perform these functions, thereby contributing to tumor formation. The main functions attributed to APC are downregulation of the Wnt pathway (through b-catenin), modulation of cell adhesion/migration, and maintenance of chromosomal stability. ...

The Wnt family of proteins is comprised of several members, all of which are cysteine- rich secreted proteins of about 38–45 kDa in size. Wnts were first identified in Drosophila, are highly conserved throughout the species, and are important in mediating cell–cell interactions during embryogenesis. In normal development, Wnts are expressed both tissue specifically and temporally (63). Deletions of Wnt in mice result in very specific phenotypes, and will be discussed in the section concerning transgenic animals. In general, the deletion of specific Wnts results in the lack of development of specific organs, stressing the importance of Wnts in development due to their signal transduction cascades during proliferation and differentiation.

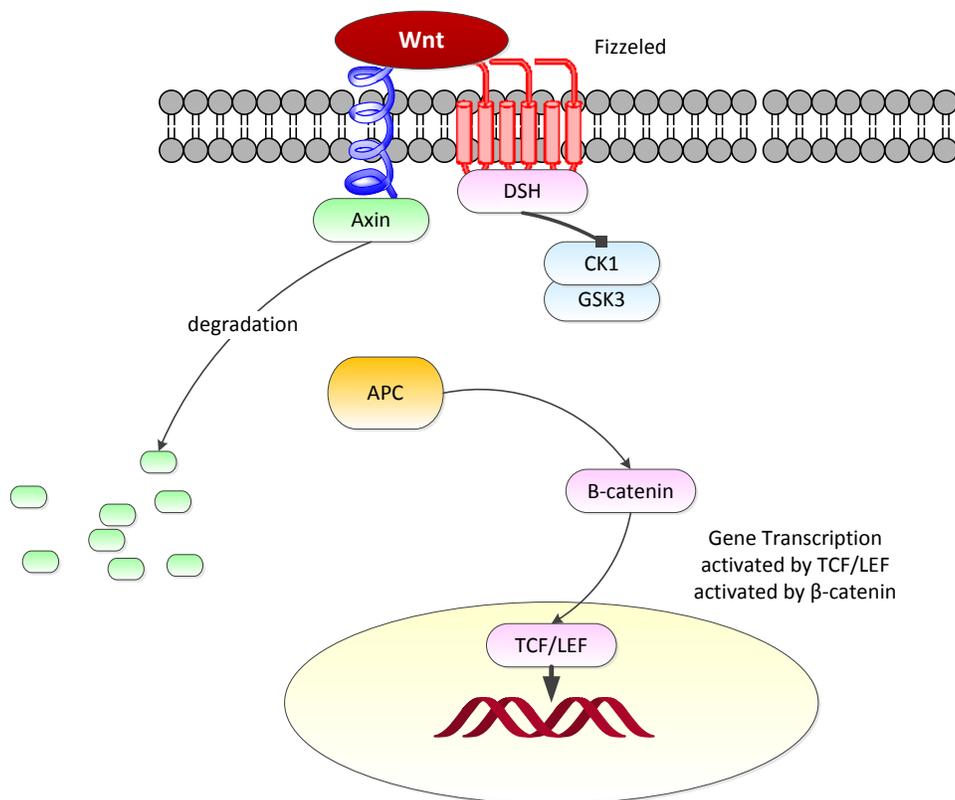
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One of the main features of Wnt signaling is the regulation of b-catenin stabilization. Although b-catenin stability can be regulated by Wnt/APC-independent mechanisms, such as via integrin-linked kinase, GBP, and the c-met receptor tyrosine kinase, APC appears to be a key player. In the absence of Wnt signals, cytoplasmic b-catenin is phosphorylated at multiple residues in its N terminus by glycogen synthase kinase 3b (GSK3b) and then targeted for degradation. The phosphorylation of b-catenin depends on a multiprotein complex that consists of APC, Axin/Conductin, and GSK3b. Phosphorylated b-catenin is recognized by the ubiquitin ligase b-TrCP/Slimb, ubiquitinated and degraded by the proteasome pathway. Axin and APC are also phosphorylated by GSK3b, resulting in enhanced b-catenin binding to the complex and thus enhanced b-catenin degradation.

We demonstrate this relationship to the Wnt pathway in the following Figures. First we show the normal breakdown of β -catenin in the cell.



Then we show below the activation of the Wnt pathway results in the β -catenin becoming a facilitator for gene transcription.



As Morin and Weeraratna state:

...more than 90% of APC mutations result in a premature stop codon, and thus in the truncation of the C terminus. A 5-bp deletion at codons 1309 and 1061 are found in 18% and 12%, respectively, of all germline mutations. These deletions, which occur in short direct repeats within APC, have also been identified in patients without any family history, suggesting that they may also arise sporadically.

Germline mutations can predict the phenotype of the disease they are likely to cause, by their location within the APC gene. A phenotype of multiple tumors (over 5000) is predicted when the mutation arises between codons 1249 and 1330, whereas mutations that are upstream or downstream of this region result in a phenotype of fewer than 1000 tumors.

As He et al (including Vogelstein) noticed in 1998:

The adenomatous polyposis coli gene (APC) is a tumor suppressor gene that is inactivated in most colorectal cancers. Mutations of APC cause aberrant accumulation of b-catenin, which then binds T cell factor-4 (Tcf-4), causing increased transcriptional activation of unknown genes. Here, the c-MYC oncogene is identified as a target gene in this signaling pathway.

Expression of c-MYC was shown to be repressed by wild-type APC and activated by b-catenin, and these effects were mediated through Tcf-4 binding sites in the c-MYC promoter. These results provide a molecular framework for understanding the previously enigmatic overexpression of c-MYC in colorectal cancers.

Thus we are not surprised that APC plays such a significant role.

6 OBSERVATIONS

Methylation is but one of the very many changes we see in cancers. Whether they are cause or effect is yet to be determined. We know, for example, that methylation is causal in MDS, myelodysplasia syndrome, a precursor often of ALL. However, the cause of this methylation is still problematic. Drugs like DNMT inhibitors, azacitidine and decitabine are methylation inhibitors that seem to work for a while in this disorder. However what they do to other cells is uncertain.

In this study which we examine, there is a multiplicity of questions.

1. What causes the methylation?

There is still a lack of clear process as to how methylation occurs. As we will note shortly there are hypothesis that it is a result from inflammatory states and others that there may be secondary effects of autoimmune conditions. The elements of the process are understood to some degree but no true full causal chain is established.

2. Why are these gene sites methylated?

Why specific CpG islands at specific genes are methylated is still unknown. Are these initial locations or are the related to other as yet to be determined sites. Are certain CpG site more susceptible? Is there some histone issue wherein the histones are demethylated opening the DNA and thus allowing CpG methylation?

3. Why not try the DNMT inhibitors if these methylations are causal for PCa?

DNMT inhibitors are used for MDS with some short term success. Can they be used here as a step towards reversal? The problem is that we do not know what DNMTI sequellae are. The unintended consequences could be significant. Can we develop cell specific DNMTIs or are they even too potent?

4. Are many cancers caused by methylation or demethylation? If so which ones and why?

We have examined several different cancers and there is a large putative collection of genes, translocations, miRNAs, SNPs, methylations, and the like. Almost weekly and perhaps soon daily we will be seeing alleged markers for every element of a cancer development. Two decades ago we saw just gene issues. Today we have a problem of ascertaining the chicken or the egg. This is both a challenge and an opportunity. The problem, however, is that each time one of these has been developed, we see a Press release frenzy as it be a sine qua non.

5. Are the observations made by the researchers causal or coincidental?

This is always a critical question. Is this specific methylation of genes the cause of ultimate PCa or the coincidental effect of some other factor?

6. Are the methylation observations drivers or therapeutic approaches?

This question is a follow on to our question regarding DNMTIs.

7. Should we be examining more cancers from an epigenetic point of view rather than a genetic mutation perspective?

We have examined a few cancers for methylation as drivers for malignancy and metastatic behavior.

8. If as we have seen anecdotally, patients determined to have widespread HGPIN on a first 24 core biopsy and then none on a second 24 core biopsy, is this possibly a demethylation result, a stem cell result, or some other factor.

We have examined HGPIN in previous analyses. Although not considered a true PCa it has been considered as a natural pre-cursor. Namely many assume that HGPIN will always turn into PCa. However we have anecdotally seen patients where the HGPIN actually regresses, to the level of being unidentifiable at on a high density core biopsy. Thus we have asked if the first biopsy actually precipitated the remission, if so how and why, or was there some other factor. Unfortunately there is inadequate data for this study.

9. What of the stem cell factor in PCa?

PCa stem cells are always a concern. We have discussed them at length and basically research continues into these cells in PCa. Yet it does beg the question; what cells need be methylated and are certain cells more likely than others? Furthermore we can ask; how do we segregate prostate cells so as to ascertain the severity of methylation?

10. PTEN mutations have been proposed as a major causal effect of PCa. How does this relate to epigenetic factors?

We have shown that PTEN is controlled in the process of p53 and in turn the MDM2 and its RAS precursor control. Thus the focus on RAS derivatives is consistent with the PTEN argument.

11. Are epigenetic factors the results of inflammatory states? Namely in a patient with for example Type 2 Diabetes and elevated inflammatory status, does this become an initiating factor for methylation?

Hartnett and Egan have recently written:

Recently, epigenetic alterations, in particular alterations in DNA methylation, have been observed during inflammation and inflammation-associated carcinogenesis. The mediators of this, the significance of these changes in DNA methylation and the effect this has on gene expression and the malignant transformation of the epithelial cells during IBD and CAC are discussed in this review. The recent advances in technologies to study genomewide DNA

methylation and the therapeutic potential of understanding these molecular mechanisms are also highlighted.

Dayeh et al focus on these types of methylation induced by Type 2 Diabetes in a recent paper as well.

Also it is worth examining the summary by Kundu and Surh on inflammation and cancer. They state:

DNA methylation, the covalent addition of a methyl group to the 5-position of cytosine base in the DNA, represents a critical epigenetic control of gene expression. The perturbation of methylation patterns as either aberrant loss of cytosine methylation in transforming genes or inappropriate gain of cytosine methylation in tumor suppressor genes has been involved in various human malignancies.

The most predominant aberrant DNA methylation is hypermethylation that typically occurs at the CpG islands located in the promoter regions of tumor suppressor genes. Promoter hypermethylation of tumor suppressor genes, such as p16, von-Hippel Lindau (VHL), adenomatous polyposis coli (APC), breast cancer susceptibility gene (BRCA1), retinoblastoma (Rb), E-cadherin (CDH1), p73, p53, and p57, results in transcriptional silencing. By analyzing the methylation status of 11 candidate cancer-related genes in cutaneous squamous cell carcinomas, Murao et al. have demonstrated that the promoter hypermethylation of CDH1, p16, Rb1 and p14 results in the loss of respective protein production.

Therefore, the epigenetic silencing of tumor suppressor genes by promoter CpG island hypermethylation perturbs cell cycle control, apoptosis, DNA repair and cell adhesion, and is recognized as an important mechanism in the tumorigenesis. However, global hypomethylation of certain genes, e.g., insulin-like growth factor-2 (IGF-2), can also result in genomic instability, accelerating malignant transformation.

As Donkena et al state:

“Oxidative stress” is the state of a cell, which is characterized by excess production of reactive oxygen species (ROS) and/or a reduction in antioxidant defenses responsible for metabolism. ROS are formed as a natural byproduct of the normal metabolism of oxygen. Under normal circumstances, the cell is able to maintain an adequate homeostasis between the formation of ROS and its removal through enzymatic pathways or via antioxidants. If, however, this balance is disturbed, then oxidative stress occurs.

This generates an imbalance of production/removal of ROS, which is either directly or indirectly involved in initiation, promotion, and progression phases of carcinogenesis. Oxygen radicals may cause damage to DNA and chromosomes, induce epigenetic alterations, interact with oncogenes or tumor suppressor genes, and impart changes in immunological mechanisms.

The extent of ROS induced oxidative damage can be exacerbated by a decreased efficiency of antioxidant defense mechanisms. Hypermethylation of a combination of genes including

APC, RASSF1A, PTGS2, PDLIM4, and MDR1 could distinguish cancer from benign prostate tissues with sensitivities of 97.3%–100% and specificities of 92%–100%. The increase in methylation of these genes with cancer progression indicates that they could be used for biomarkers for both diagnosis and risk assessment

Furthermore, we showed significant differences in the frequency of methylation at individual CpG sites of PITX2, PDLIM4, KCNMA1, GSTP1, FLNC, EFS, and ECRG4 in recurrent and nonrecurrent subtypes of prostate tumors. Indeed, hypermethylation of a CpG island in PITX2 portended an increased risk of prostate cancer recurrence and was a predictor of distant disease recurrence in tamoxifen-treated, node-negative breast cancer patients.

12. HGPIN has always been an issue of concern. In some cases it appears to revert to fully benign states and in others it progresses to PCa. The question is: How useful would such a test be in HGPIN conditions?

The concern always is the HGPIN cells are confined to the glandular portions of luminal and basal cells. Is it necessary to get HGPIN cells to test or can the test be performed on other cells? Specifically how large a sample is necessary to get adequate sensitivity and specificity?

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