Disease Markers 23 (2007) 73–87
IOS Press

The role of RASSF1A methylation in cancer

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Abstract. Tumour suppressor gene inactivation is critical to the pathogenesis of cancers; such loss of function may be mediated by irreversible processes such as gene deletion or mutation. Alternatively tumour suppressor genes may be inactivated via epigenetic processes a reversible mechanism that promises to be more amenable to treatment by therapeutic agents. The CpG dinucleotide is under-represented in the genome, but it is found in clusters within the promoters of some genes, and methylation of these CpG islands play a critical role in the control of gene expression. Inhibitors of the DNA methyltransferases DNMT1 and DNMT3b have been used in a clinical setting, these nucleotide analogues lack specificity but the side effects of low dose treatments were minimal and in 2004 Vidaza (5-azacitidine) was licensed for use in myelodysplastic syndrome. Methylation inhibitors are also entering trials in conjunction with another class of epigenetic modifiers, the histone deacetylase inhibitors and this epigenetic double bullet offers hope of improved treatment regimes. Recently there has been a plethora of reports demonstrating epigenetic inactivation of genes that play important roles in development of cancer, including Ras-association domain family of genes. Epigenetic inactivation of *RASSF1A* (Ras-association domain family 1, isoform A) is one of the most common molecular changes in cancer. Hypermethylation of the *RASSF1A* promoter CpG island silences expression of the gene in many cancers including lung, breast, prostate, glioma, neuroblastoma and kidney cancer. Several recent studies have illustrated the diagnostic and prognostic potential of *RASSF1A* methylation. This presents *RASSF1A* methylation as an attractive biomarker for early cancer detection which, for most cancers, results in improved clinical outcome. DNA methylation analysis is applicable to a range of body fluids including serum, urine, bronchioalveolar lavage and sputum. The ease with which these body fluids can be acquired negates the need for invasive procedures to obtain biopsy material. This review will discuss the feasibility of using *RASSF1A* methylation as a diagnostic and prognostic marker in cancer management.

Keywords: RASSF1A, tumour suppressor gene, 3p21.3, ras association domain, methylation, cancer

1. Introduction

Cancer mortality could be greatly reduced by earlier detection since existing surgical and treatment strategies are more effective against early stage tumours. In the case of lung cancer around two-thirds of patients present with advanced metastatic tumours at the time of diagnosis [125]. This is also true for ovarian cancer patients 70% of whom have advanced disease (stage III or IV) and 5 year survival of only 15–20% upon presentation [57]. DNA methylation has several advantages over commonly used biomarkers such as cytology or the assay of tumour-derived RNA or protein. DNA methylation is a molecular change more readily detectable than gross cytogenetic or cellular abnormalities which can also be subjective. Other techniques such as radiography rely on a visible tumour mass, which can make them unsuitable for early detection. Some screening strategies rely on the detection of tumour-derived RNA or protein. However, DNA is much more stable and unlike protein can be amplified for increased sensitivity and is not dependent on levels of gene expression. This also makes the use of DNA more suitable when a limited amount of tissue/fluid is available. DNA methyla-

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tion associated with particular genes is one of the earliest detectable changes and in some cases may even precede tumour-formation. DNA methylation may also provide prognostic information. Furthermore, tumour-associated hypermethylation usually occurs in discrete CpG island promoter regions. This minimises the region of analysis when compared to mutation screening involving several exons. Since DNA methylation is heritable, a gene that acquires methylation will usually remain methylated throughout tumour progression. Finally, some cancer biomarkers such as allelic loss, experimentally rely on loss of signal as opposed to DNA methylation which is gain of signal [88,124].

A further advantage of DNA methylation analysis is its applicability to a range of body fluids. The presence of tumour-derived DNA within the blood stream (either within tumour cells or as free DNA) has been known for some time [73]. Tumour DNA within the bloodstream arises following vascular invasion or absorption of DNA from apoptotic or necrotic cells at the tumour site. Tumour-derived DNA may also be found in bodily fluids that drain from the organ of interest including urine, bronchioalveolar lavage, nipple aspirates, mouth and throat rinsings, nasopharyngeal swabs and stool samples. The ease with which body fluids can be acquired negates the need for invasive procedures to obtain biopsy material. A range of techniques are available to study DNA methylation. These include CoBRA (Combined Bisulphite Restriction Analysis) and bisulphite sequencing, methylation-specific PCR (MSP) and high throughput quantitative MethyLight assays [34,48,116,129].

Recently we and others have cloned and characterised the *RASSF1* gene and shown frequent inactivation of the A isoform by promoter CpG island hypermethylation in a broad spectrum of tumour types. Several studies reveal that *RASSF1A* methylation can also be detected in several body fluids in cancer patients highlighting its potential as a disease marker.

2. RASSF1A inactivation in cancer

Allelic losses of 3p are one of the most frequently detected genetic alterations in many cancers including lung, kidney and breast cancers. Loss of heterozygosity studies and the identification of overlapping homozygous deletions in lung and breast tumour cell lines indicated a critical region at 3p21.3 that may harbour one or more tumour suppressor genes (TSGs). Eight genes, namely *CACNA2D2, PL6/Placental protein 6, 101F6, TUSC4/NPRL2, ZMYND10/BLU, RASSF1, TUSC2/FUS1* and *HYAL2* were cloned from the minimal 120 kb overlapping region. Despite extensive investigation only rare mutations and polymorphisms of those genes were identified. Interestingly however, expression of RASSF1A, but not its alternative splice form RASSF1C, was lost or downregulated in most lung tumour cell lines [26,74]. The principal cause of this loss of expression was promoter CpG island hypermethylation. The Ras-association domain family member 1 (*RASSF1*) gene is comprised of eight exons and generates seven transcripts, designated RASSF1A-G, via differential promoter usage and alternative splicing. Two major isoforms RASSF1A and RASSF1C, are transcribed from two separate CpG island promoter regions (See figure 1a). Both RASSF1A and RASSF1C proteins contain a C-terminal Ras-association domain and a putative ATM phosphorylation site. In addition to this RASSF1A also contains an N-terminal diacylglycerol binding/protein kinase C conserved 1 (C1) domain. *RASSF1A* inactivation by methylation was originally described in lung and breast cancers [26]. Since then it has emerged that *RASSF1A* is one of the most frequently hypermethylated genes so far described in human cancer. Inactivation is frequently observed in a broad spectrum of tumours (summarised in Table 1 and Fig. 2). Associated loss of expression has been shown in lung, breast, bladder, gastric, cholangiocarcinoma and oesophageal sqaumous cell carcinoma primary tumours [12,13,26,67,70,126]. While allelic loss at the *RASSF1* locus has been shown to be a frequent 'second hit' [3,5,67,85], mutation is described as an occasional event [3,26,68,74,80]. Recently however, using a more sensitive methodology a high incidence of *RASSF1A* mutations were found in primary NPC tumours [93]. In some cases, such as in medulloblastoma, biallelic inactivation of *RASSF1A* by promoter hypermethylation has been observed [80].

3. Human homologues of RASSF1

Homology searches of the human genome have identified several other members of the RASSF gene family that also contain a Ras-association domain. These are *RASSF2* (20pter-p12.1), *RASSF3* (12q14.1), *RASSF4/AD037* (10p11.21), *RASSF5/NORE1* (1q32.1) and *RASSF6* (4q21.21), (Fig. 1b). Interestingly some of these additional members are also inactivated by promoter hypermethylation. Tumour-specific *RASSF2* promoter hypermethylation frequently inactivates

Table 1
RASSF1A methylation occurs in a broad spectrum of tumours. Frequencies (given as percentages) can differ significantly between tumour types

Cancer	RASSF1A methylation	
	Percentage of RASSF1A methylation in primary tumours	Reference
Anal SCC	15 (19/127)	[135]
Bladder	62 (34/55)	[70]
	35 (34/98)	[82]
	48 (19/40)	[15]
	51 (23/45)	[33]
Breast	62 (28/45)	[28]
	49 (19/39)	[12]
	65 (11/17)	[53]
Biliary tract	27 (10/37)	[115]
Cervical SCC	30 (10/33)	[134]
	10 (4/42)	[69]
	0 (0/31)	[24]
Cervical adenosqaumous	21 (4/19)	[69]
Cervical adenocarcinoma	12 (2/17)	[134]
	24 (8/34)	[69]
	45 (9/20)	[24]
Cholangiocarcinoma	69 (9/13)	[126]
	65 (47/72)	[131]
	85 (28/33)	[18]
Colorectal	20 (45/222)	[117]
	45 (13/29)	[122]
	16 (24/149)	[71]
Ependymoma	86 (30/35)	[45]
Esophageal SCC	52 (25/48)	[67]
	24 (13/55)	[130]
Ewing's sarcoma	0 (0/8)	[46]
Gastric EBV+	67 (14/21)	[60]
Gastric EBV−	4 (2/56)	[60]
Gastric	43 (39/90)	[13]
Glioma	57 (36/63)	[49]
	54 (25/46)	[56]
	57 (12/21)	[7]
	57 (16/28)	[97]
Head and neck	0 (0/32)	[81]
	15 (7/46)	[29]
	17 (2/24)	[52]
	8 (6/80)	[47]
	12 (5/42)	[91]
Hepatoblastoma	19 (5/27)	[46]
Hepatocellular	85 (70/83)	[136]
	100 (29/29)	[133]
	95 (41/43)	[137]
Hodgkin's lymphoma	65 (34/52)	[89]
Kidney	91 (39/43)	[30]
	26 (44/165)	[85]
	46 (23/50)	[32]
Lung: SCLC	72 (21/29)	[3]
	79 (22/28)	[28]
	84 (36/43)	[114]
Lung: NSCLC	34 (14/41)	[3]
	30 (32/107)	[12]
	32 (35/110)	[111]
Leukaemia (acute)	15 (3/20)	[46]
Medulloblastoma	79 (27/34)	[80]

Table 1, continued

Cancer	RASSF1A methylation	
	Percentage of RASSF1A methylation in primary tumours	Reference
Melanoma	41 (14/44)	[108]
	15 (3/20)	[55]
Meningioma	17 (2/12)	[56]
Mesothelioma	32 (21/66)	[113]
Multiple myeloma	28 (9/32)	[90]
	15 (17/113)	[104]
	0 (0/56)	[44]
Nasopharyngeal	67 (20/30)	[16]
	67 (14/21)	[79]
Neuroblastoma	55 (37/67)	[5]
	52 (14/27)	[46]
	84 (26/31)	[8]
Osteosarcoma	0 (0/11)	[46]
Ovarian	40 (8/20)	[132]
	50 (25/50)	[57]
	41 (20/49)	[98]
Pancreatic	62 (47/75)	[27]
Phaeochromocytoma	22 (5/23)	[5]
Pituitary adenoma	28 (20/52)	[95]
Primitive neuroectodermal tumours (supratentorial)	79 (19/24)	[86]
Prostate	54 (53/101)	[83]
	71 (37/52)	[78]
	99 (117/118)	[59]
Rhabdomyosarcoma	61 (11/18)	[46]
Retinoblastoma	59 (10/17)	[46]
	82 (56/68)	[23]
Salivary gland adenoid cystic carcinoma	40 (25/60)	[76]
Schwannoma	10 (1/10)	[56]
Testicular nonseminoma	83 (15/18)	[54]
	21 (9/44)	[64]
Testicular seminoma	40 (4/10)	[54]
Thyroid	71 (27/38)	[102]
	37 (19/51)	[128]
Wilms' tumour	71 (22/31)	[36]
	54 (21/39)	[122]

RASSF2A expression in colorectal carcinomas at frequencies ranging from 42% to 70%. Furthermore, in colorectal carcinoma *RASSF2A* inactivation is an early event detectable in adenoma polyps [4,51]. Inactivation of *RASSF2A* in colorectal carcinomas is a much earlier and more frequent event than inactivation of *RASSF1A* (15–45%; [4,51,117,122]). Hence, *RASSF2* methylation may provide a much more effective marker for early detection and diagnosis of colorectal carcinomas. *RASSF2* methylation has also been found in gastric cancer at frequencies ranging from 29% to 79% [38]. Whilst RASSF4/AD037 is expressed in a variety of normal tissues, it is lost or downregulated in a variety of tumour cell lines and primary tumours. In some cases this loss of expression is caused by promoter hypermethylation [22,35]. Expression of NORE1A, but not NORE1B, is lost or downregulated in cancer due to promoter hypermethylation, whilst mutation of the *NORE1* gene is a rare event [17,50,58]. *RASSF3A* is not methylated in human tumours [4,49,51], and *RASSF6* is not methylated in colorectal cancer [4].

4. RASSF1A function

Recent investigation of RASSF1A has revealed a protein with diverse functions including the regulation of apoptosis and of microtubule dynamics during mitotic progression. Re-expression of RASSF1A also suppresses growth in *in vitro* and *in vivo* systems in lung, breast, kidney, prostate, NPC and glioma cancer cells [12,21,26,30,49,68]. Overexpression of

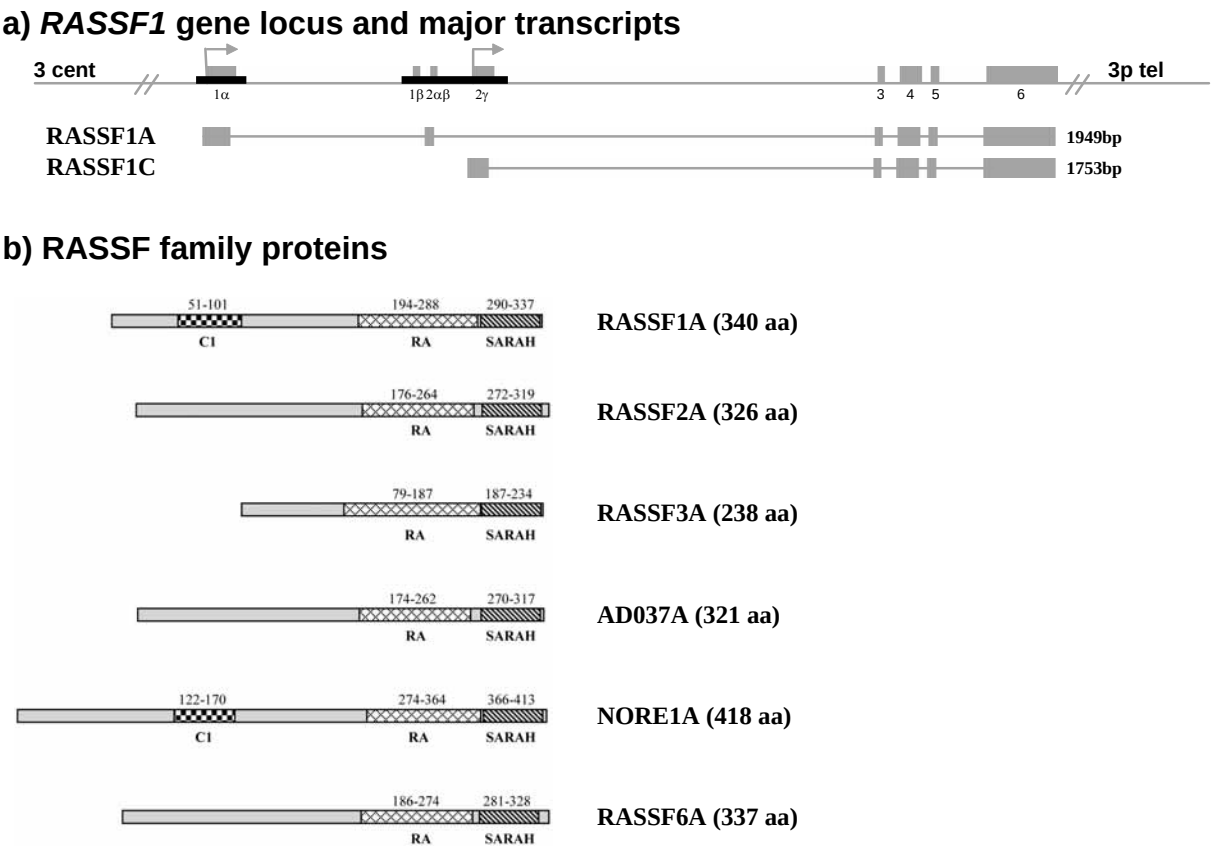

Fig. 1. a) The *RASSF1* gene locus and major transcripts. *RASSF1* isoforms are generated by differential promoter usage (arrows) and alternative splicing. The two promoter associated CpG islands are shown in black, and the major transcripts associated with these promoters are also depicted. b) The domain structures of the A isoforms of the RASSF family of proteins. Putative functional domains (predicted using Prosite) of RASSF1 (AAD44174), RASSF2 (AAN59975), RASSF3 (AAO61687), AD037 (AAH32593), NORE1 (NP 872604) and RASSF6 (NP 803876) are shown: RA, RalGDS/AF6 Ras association domain; C1, DAG/diacylglycerol binding domain; and SARAH, Sav/RASSF/Hpo interaction domain.

RASSF1A results in cell cycle arrest and is accompanied by dramatic changes in gene expression [1, 20]. This includes changes to the expression of important cell cycle regulatory genes such as cyclin D [105] but also to genes involved in diverse functions including transcription, cytoskeletal organisation, angiogenesis, signalling, cell adhesion, cell migration and apoptosis. RASSF1A regulates apoptosis via at least two pathways. RASSF1A binds the proapoptotic serine/threonine kinase MST1 [62]. NORE1A and RASSF1A are constitutively complexed with MST1 and serve as sensory modules to detect pro-apoptotic signals initiated through Ras pathways [62,94]. Although RASSF2, NORE1/RASSF5 and AD037/RASSF4 bind to Ras proteins directly RASSF1A associates with K-Ras indirectly through NORE1A [35,92,118,119,121]. The RASSF1A-MST1 complex may also indirectly associate with Ras via CNK1 [96]. RASSF1A may also regulate apoptosis through MOAP-1 (Modulator of Apoptosis 1). MOAP-1 associates with Bcl-2 family members Bax and Bcl-2 and initiates caspase-dependent apoptosis when overexpressed [109]. Following apoptotic stimulation, RASSF1A associates with MOAP-1, promoting Bax conformational change, integration of Bax into the mitochondrial membrane and the release of cytochrome-c. In support of this, the effects of RASSF1A on Bax conformation were reversed by siRNA directed against RASSF1A [6]. RASSF1A is also involved in the regulation of cytoskeletal dynamics and co-localises with microtubules, spindles and centrosomes during metaphase and promotes microtubule stability and polymerisation [25,77,120]. RASSF1A microtubule association may be mediated in part by interaction with MAP1B and C19ORF5 [25]. RASSF1A also binds Cdc20, which negatively regulates APC

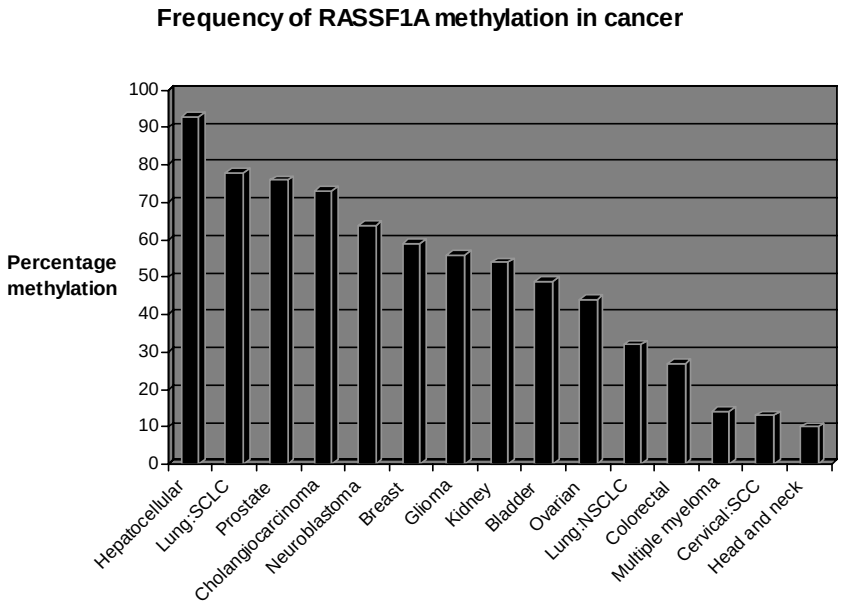

Fig. 2. Frequency of *RASSF1A* methylation in several tumour types. Several of the most common cancers are shown. Frequencies represent the average of at least three independent studies on a given tumour type (see Table 1).

(anaphase promoting complex). The Cdc20-APC complex is a central component of the spindle assembly checkpoint during mitosis [107]. RASSF1A can also inhibit G_1-S phase transition by interacting with $p120^{E4F}$, a protein known to associate with pRb, p53 and $p14^{ARF}$ [39,40,99,101]. For a more extensive review of RASSF1 functions, see [2].

5. RASSF1A methylation as a cancer biomarker

Methylation of *RASSF1A* seems an ideal cancer biomarker for three main reasons. Firstly, methylation occurs in a very broad spectrum of tumour types. Secondly, the frequency of methylation is often moderate to very high (Table 1) thus providing a high frequency of diagnostic coverage. Thirdly, methylation of *RASSF1A* is rare in normal tissues providing a marker with a high specificity. Many studies have shown hypermethylation of *RASSF1A* in cancer may have several clinical utilities. These include its use as a diagnostic marker, as a marker for early detection or patient prognosis, as a predictive marker in benign growths from 'at risk' patients or even as a marker for resistance to some treatments.

5.1. Diagnostic marker

For lung cancer surgical intervention is more successful when tumours are detected early and are still resectable. One strategy for early detection could be the screening of populations at risk such as smokers. The lifetime risk of never smokers developing lung cancer is 1×10^{-4} whereas for current and former chronic smokers the yearly risk is 0.3% [11]. Bronchial aspirates from smokers and never smokers with lung cancer showed frequencies of *RASSF1A* methylation of 21% and 1% respectively. Amongst smokers the frequency of methylation was 10% for $\leqslant 30$ pack years smoked versus 26% for > 30 pack years smoked [103]. Methylation was also significantly associated with the number of pack years smoked during the lifetime ($p \leqslant 0.05$). Another population at risk of developing lung cancer are lung cancer survivors (6.0% risk per patient year, 5-year survival of 60% [11]). A recent investigation of *RASSF1A* methylation in plasma and sputum revealed a frequency of 7% in current and former smokers whereas in lung cancer survivors it was 25% ($p \leqslant 0.01$) [11]. Using a panel of four genes commonly methylated in cancer, including *RASSF1A*, methylation of at least one gene could be detected in the upper aerodigestive tract from 48% of current smokers without cancer [138]. This study included samples obtained from bronchial brushes, bronchoalveolar lavage and oropharyngeal brushes with *RASSF1A* methylation detected in 6%, 5% and 2% of the samples respectively. In a separate study of sputum from current and former smokers some of whom later developed cancer *RASSF1A* methylation could be detected [53]. *RASSF1A* methy-

lation has also been detected in the tumour and corresponding bronchoalveolar lavages in 29% (5/17) of methylated lung cancer cases and in combination with 5 other tumour-related genes could diagnose lung cancer in 68% (21/31) of patients [112]. These data suggest regular screening for *RASSF1A* methylation in sputum, bronchoalveolar lavages and serum from populations at risk, particularly smokers and lung cancer survivors, may enable earlier detection of lung cancer and the reduction of lung cancer mortality. Ramirez et al. [97] showed 34% (17/51) NSCLC tumours showed *RASSF1A* methylation with concomitant methylation observed in the corresponding serum.

In a recent report, methylation analysis of a panel of just 3 genes (*RASSF1A*, *APC* and *DAPK1*) was sufficient to differentiate normal and tumour tissue in 94% of breast cancer cases. Dulaimi et al. [31] also showed 76% of corresponding serum DNA was also positive for methylation illustrating the potential sensitivity of these methylation markers in early detection of breast cancer [31]. Furthermore, in breast cancer patients where *RASSF1A* methylation is undetectable in plasma, methylation may be detected in tumour DNA eluted from the surface of erythrocytes and leukocytes suggesting a further measure to increase sensitivity of detection [100]. Alternatively, breast cancer has also been detected in nipple aspirate fluid in 82% (18/22) cases including ductal carcinoma in situ and stage I cancer by CpG island hypermethylation [66]. Analysis of urine DNA represents a simple method for kidney and bladder cancer detection. Investigation of 45 urothelial cancer patients and 12 normal healthy individuals using a panel of just three genes (*RASSF1A*, *APC* and $p14^{\mathrm{ARF}}$) gave a diagnostic coverage of 100%, sensitivity of 87% and specificity of 100% [33]. More importantly, methylation analysis identified 16 cases that had negative cytology. In a different study *RASSF1A* methylation was detected in the urine of 50% (7/14) bladder cancer patients, but not in normal control samples. This study also showed methylation analysis of urine DNA was more sensitive than conventional cytology especially for low-grade tumours [15]. Battagli et al. [10] found at least one of a panel of six genes (*VHL*, *p16*$^{\mathrm{INK4A}}$, $p14^{\mathrm{ARF}}$, *APC*, *RASSF1A* and *TIMP3*) were methylated in all 50 kidney tumours investigated. Furthermore, the same pattern of methylation was observed in corresponding urine DNA in 88% (44/50) of cases, including 27/30 cases of stage I disease. The diagnostic potential of DNA hypermethylation has also been explored in ovarian cancer. In one study the use of 6 genes (*RASSF1A, BRCA1, APC, DAPK1, p14*$^{\mathrm{ARF}}$ and $p16^{\mathrm{INK4A}}$) gave 100% diagnostic coverage (50/50 tumours) with *RASSF1A* methylation detected in 50% cases. Furthermore *RASSF1A* methylation was detected in patient serum from 84% (21/25) methylated tumour cases including several stage I tumours. Of the 50 tumours investigated serum or peritoneal fluid was positive for methylation in 88% of cases thus offering a much greater diagnostic sensitivity than conventional cytology. In some cases methylation was detected in the serum of patients negative for the CA-125 serum marker [57]. In general however, DNA hypermethylation would provide a very powerful adjunct to conventional diagnostic methods such as cytology and histology. For example, a study of *RASSF1A, p16*$^{\mathrm{INK4A}}$ and *APC* methylation in bronchial aspirates showed that cytology, quantitative MSP and histology could detect lung cancer in 44% (37/85), 53% (45/85) and 59% (50/85) of cases respectively. When combined however diagnostic sensitivity extended to 81% (69/85) of patients [103].

A novel approach of detecting endometrial cancer was recently described with the use of DNA collected from tampons [41]. In this study, hypermethylation of 3 or more of 5 candidate genes, including *RASSF1A*, was a significant indicator of endometrial cancer ($p = 0.001$) with a sensitivity and specificity of 100% and 97.2% respectively. Those patients without endometrial cancer that showed hypermethylation of 3 or more genes were shown to have cervical cancer, endometrium polyps or fibroids. *RASSF1A* methylation was also detectable in 57% (16/28) glioma tumours and in 50% (14/28) of corresponding patient serum [97]. However, some other studies have shown limited success in the detection of *RASSF1A* methylation in serum. For example, methylation was detected in 65% (34/52) of Hodgkin's lymphoma tumours but in only 2/22 corresponding serum [89]. Methylation of *CDH1, p16*$^{\mathrm{INK4A}}$, *DAPK1* and $p15^{\mathrm{INK4B}}$ could be detected in 46%, 42%, 20% and 20% of plasma from NPC patients respectively, yet only 5% showed *RASSF1A* methylation [127]. In a further study of NPC *RASSF1A* methylation was detected in 67% (20/30) of tumour samples and in 3% of corresponding plasma. However, in nasopharyngeal swabs and mouth and throat rinsings methylation was detected at frequencies of 33% and 37% respectively illustrating the importance of selecting the correct body fluid [16]. Taken together, these studies show that *RASSF1A* methylation can be detected in a range of body fluids from cancer patients and offers an exciting new approach to cancer diagnosis. The sensitivity of methylation assays in these body fluids compares favourably with conventional diagnostic methods.

5.2. Predictive

Several studies have illustrated the use of *RASSF1A* methylation in benign growths or hyperplastic lesions as a means of predicting cancer risk. In benign breast tissues from unaffected women at high-risk for breast cancer *RASSF1A* methylation was found in 70% of samples, but in only 29% of samples from women at low/intermediate risk. Demonstrating that in benign breast epithelium *RASSF1A* promoter methylation is associated with epidemiological markers of increased breast cancer risk. In particular, biopsies from women with a previous history of benign breast growths were statistically more likely to have *RASSF1A* methylation [75]. *RASSF1A* methylation is also prevalent in breast epithelial hyperplasia, papilloma samples and ductal carcinoma in situ but not in normal breast tissues [53,72]. Early prediction of prostate cancer may also be possible since 19–28% of benign prostate hyperplasia (BPH) samples also show methylation [9, 106] with some studies describing up to 100% in tumours and BPH samples [59,68]. Precancerous intestinal metaplasia lesions may also be useful for predicting gastric cancer risk since a subgroup show *RASSF1A* methylation [110]. This suggests that *RASSF1A* methylation in benign growths or hyperplastic lesions often indicates an increased risk of some forms of cancer which may be detected at the earliest stages with regular surveillance.

5.3. Prognosis

For some cancers an association between *RASSF1A* methylation and adverse patient survival has been observed. Burbee et al. [12] found that NSCLC patients with *RASSF1A* methylation had a mean overall survival of 37 months compared with 52 months for patients without *RASSF1A* methylation ($p = 0.0463$) [12]. In lung adenocarcinoma *RASSF1A* methylation was significantly associated with vascular invasion, pleural involvement, poor tumour differentiation, decreased patient survival time and allelic loss at 3p21.3 [111]. Since all tumours investigated were stage I this study suggests *RASSF1A* methylation would provide a powerful marker for patient prognosis at an early stage of lung adenocarcinoma development. In a study of 119 well-characterised NSCLC tumours *RASSF1A* methylation was also more frequently observed in poorly-differentiated tumours (50%) than in tumours of moderate (26%) or high (0%) differentiation ($p = 0.04$). More importantly, methylation of $p16^{\mathrm{INK4A}}$ was associated with stage I/II disease whereas *RASSF1A* methylation was associated with stage IIIA disease [123]. This contradicts the earlier report by Tomizawa et al. [111] and suggests that *RASSF1A* methylation occurs during NSCLC progression whereas $p16^{\mathrm{INK4A}}$ inactivation occurs in the early stages of lung cancer development. Wang et al. [123] also show $p16^{\mathrm{INK4A}}$ and *RASSF1A* methylation were powerful prognostic indicators. Patients with stage I/II tumours containing $p16^{\mathrm{INK4A}}$ methylation had a significantly poorer 5 year survival rate compared to those without $p16^{\mathrm{INK4A}}$ methylation. However, *RASSF1A*, and $p16^{\mathrm{INK4A}}$, methylation in stage IIIA tumours were profound indicators of poor survival. All 11 stage IIIA patients with methylation of both genes died within 3 years post-surgery, whereas 21 (62%) with methylation of either $p16^{\mathrm{INK4A}}$ or *RASSF1A* died within 5 years and only 5 (29%) with methylation of neither died within 6.5 years ($p = 0.0001$). *RASSF1A* methylation remained a very strong prognostic indicator irrespective of whether patients had received adjuvant radiotherapy [123]. In contrast to this, a more recent study of 116 cases of NSCLC showed that *RASSF1A* methylation was not related to poor prognosis, including tumour stage, recurrence, lymphatic permeation and smoking duration [19]. However, a significant association between *RASSF1A* methylation, age at which smoking began (below 19 years) and decreased NSCLC patient survival time has been observed [63]. Interestingly, another study showed that earlier recurrence of lung cancer (but not tumour grade, stage, histological type or patient survival) is associated with *RASSF1A* methylation [37]. With regular surveillance this may allow early detection of recurrent tumours. However, additional studies will be required to clarify the prognostic value of *RASSF1A* methylation in lung cancer. In prostate cancer *RASSF1A* methylation status may be informative of disease progression. Tumours with a high Gleason score or high serum prostate-specific antigen (both features of poor prognosis and advanced prostate cancer) showed a significantly higher frequency of *RASSF1A* methylation [61,78,83]. However, some studies show that hypermethylation of *GSTP1, APC* and *PTGS2* in prostate cancer is more informative of prognosis [9, 59] whereas *RASSF1A* methylation was only associated with advanced stage [59]. Many studies have shown that *RASSF1A* methylation, as well as methylation of some other tumour-related genes, occurs significantly more frequently in tumours of a higher-grade, later stage or in invasive or metastatic tumours. This has been shown in many types of cancer including

breast cancer, bladder cancer, salivary adenoid cystic carcinoma, glioma, pituitary adenomas, pancreatic endocrine tumours and gastric cancer indicating *RASSF1A* methylation may be used as a marker for tumour progression and metastasis [13,43,49,70,76,82, 84,95]. Methylation of *MGMT*, *RASSF1A* and *DAPK1* is also significantly associated with progression from primary melanoma to metastatic melanoma [55]. Many other studies provide further evidence that *RASSF1A* methylation is a marker for disease progression. For example, a study of transitional cell carcinomas (comprising 116 bladder and 164 upper-tract tumours) revealed a significantly higher frequency of *RASSF1A* methylation in poorly-differentiated ($p = 0.017$) and more rapidly progressing tumours (0.0076) [14]. Mortality was also higher in tumours with *RASSF1A* methylation ($p = 0.018$). Methylated *RASSF1A* and/or *APC* DNA in serum from breast cancer patients is strongly associated with metastasis, tumour size and increased relative risk for death [87]. A total of 11% (7/66) of patients with methylation of *RASSF1A* or *APC* died compared with 53% (10/19) of patients with methylation of both ($p = 0.001$). In summary, *RASSF1A* methylation in tumour tissue and corresponding body fluids often correlates with advanced tumour stage and grade, metastasis, poor tumour differentiation and adverse survival.

5.4. Drug resistance

Testicular nonseminoma germ cell tumours (NSGCT) are particularly sensitive to cisplatin-based chemotherapy. However, 20–30% of metastatic tumours acquire resistance to such therapy. A recent study showed hypermethylation of *RASSF1A* and *HIC1* was much more frequent in cisplatin resistant versus cisplatin sensitive NSGCT [65]. Interestingly the frequency of both *RASSF1A* and *HIC1* methylation increased following each regimen of cisplatin chemotherapy. Very recently *RASSF1A* methylation in the serum of breast cancer patients was identified as a surrogate marker for the monitoring of response to adjuvant tamoxifen treatment [42]. Persistence of *RASSF1A* methylation post-surgery and throughout treatment indicated resistance to tamoxifen whereas loss of methylation indicated a response. Thus, *RASSF1A* methylation may offer a marker for cisplatin and tamoxifen resistance in some tumours and could be monitored throughout the course of treatment.

6. Conclusion

Whilst global hypomethylation is a characteristic of many cancers, the specific hypermethylation of the CpG islands associated with certain tumour suppressor genes may be exploitable for the generation of assays to determine clinical risk. However a current problem with methylation as a biomarker is its potential lack of specificity, for example in the colon the genome becomes increasingly methylated as an individual ages and whilst this may reflect the increased risk of colon cancer experienced by older people, it may also confound techniques that rely on an absence of methylation to predict reduced risk. The use of panels of carefully chosen markers specific for the tumour type and taking into account the demographics of the population to be tested will be an essential prerequisite to successful clinical application of these assays. Regardless of the role of DNA hypermethylation in cancer these epigenetic changes will become useful in the clinic as diagnostic or prognostic markers. The value of using methylation of *RASSF1A* and other tumour-related genes as cancer biomarkers depends on whether these markers could offer earlier detection, greater reliability and sensitivity or be more informative of patient prognosis when compared with existing screening strategies. As discussed this appears the case for at least some cancers. Furthermore, as an adjunct to existing cancer detection methods screening for hypermethylation would provide a non-invasive, rapid and cost effective means of increasing the sensitivity and reliability of cancer diagnosis from a range of readily available body fluids. When compared with existing screening strategies methylation analysis can provide a more informative and powerful prognostic indicator with a greater level of sensitivity for some cancers. However, the use of hypermethylation as a cancer biomarker requires a panel of several carefully chosen genes that offer the greatest diagnostic coverage and prognostic information for a given cancer. Given the frequencies of *RASSF1A* methylation, the broad spectrum of tumour types in which this occurs and its potential prognostic value, *RASSF1A* should be considered for inclusion in any such panel of candidate genes.

Acknowledgments

Research in F. LATIF laboratory funded in part by Breast Cancer Campaign, Cancer Research UK and Birmingham Children's Hospital Research Foundation.

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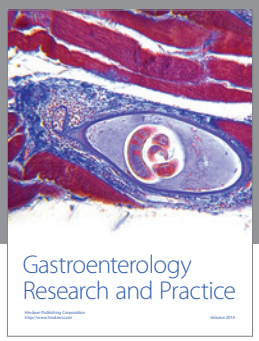

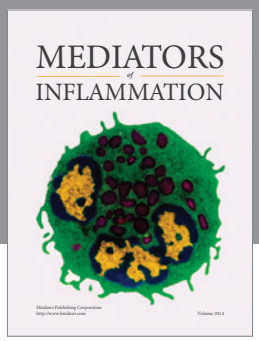

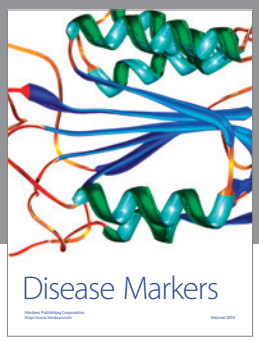

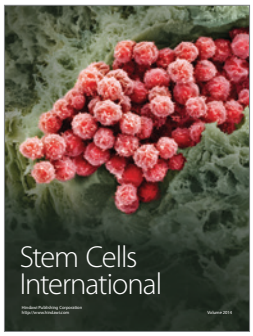

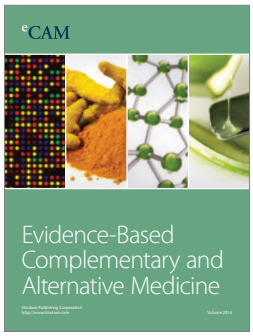

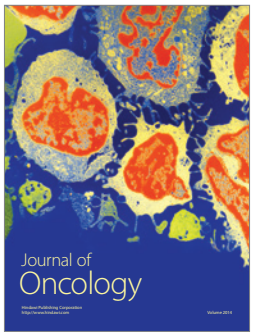

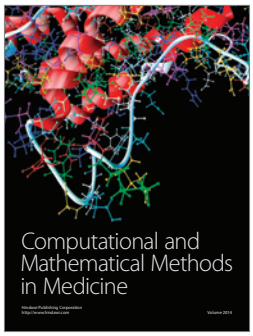

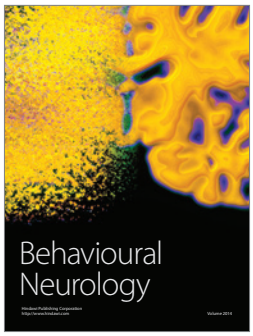

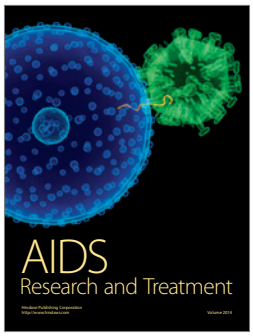

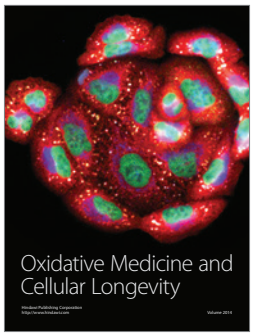

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