REVIEW

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Clinical utility of *RASSF1A* methylation in human malignancies

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The high frequency of *RASSF1A* methylation has been noted in a vast number of patients in a broad spectrum of malignancies, suggesting that *RASSF1A* inactivation is associated with cancer pathogenesis. However, whether this recurrent incidence of *RASSF1A* hypermethylation in human malignancies and its association with more aggressive tumour phenotype is a frequent event across different cancer types has not yet been discussed. In this review, we interrogated existing evidence for association of *RASSF1A* hypermethylation with clinicopathological characteristics that can indicate more invasive lesions.

One of the greatest challenges facing modern oncology is the development of biomarkers that will improve prognostication as well as prediction for the use of targeted therapies. Adequate biomarkers that define the molecular complexity of cancer could improve both diagnosis and treatment, leading to significant advances in cancer patient care. It has long been envisioned that such biomarkers will help distinguish between indolent and aggressive cancers which, in an advent of improved cancer screening, will become increasingly important with greater success in identification of earlier low-grade tumours. Molecular biomarkers that enable the sensing of malignant transformation and cancer progression will undoubtedly have strong potential as prognostic biomarkers and could lead to improvements in cancer screening and management strategies for cancer patients. However, surprisingly few such biomarkers are currently available or are in development following solid clinical confirmation. Here we review the clinical evidence for one strong emerging candidate biomarker, *RASSF1A*, that has been implicated across all major solid tumours as a prognostic marker for poor survival and is showing signs of predictive power to certain treatments (Hesson *et al*, 2007). Interestingly, given the current concentration on screening of patients for genomic mutations, this is an epigenetic event indicating the potential for more comprehensive analysis of patient material in providing biomarker delivery.

RASSF1A is one of the most frequently epigenetically inactivated tumour-suppressor genes in sporadic human malignancies (Donninger *et al*, 2007; Hesson *et al*, 2007; Van der Weyden and Adams, 2007). As a component of key cancer pathways, namely Ras/PI3K/AKT, Ras/RAF/MEK/ERK and Hippo pathways, inactivation of *RASSF1A* is an important factor contributing to pathogenesis and progression of solid tumours (Guo *et al*, 2007; Van der Weyden and Adams, 2007). Originally discovered in the search for a tumour suppressor on chromosome 3p21, subsequent analysis found that epigenetic inactivation of the RASSF1 promoter region by DNA methylation was more widespread in lung cancer than loss of heterozygosity (Kok *et al*, 1987; Dammann *et al*, 2000). Methylation of the *RASSF1A* gene is rare in normal tissues, whereas the frequency of methyl-cytosine in the promoter spanning CpG island increases in tumour tissue and is one of the highest described, leading to multiple correlations of the biomarker with increased risk of lung cancer (Donninger *et al*, 2007).

High frequencies of *RASSF1A* promoter 'hypermethylation' have subsequently been reported in a number of different malignancies. *RASSF1A* hypermethylation frequency ranges up to 99% in tumours compared with 0% in normal surrounding tissue, with the highest frequencies of up to 88, 95 and 99% being reported in lung, breast and prostate cancers, respectively (reviewed in Donninger *et al*, 2007). The high frequency of *RASSF1A* promoter methylation has also been associated with cancer pathogenesis and more aggressive clinical phenotype. Additionally, a number of studies have successfully demonstrated that *RASSF1A* methylation status can be derived from cell-free circulating tumour DNA (ctDNA; Wang *et al*, 2007; Chan *et al*, 2008; Göbel *et al*, 2011; Ponomaryova *et al*, 2013). ctDNA offers an alternative diagnostic material for clinical use as it is more readily accessible for analysis than tumour material. Together, *RASSF1A* methylation status holds a strong potential for clinical utility as an attractive biomarker for cancer risk and prognosis.

For the purpose of this review, we selected studies with cohort sizes of $\geqslant 50$ patients which reported any clinicopathological

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features associated with *RASSF1A* methylation. We categorised different clinicopathological features such as (i) cancer risk (assessed in case–control studies only), (ii) advanced stage and/or grade, (iii) local recurrence or distal metastasis, (iv) poor overall survival and (v) poor disease-free survival. Up until December 2014, 76 studies in 11 different cancer types, inclusive of 8 meta-analyses, reported clinical significance of *RASSF1A* promoter hypermethylation (Table 1).

BREAST CANCER

A total of 8 individual reports and 1 meta-analysis of 1759 breast cancer patients lend strong support to *RASSF1A* promoter hypermethylation involvement in the tumourogenesis of breast cancer (Shinozaki *et al*, 2005; Bagadi *et al*, 2008; Euhus *et al*, 2008; Karray-Chouayekh *et al*, 2010; Buhmeida *et al*, 2011; Göbel *et al*, 2011; Jiang *et al*, 2012; Wang *et al*, 2012; Xu *et al*, 2012; Stuopelytė *et al*, 2013; Hagrass *et al*, 2014). The study of tumour-suppressor gene methylation frequency, including *RASSF1A*, in benign and malignant tissues of 69 breast cancer patients and breast tissues of 95 unaffected women by Euhus *et al* (2008), demonstrated that promoter methylation of *RASSF1A* is the most frequent among all tumour-suppressor genes tested and correlates with increased breast cancer risk (odds ratio (OR) 5.28), indicating that assessment of *RASSF1A* promoter methylation in benign tissues could improve breast cancer risk stratification.

Metastasis is a primary cause of death in around 90% of cancer patients (Mehlen and Puisieux, 2006), therefore it is of great importance to identify clinically relevant biomarkers that can identify groups of patients with high risk of metastatic disease. Strikingly, *RASSF1A* hypermethylation is strongly associated with poor prognosis and adverse cancer outcome in 7 individual studies and one meta-analysis of 1795 cases. Specifically, breast tumours with inactivated *RASSF1A* associated with advanced stage (Karray-Chouayekh *et al*, 2010; Hagrass *et al*, 2014), lymph node metastasis (Bagadi *et al*, 2008; Hagrass *et al*, 2014), higher risk of recurrence (Jiang *et al*, 2012), shorter progression-free survival (Buhmeida *et al*, 2011; Göbel *et al*, 2011; Xu *et al*, 2012) and poor overall survival (Karray-Chouayekh *et al*, 2010; Göbel *et al*, 2011; Jiang *et al*, 2012; Wang *et al*, 2012; Xu *et al*, 2012). Together, this evidence strongly suggests that epigenetic inactivation of the *RASSF1A* gene is a critical event in progression of breast cancer and that *RASSF1A* promoter methylation could serve as a biomarker for more aggressive breast tumours with high risk of metastasis. Additionally, evidence exists suggesting that *RASSF1A* methylation could be utilised in the clinic for monitoring response to adjuvant therapy, whereby depletion of *RASSF1A* methylation in ctDNA has been associated with good response to adjuvant regimens (Fiegl *et al*, 2005; Avraham *et al*, 2012).

Oestrogen receptor (ER) status is one of the most important prognostication factors in breast cancer, whereby ER-positive tumours are considered less aggressive (Reis-Filho and Pusztai, 2011). Interestingly, a study of 193 breast cancer patients by Xu *et al* (2012), which reported association of *RASSF1A* methylation with poor progression-free and overall survival, demonstrated that higher median *RASSF1A* methylation was observed in ER- and progesterone receptor (PR)-positive tumours. Similarly, in a study of 72 breast cancer patients Stuopelytė *et al* (2013) reported that *RASSF1A* methylation is more frequent in less aggressive, ER-positive tumours of low grade and with low proliferative potential. The prevalence of *RASSF1A* hypermethylation in hormone receptor-positive tumours was also reported in relatively larger breast cancer studies of 151 and 765 patients (Shinozaki *et al*, 2005; Cho *et al*, 2012); however, no clinical associations that would indicate more aggressive phenotype in tumours with hypermethylated *RASSF1A* were found in these patient cohorts. Inactivation of *RASSF1A* and its association with more aggressive phenotype is not restricted to hormone receptor-positive breast tumours. In a study of 120 patients, Hagrass *et al* (2014) reported that *RASSF1A* methylation associates with invasive carcinoma, advanced stage and lymph node metastasis in ER-, PR- and HER2-negative breast tumours. Therefore, further investigation in much larger patient cohorts is needed to better understand the possible interaction of *RASSF1A* inactivation with hormone receptor status and prognosis.

Taken together, the body of evidence gives strong support to the hypothesis that inactivation of *RASSF1A* in breast tumours leads to more aggressive phenotype, likely independent of hormone receptor status, and it can be speculated that RASFF1A hypermethylation could identify a subgroup of ER-positive breast cancer patients with more aggressive tumours with a high risk of metastasis.

LUNG CANCER

Apparent correlation of *RASSF1A* methylation with clinical characteristics of invasive tumours is also evident in lung cancer (Table 1). Association of high levels of *RASSF1A* promoter methylation with cancer risk has been demonstrated in two independent clinical studies (Hsu *et al*, 2007; Li *et al*, 2012) and one meta-analysis of 2008 cases (Huang *et al*, 2014). *RASSF1A* methylation associates with elevated risk of lung cancer with reported OR ranging from 7.5, in a study of 56 lung cancer cases and 52 healthy controls, through OR 9.9 in a study of 63 non-small cell lung patients and 36 controls, to OR 16.2 reported in a meta-analysis of 2008 cases and 1239 controls (Hsu *et al*, 2007; Li *et al*, 2012; Huang *et al*, 2014).

Lung tumours with hypermethylated *RASSF1A* methylation are poorly differentiated (Tomizawa *et al*, 2002; Wang *et al*, 2007) and associate with advanced stage (Wang *et al*, 2007; Lee *et al*, 2012) and local recurrence (Tomizawa *et al*, 2002; Endoh *et al*, 2003; Kubo *et al*, 2009; Buckingham *et al*, 2010). Similarly to breast cancer, a strong body of evidence supports an association of *RASSF1A* hypermethylation with adverse outcome of lung cancer, whereby 8 independent studies (Burbee *et al*, 2001; Kim *et al*, 2003a, 2003b; Wang *et al*, 2004; Fischer *et al*, 2007; Yanagawa *et al*, 2007; De Fraipont *et al*, 2012) and a meta-analysis of a total of 2802 lung cancer patients (Wang *et al*, 2011) demonstrate significantly shorter overall survival in those patients whose tumours had inactivated *RASSF1A* by promoter methylation. Additionally, poor progression-free survival of patients with hypermethylated *RASSF1A* was demonstrated in two independent studies of non-small cell lung cancer patients (De Fraipont *et al*, 2012; Ko *et al*, 2013).

Although some studies included more aggressive small cell lung carcinomas (Wang *et al*, 2007; Kubo *et al*, 2009), the majority of reports were wholly conducted in non-small cell lung carcinoma (NSCLC) specimens (Burbee *et al*, 2001; Tomizawa *et al*, 2002; Endoh *et al*, 2003; Kim *et al*, 2003a, 2003b; Wang *et al*, 2004, 2011; Yanagawa *et al*, 2007; Buckingham *et al*, 2010; Ko *et al*, 2013). Therefore, it could be speculated that *RASSF1A* methylation may be a good predictor of non-small cell lung cancer outcome as it could contribute to identification of a subset of more aggressive tumours that progress to metastatic disease. Intriguingly, *RASSF1A* methylation has been reported as a good predictor of response to chemotherapy, whereby Fischer *et al* (2007) reported in the study of 92 NSCLC patients treated with gemcitabine that *RASSF1A* hypermethylation is a good predictor of overall survival, as those patients who demonstrated partial response to the administered chemotherapy and had tumours with hypermethylated *RASSF1A*

Table 1. Clinical associations of *RASSF1A* promoter methylation

		Clinicopathological associations of *RASSF1A* hypermethylation						
Cancer type	**Cohort size**	**Risk**	**Advanced stage and/or high grade**	**Local recurrence or distal metastasis**	**Poor overall survival**	**Poor disease-free survival**	**Other**	**Reference**
Bladder cancer	55		•					Lee *et al*, 2001
	98		•	•				Maruyama *et al*, 2001
	58		•	•				Jarmalaite *et al*, 2008
	543[a]	•	•					Gao *et al*, 2012
	101			•				Ha *et al*, 2012
	115		•			•		Kim *et al*, 2012
	64			•				Meng *et al*, 2012
Brain cancer	63		•					Hesson *et al*, 2004
	56				•			Yang *et al*, 2004
	52		•					Qian *et al*, 2005
	71		•		•			Stutterheim *et al*, 2012
Breast cancer	69	•						Euhus *et al*, 2008
	54			•				Bagadi *et al*, 2008
	78		•		•			Karray-Chouayekh *et al*, 2010
	100					•		Buhmeida *et al*, 2011
	428				•	•		Göbel *et al*, 2011
	1795[a]			•	•			Jiang *et al*, 2012
	65				•			Wang *et al*, 2012
	193				•	•	ER/PR + ve tumours	Xu *et al*, 2012
	120		•	•			ER/PR/HER2 − ve tumours	Hagrass *et al*, 2014
Gastrointestinal cancer	63					•		Chan *et al*, 2008
	97		•	•	•			Honda *et al*, 2008
	92		•					Guo *et al*, 2009
	56				•			Arai *et al*, 2010
	124		•	•				Mao *et al*, 2011
	141			•	•			Yao *et al*, 2012
	62		•	•				Sinha *et al*, 2013
	228	•						Zhou *et al*, 2013
	74			•	•	•		Honda *et al*, 2013
	1205[a]	•						Li *et al*, 2014
	1215[a]	•						Shi *et al*, 2014
	630[a]	•						Wang *et al*, 2014a
	1505[a]		•	•				Wang *et al*, 2014b
Gynecological cancer	70		•	•				Jo *et al*, 2006

Table 1. (Continued)

		Clinicopathological associations of *RASSF1A* hypermethylation						
Cancer type	**Cohort size**	**Risk**	**Advanced stage and/or high grade**	**Local recurrence or distal metastasis**	**Poor overall survival**	**Poor disease-free survival**	**Other**	**Reference**
	76		•					Liao *et al*, 2008
	60		•					Neyaz *et al*, 2008
	62		•					Pallarés *et al*, 2008
	110				•			Mitra *et al*, 2012
Head and neck cancer	60		•	•				Li *et al*, 2005
	50				•			Ghosh *et al*, 2008
	69				•			Lee *et al*, 2008
	68		•	•			Early age of onset	Fendri *et al*, 2009
	482					•		Huang *et al*, 2009
	189		•					Yang *et al*, 2014
	167		•		•	•		Zhang *et al*, 2014
Lung cancer	107				•			Burbee *et al*, 2001
	110			•			Poor differentiation	Tomizawa *et al*, 2002
	100			•				Endoh *et al*, 2003
	242				•			Kim *et al*, 2003a
	204				•			Kim *et al*, 2003b
	119				•			Wang *et al*, 2004
	92				•			Fischer *et al*, 2007
	63	•						Hsu *et al*, 2007
	70		•				Poor differentiation	Wang *et al*, 2007
	101				•			Yanagawa *et al*, 2007
	100			•				Kubo *et al*, 2009
	132			•				Buckingham *et al*, 2010
	2802[a]				•			Wang *et al*, 2011
	528				•	•		De Fraipont *et al*, 2012
	206		•		•			Lee *et al*, 2012
	56	•						Li *et al*, 2012
	328					•		Ko *et al*, 2013
	2008[a]	•						Huang *et al*, 2014
Melanoma	122		•					Tanemura *et al*, 2009
Prostate cancer	52		•					Liu *et al*, 2002
	101		•					Maruyama *et al*, 2002
	118		•					Jerónimo *et al*, 2004
	131		•					Kawamoto *et al*, 2007

Table 1. (Continued)

Cancer type	Clinicopathological associations of *RASSF1A* hypermethylation						Reference
	Cohort size	Risk	Advanced stage and/or high grade	Local recurrence or distal metastasis	Poor overall survival	Poor disease-free survival	Other	
	219		•					Liu *et al*, 2011
	253			•				Daniunaite *et al*, 2014
	1123[a]		•					Ge *et al*, 2014
	71			•				Litovkin *et al*, 2014
Renal cancer	179		•		•			Kawai *et al*, 2010
	84				•	•		Ohshima *et al*, 2012
Sarcoma	84				•			Seidel *et al*, 2005
	105				•			Danielsen *et al*, 2014

Abbreviations: ER = oestrogen receptor; HER = human epidermal growth factor receptor 2; PR = progesterone receptor.
[a]Meta-analysis.

had significantly longer survival time. Additionally, De Fraipont *et al* (2012) in a study of 528 NSCLC patients treated with either gemcitabine or paclitaxel demonstrated significant differences in disease-free survival of patients whose tumours had methylated *RASSF1A*, whereby those patients who received paclitaxel chemotherapy had longer survival than those patients who were treated with gemcitabine. Altogether, the vast clinical evidence presented in lung cancer studies lends strong support to the clinical utility of *RASSF1A* methylation.

GASTROINTESTINAL CANCER

Correlation of *RASSF1A* methylation with cancer risk is best validated in gastrointestinal (GI) cancer. Zhou *et al* (2013) in a study of 112 oesophageal squamous cell carcinomas (ESCC), 116 gastric cardia adenocarcinomas (GCA) and 235 normal controls reported that *RASSF1A* promoter methylation associates with 5.9 OR of development of ESCC and 7.5 OR for GCA. This association has been recently corroborated in three different meta-analyses in 1205 liver (Li *et al*, 2014), 1215 gastric (Shi *et al*, 2014) and 630 colorectal (Wang *et al*, 2014a) tumours, indicating that *RASSF1A* methylation is strongly associated with the pathogenesis of GI cancer (Li *et al*, 2014; Shi *et al*, 2014; Wang *et al*, 2014a). Nonetheless, the role of epigenetic inactivation of *RASSF1A* does not restrict to the onset of GI malignancies. Honda *et al* (2008) in a study of 97 hepatoblastoma patients demonstrated that *RASSF1A* methylation is an independent predictor of outcome in both early- and advanced-stage patients, suggesting that *RASSF1A* inactivation associates with a more aggressive tumour phenotype. Altogether, four independent studies in liver cancer and one in gastric cancer demonstrated that *RASSF1A* hypermethylation is linked with poor disease-free (Chan *et al*, 2008; Honda *et al*, 2013) and overall survival (Honda *et al*, 2008; Arai *et al*, 2010; Yao *et al*, 2012). Furthermore, the liver and gastric malignancies with inactivated *RASSF1A* appear to have more clinicopathological characteristics that indicate more aggressive phenotype, such as advanced stage (Honda *et al*, 2008; Guo *et al*, 2009), lymph node involvement (Yao *et al*, 2012) and metastasis (Honda *et al*, 2008, 2013). There are no reports to date on the correlation of *RASSF1A* promoter methylation with the outcome of colorectal and oesophageal cancer; however, the body of evidence suggests that inactivation of *RASSF1A*, similar to liver and gastric lesions, is an adequate clinical marker of more invasive colorectal and oesophageal tumours with advanced stage, high grade, regional lymph involvement and distant metastases (Mao *et al*, 2011; Sinha *et al*, 2013; Wang *et al*, 2014b).

OTHER CANCERS

Association of *RASSF1A* hypermethylation with cancer risk, beyond breast, GI and lung malignancies, as discussed above, has been also described in bladder cancer (Gao *et al*, 2012). Gao *et al* (2012) in a meta-analysis of 543 cases and 217 controls pooled from 10 different studies reported an increased risk of bladder cancer with OR of 7.29 in tumours with hypermethylated *RASSF1A*. Further evidence to support *RASSF1A* hypermethylation as a marker of accelerated tumourogenesis comes from a study of 68 nasopharyngeal carcinomas by Fendri *et al* (2009), whereby the authors reported an early age of onset of those patients whose tumours had hypermethylated *RASSF1A*.

A strong association of *RASSF1A* with more invasive characteristics of tumours has been noted in prostate cancer, whereby tumours with *RASSF1A* promoter methylation associate with high Gleason and PSA scores, advanced stage in five independent studies (Liu *et al*, 2002, 2011; Maruyama *et al*, 2002; Jerónimo *et al*, 2004;

www.bjcancer.com | DOI:10.1038/bjc.2015.221

Kawamoto *et al*, 2007) and in a meta-analysis of 1123 cases (Ge *et al*, 2014). Recently, higher risk of biochemical recurrence has also been described in association with *RASSF1A* hypermethylation in prostate cancer (Daniunaite *et al*, 2014; Litovkin *et al*, 2014).

RASSF1A methylation has been linked to advanced stage and high grade tumours of bladder (Lee *et al*, 2001; Maruyama *et al*, 2001; Jarmalaite *et al*, 2008; Gao *et al*, 2012; Kim *et al*, 2012), endometrium (Jo *et al*, 2006; Liao *et al*, 2008; Pallarés *et al*, 2008), cervix (Neyaz *et al*, 2008), head and neck (Li *et al*, 2005; Fendri *et al*, 2009; Yang *et al*, 2014; Zhang *et al*, 2014), melanoma (Tanemura *et al*, 2009), kidney (Kawai *et al*, 2010) and brain tumours, such as glioma (Hesson *et al*, 2004), neuroblastoma (Stutterheim *et al*, 2012) and pituitary adenomas (Qian *et al*, 2005). Some of these tumours with advanced stage or high grade and hypermethylated *RASSF1A*, such as bladder (Maruyama *et al*, 2001; Jarmalaite *et al*, 2008; Meng *et al*, 2012), endometrium (Jo *et al*, 2006) and head and neck (Li *et al*, 2005; Fendri *et al*, 2009) are also associated with local and distal metastases, whereas neuroblastomas (Yang *et al*, 2004; Stutterheim *et al*, 2012), bladder (Kim *et al*, 2012), head and neck (Ghosh *et al*, 2008; Lee *et al*, 2008; Zhang *et al*, 2014), kidney (Kawai *et al*, 2010; Ohshima *et al*, 2012) and cervical tumours (Mitra *et al*, 2012) associate with shorter overall survival. Additionally, *RASSF1A* hypermethylation as a prognostic marker of poor outcome has been also reported in sarcomas (Seidel *et al*, 2005; Danielsen *et al*, 2014). Together, these studies lend strong support to the use of *RASSF1A* hypermethylation as a prognostic biomarker of poor outcome and indicate that inactivation of *RASSF1A* has a key role in cancer progression. Indeed, studies in head and neck and renal cancers demonstrate that those tumours with high levels of *RASSF1A* methylation not only have poor outcome but progress to metastatic disease significantly faster than other tumours (Huang *et al*, 2009; Ohshima *et al*, 2012; Zhang *et al*, 2014).

RASSF1A POLYMORPHIC VARIANT A133S

Germ-line biomarkers, such as single-nucleotide polymorphisms (SNPs), similar to biomarkers derived from ctDNA are derived from stable and more readily accessible material and offer a promising clinical utility. Genetic screening using phenotype-specific SNP panels for retinal degradation has already been clinically validated and offers low-cost, high-quality molecular diagnoses (Katsanis and Katsanis, 2013). SNP of *RASSF1A* A133S alters the activity of *RASSF1A* and has been associated with increased risk of gastric cardia adenocarcinoma (Zhou *et al*, 2013), hepatocellular carcinoma (Bayram, 2012) lung adenocarcinoma (Kanzaki *et al*, 2006) early age of onset of breast cancer (Gao *et al*, 2008) and soft tissue sarcomas (Yee *et al*, 2012). Additionally, the polymorphic variant of *RASSF1A* negatively affects overall survival of soft tissue sarcomas (Yee *et al*, 2012) and accelerates progression of clear cell renal cell carcinoma (Kawai *et al*, 2012). Thus it is likely that inherited polymorphisms of *RASSF1A* could be used in combination with epigenetic inactivation of *RASSF1A* to better define patient populations at different risk of particular cancers.

SUMMARY AND CONCLUSIONS

Evidence for some of the noted clinical associations of *RASSF1A* methylation comes only from single cohort studies, and further investigation in large cohort studies is needed for validation. Additionally, clinical evidence on the impact of *RASSF1A* inactivation on risk and outcome of tumours with high frequency of *RASSF1A* methylation, such as pancreatic tumours, is lacking. Nonetheless, association of *RASSF1A* promoter methylation with one or more clinicopathological characteristics has been validated in at least two independent studies for as many as 10 types of malignancies out of a total of 11 different cancer types that had been linked with *RASSF1A* promoter methylation (Table 2).

RASSF1A hypermethylation has been associated with cancer risk in a number of malignancies, suggesting its utility in monitoring premalignant tissues. However, existing evidence demonstrates that *RASSF1A* methylation status as a marker for cancer susceptibility is most likely to find its use in detection of early-stage GI and lung cancers (Table 2).

To explore the potential of *RASSF1A* hypermethylation as a candidate biomarker for aggressive tumours with poor outcome, we explored existing literature for any associations of *RASSF1A* epigenetic inactivation with clinical indicators of such phenotype, including poor overall survival and poor disease-free survival as well as advanced stage and/or grade and local recurrence and/or distal metastasis. Association of *RASSF1A* hypermethylation with adverse outcome has been substantiated in seven different types of malignancies, namely, brain, breast, GI, head and neck, lung and renal cancers and sarcomas (Table 2). The evidence is particularly strong in breast and lung cancers where as many as five independent reports in breast cancer cohorts and nine in lung cancer cohorts described *RASSF1A* hypermethylation as an independent predictor of cancer outcome. Additionally, high levels of *RASSF1A* methylation in breast, lung, GI and head and neck lesions has been also associated with shorter progression-free survival, suggesting that inactivation of *RASSF1A* has an important role in progression to the metastatic disease.

Advanced stage or high tumour grade, and particularly the presence of local and distant metastases at the time of diagnosis, are good indicators of the invasive potential of primary tumours. Indeed, associations with these indicators and inactivation of *RASSF1A* were reported in five out of the seven cancers where *RASSF1A* hypermethylation associated with adverse prognosis, with only three cancers lacking clear significant associations of *RASSF1A* hypermethylation with poor survival (Table 2).

The evidence discussed in this review gives strong support to the utility of *RASSF1A* promoter methylation as a biomarker for cancer risk as well as more invasive malignancies with poor outcome. Nonetheless, a number of reports in breast (Shinozaki *et al*, 2005; Cho *et al*, 2012), gynaecological (Pan *et al*, 2009; Montavon *et al*, 2012), GI (Kim *et al*, 2009; Okamoto *et al*, 2011) and lung (Safar *et al*, 2005; Chen *et al*, 2006; Brock *et al*, 2008; Niklinska, 2009) malignancies fail to identify any significant clinical association with *RASSF1A* promoter methylation. For instance, Niklinska *et al* (2009), in a study of 70 NSCLC patients did not find any associations of *RASSF1A* hypermethylation with overall survival. Similarly, advanced stage and lymph node metastases have been reported in GI malignancies (Table 1), including oesophageal cancer (Mao *et al*, 2011); however, Kim *et al* (2009) did not detect any significant association with *RASSF1A* hypermethylation in 50 oesophageal patients. Epigenetic inactivation of tumour-suppressor genes is a frequent event in human malignancies (Jones and Baylin, 2002). Indeed, methylation status of a number of other classic tumour-suppressor genes has been also extensively investigated, often in conjunction with *RASSF1A* gene methylation. Interestingly, in the above-mentioned study by Kim *et al* (2009), *RASSF1A* methylation was relatively low at 14%, whereas the *APC* gene, with observed methylation frequency of 46%, was identified as an independent predictor of outcome in the investigated cohort. Intriguingly, Safar *et al* (2005) in a study of clinical association with methylation status of a panel of 8 genes in the 105 NSCLC patients revealed that, although methylation of individual genes, including *RASSF1A*, cannot be used as independent predictors of outcome, combined methylation status of *RASSF1A*, *APC* and *ATM* stratifies patients into groups with

Table 2. Summary of clinical associations of *RASSF1A* promoter methylation

Cancer type	Bladder cancer	Brain cancer	Breast cancer	Gastrointestinal cancer	Gynecological cancer	Head and neck cancer	Lung cancer	Prostate cancer	Renal cancer	Sarcoma
Clinicopathological associations of RASSF1 hypermethylation										
Risk				+ + + +			+ + +			
Advanced stage and/or high grade	+ + + +	+ + +	+ +	+ + + +	+ + + +	+ + + +	+ + +	+ + + + (6)		
Local recurrence or distal metastasis	+ + +		+ + +	+ + + + (6)		+ +	+ + +	+ +		
Poor overall survival		+ +	+ + + (5)	+ + + +		+ + +	+ + + + (9)		+ +	+ +
Poor disease-free survival			+ + +	+ +		+ +	+ +			

Association reported in: + +, 2 studies; + + +, 3 studies; + + + +, ⩾4 studies.

different clinical outcomes. Furthermore, the possibility exists that utility of *RASSF1A* methylation as an independent biomarker for cancer risk and outcome could be affected by Ras activation; evidence suggests that inactivation of *RASSF1A* is mutually exclusive with K-Ras mutation, where tumours with methylated *RASSF1A* had predominantly wt K-Ras (Dammann *et al*, 2003; Miranda *et al*, 2006; Cao *et al*, 2013). However, other reports demonstrate no correlation between *RASSF1A* inactivation and K-Ras mutation (Liu *et al*, 2007; Pijnenborg *et al*, 2007). More clinical studies are therefore needed to determine the association of Ras pathway deregulation with *RASSF1A* inactivation and its potential as a cancer biomarker.

Although it is possible that underlying differences in molecular composition and origin of malignancies might determine whether inactivation of *RASSF1A* can be a suitable predictor of clinical outcome, substantial variability exists in the definition of 'methylated' *vs* 'non-methylated' calls, which may also affect the power and consistency. Variable methylation positivity of individual CG sites within relatively large CpG island locus of *RASSF1A* promoter and different methods used in many studies to assess DNA methylation status pose a significant hurdle that is likely to contribute to some inconsistency in the reported results.

In order to validate *RASSF1A* hypermethylation as an effective biomarker for cancer diagnostics, it is vital to clarify those CpG sites that contribute to the clinical phenotype across all tumour types. Given the substantial evidence outlined above, a definitive understanding of the true epigenetic signal at the *RASSF1A* promoter will undoubtedly improve the associations and be of great clinical benefit, potentially as the first broad pan-cancer biomarker of advanced disease. Altogether, the body of evidence suggests that epigenetic inactivation of the *RASSF1A* gene strongly associates with tumorigenesis and cancer risk and is a good candidate biomarker that could be utilised for diagnostic and therapeutic purposes.

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