Clinical utility of *RASSF1A* methylation in human malignancies

A M Grawenda[1] and E O'Neill[*,1]

[1]CRUK/MRC Oxford Institute, Department of Oncology, University of Oxford, Oxford, UK

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 ⩾50 patients which reported any clinicopathological

---

*Correspondence: Professor E O'Neill; E-mail: Eric.oneill@oncology.ox.ac.uk

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

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
							Clinicopathological associations of RASSF1A hypermethylation	
Bladder cancer	55		•					Lee et al, 2001
	98		•	•				Maruyama et al, 2001
	58		•	•				Jarmalaite et al, 2008
	543ᵃ	•	•					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
	1795ᵃ			•	•			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
	1205ᵃ	•						Li et al, 2014
	1215ᵃ	•						Shi et al, 2014
	630ᵃ	•						Wang et al, 2014a
	1505ᵃ		•	•				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)

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
	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;

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.

REFERENCES

Arai Y, Honda S, Haruta M, Kasai F, Fujiwara Y, Ohshima J, Sasaki F, Nakagawara A, Horie H, Yamaoka H, Hiyama E, Kaneko Y (2010) Genome-wide analysis of allelic imbalances reveals 4q deletions as a poor prognostic factor and MDM4 amplification at 1q32.1 in hepatoblastoma. *Genes Chromosomes Cancer* **49**: 596–609.

Avraham A, Uhlmann R, Shperber A, Birnbaum M, Sandbank J, Sella A, Sukumar S, Evron E (2012) Serum DNA methylation for monitoring response to neoadjuvant chemotherapy in breast cancer patients. *Int J Cancer* **131**: E1166–E1172.

Bagadi SAR, Prasad CP, Kaur J, Srivastava A, Prashad R, Gupta SD, Ralhan R (2008) Clinical significance of promoter hypermethylation of RASSF1A, RARbeta2, BRCA1 and HOXA5 in breast cancers of Indian patients. *Life Sci* **82**: 1288–1292.

Bayram S (2012) RASSF1A Ala133Ser polymorphism is associated with increased susceptibility to hepatocellular carcinoma in a Turkish population. *Gene* **498**: 264–269.

Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glöckner S, Piantadosi S, Gabrielson E, Pridham G, Pelosky K, Belinsky Sa, Yang SC, Baylin SB, Herman JG (2008) DNA methylation markers and early recurrence in stage I lung cancer. *N Engl J Med* **358**: 1118–1128.

Buckingham L, Penfield Faber L, Kim A, Liptay M, Barger C, Basu S, Fidler M, Walters K, Bonomi P, Coon J (2010) PTEN, RASSF1 and DAPK site-specific hypermethylation and outcome in surgically treated stage I and II nonsmall cell lung cancer patients. *Int J Cancer* **126**: 1630–1639.

Buhmeida A, Merdad A, El-Maghrabi J, Al-Thobaiti F, Ata M, Bugis A, Syrjänen K, Abuzenadah A, Chaudhary A, Gari M, Al-Qahtani M, Dallol A (2011) RASSF1A methylation is predictive of poor prognosis in female breast cancer in a background of overall low methylation frequency. *Anticancer Res* **31**: 2975–2981.

Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar AF, Lerman MI, Zabarovsky E, White M, Minna JD

(2001) Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* **93**: 691–699.

Cao D, Chen Y, Tang Y, Peng XC, Dong H, Li LH, Cheng K, Ge J, Liu JY (2013) Loss of RASSF1A expression in colorectal cancer and its association with k-ras status. *Biomed Res Int* **2013**: 1–8.

Chan K, Lai P, Mok T, Chan HL, Ding C, Yeung SW, Lo YM (2008) Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. *Clin Chem* **54**: 1528–1536.

Chen H, Suzuki M, Nakamura Y, Ohira M, Ando S, Iida T, Nakajima T, Nakagawara A, Kimura H (2006) Aberrant methylation of RASGRF2 and RASSF1A in human non-small cell lung cancer. *Oncol Rep* **15**: 1281–1285.

Cho YH, Shen J, Gammon MD, Zhang Y-J, Wang Q, Gonzalez K, Xu X, Bradshaw PT, Teitelbaum SL, Garbowski G, Hibshoosh H, Neugut AI, Chen J, Santella RM (2012) Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. *Breast Cancer Res Treat* **131**: 197–205.

Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP (2000) Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* **25**: 315–319.

Dammann R, Schagdarsurengin U, Liu L, Otto N, Gimm O, Dralle H, Boehm BO, Pfeifer GP, Hoang-Vu C (2003) Frequent RASSF1A promoter hypermethylation and K-ras mutations in pancreatic carcinoma. *Oncogene* **22**: 3806–3812.

Danielsen SA, Lind GE, Kolberg M, Høland M, Bjerkehagen B, Sundby Hall K, van den Berg E, Mertens F, Smeland S, Picci P, Lothe RA (2014) Methylated RASSF1A in malignant peripheral nerve sheath tumors identifies neurofibromatosis type 1 patients with inferior prognosis. *Neuro Oncol* **17**(1): 63–69.

Daniunaite K, Jarmalaite S, Kalinauskaite N, Petroska D, Laurinavicius A, Lazutka JR, Jankevicius F (2014) Prognostic value of RASSF1 promoter methylation in prostate cancer. *J Urol* **192**(6): 1849–1855.

De Fraipont F, Levallet G, Creveuil C, Bergot E, Beau-Faller M, Mounawar M, Richard N, Antoine M, Rouquette I, Favrot MC, Debieuvre D, Braun D, Westeel V, Quoix E, Brambilla E, Hainaut P, Moro-Sibilot D, Morin F, Milleron B, Zalcman G (2012) An apoptosis methylation prognostic signature for early lung cancer in the IFCT-0002 trial. *Clin Cancer Res* **18**: 2976–2986.

Donninger H, Vos M, Clark G (2007) The RASSF1A tumor suppressor. *J Cell Sci* **120**(Pt 18): 3163–3172.

Endoh H, Yatabe Y, Shimizu S, Tajima K, Kuwano H, Takahashi T, Mitsudomi T (2003) RASSF1A gene inactivation in non-small cell lung cancer and its clinical implication. *Int J Cancer* **106**: 45–51.

Euhus DM, Bu D, Milchgrub S, Xie X-J, Bian A, Leitch AM, Lewis CM (2008) DNA methylation in benign breast epithelium in relation to age and breast cancer risk. *Cancer Epidemiol Biomarkers Prev* **17**: 1051–1059.

Fendri A, Masmoudi A, Khabir A, Sellami-Boudawara T, Daoud J, Frikha M, Ghorbel A, Gargouri A, Mokdad-Gargouri R (2009) Inactivation of RASSF1A, RARβ2 and DAP-kinase by promoter methylation cosrrelates with lymph node metastasis in nasopharyngeal carcinoma. *Cancer Biol* **8**: 444–451.

Fiegl H, Millinger S, Mueller-holzner E, Marth C, Ensinger C, Berger A, Klocker H, Goebel G, Widschwendter M (2005) Circulating tumor-specific DNA: a marker for monitoring efficacy of adjuvant therapy in cancer patients. *Cancer Res* (2005) **65**(4): 1141–1145.

Fischer JR, Ohnmacht U, Rieger N, Zemaitis M, Stoffregen C, Manegold C, Lahm H (2007) Prognostic significance of RASSF1A promoter methylation on survival of non-small cell lung cancer patients treated with gemcitabine. *Lung Cancer* **56**: 115–123.

Gao B, Xie X, Huang C, Shames D, Chen T, Lewis C, Bian A, Zhang B, Olopade O, Garber J, Euhus D, Tomlinson G, Minna J (2008) RASSF1A polymorphism A133S is associated with early onset breast cancer in BRCA1/2 mutation carriers. *Cancer Res* **68**(1): 22–25.

Gao T, Wang S, He B, Pan Y, Song G, Gu L, Chen L, Nie Z, Xu Y, Li R (2012) The association of RAS association domain family Protein1A (RASSF1A) methylation states and bladder cancer risk: a systematic review and meta-analysis. *PLoS One* **7**: e48300.

Ge Y-Z, Xu L-W, Jia R-P, Xu Z, Feng Y-M, Wu R, Yu P, Zhao Y, Gui Z-L, Tan S-J, Song Q (2014) The association between RASSF1A promoter methylation and prostate cancer: evidence from 19 published studies. *Tumour Biol* **35**: 3881–3890.

Ghosh S, Ghosh A, Maiti GP, Alam N, Roy A, Roy B, Roychoudhury S, Panda CK (2008) Alterations of 3p21.31 tumor suppressor genes in head and neck

squamous cell carcinoma: correlation with progression and prognosis. *Int J Cancer* **123**: 2594–2604.

Göbel G, Auer D, Gaugg I, Schneitter A, Lesche R, Müller-Holzner E, Marth C, Daxenbichler G (2011) Prognostic significance of methylated RASSF1A and PITX2 genes in blood- and bone marrow plasma of breast cancer patients. *Breast Cancer Res Treat* **130**: 109–117.

Guo C, Tommasi S, Liu L, Yee JK, Dammann R, Pfeifer GP (2007) RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats Tumor-Suppressor Network. *Curr Biol* **17**: 700–705.

Guo W, Dong Z, Chen Z, Yang Z, Wen D, Kuang G, Guo Y, Shan B (2009) Aberrant CpG island hypermethylation of RASSF1A in gastric cardia adenocarcinoma. *Cancer Invest* **27**: 459–465.

Ha YS, Jeong P, Kim JS, Kwon WA, Kim IY, Yun SJ, Kim GY, Choi YH, Moon SK, Kim W (2012) Tumorigenic and prognostic significance of RASSF1A expression in low-grade (WHO grade 1 and grade 2) nonmuscle-invasive bladder cancer. *Urology* **79**: 1411.e1–e6.

Hagrass Ha, Pasha HF, Shaheen MA, Abdel Bary EH, Kassem R (2014) Methylation status and protein expression of RASSF1A in breast cancer patients. *Mol Biol Rep* **41**: 57–65.

Hesson L, Bièche I, Krex D, Criniere E, Hoang-Xuan K, Maher ER, Latif F (2004) Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. *Oncogene* **23**: 2408–2419.

Hesson LB, Cooper WN, Latif F (2007) The role of RASSF1A methylation in cancer. *Dis Markers* **23**: 73–87.

Honda S, Haruta M, Sugawara W, Sasaki F, Ohira M, Matsunaga T, Yamaoka H, Horie H, Ohnuma N, Nakagawara A, Hiyama E, Todo S, Kaneko Y (2008) The methylation status of RASSF1A promoter predicts responsiveness to chemotherapy and eventual cure in hepatoblastoma patients. *Int J Cancer* **123**: 1117–1125.

Honda S, Miyagi H, Suzuki H, Minato M, Haruta M, Kaneko Y, Hatanaka KC, Hiyama E, Kamijo T, Okada T, Taketomi A (2013) RASSF1A methylation indicates a poor prognosis in hepatoblastoma patients. *Pediatr Surg Int* **29**: 1147–1152.

Hsu H-S, Chen T-P, Hung C-H, Wen C-K, Lin R-K, Lee H-C, Wang Y-C (2007) Characterization of a multiple epigenetic marker panel for lung cancer detection and risk assessment in plasma. *Cancer* **110**: 2019–2026.

Huang K, Huang S, Chen I, Liao C, Wang H, Hsieh L (2009) Methylation of RASSF1A, RASSF2A, and HIN-1 is associated with poor outcome after radiotherapy, but not surgery, in oral squamous cell carcinoma. *Clin Cancer* **15**: 4174–4180.

Huang Y, Wu W, Wu K, Xu X, Tang W (2014) Association of RASSF1A promoter methylation with lung cancer risk: a meta-analysis. *Asian Pac J Cancer Prev* **15**: 10325–10328.

Jarmalaite S, Jankevicius F, Kurgonaite K, Suziedelis K, Mutanen P, Husgafvel-Pursiainen K (2008) Promoter hypermethylation in tumour suppressor genes shows association with stage, grade and invasiveness of bladder cancer. *Oncology* **75**: 145–151.

Jerónimo C, Henrique R, Hoque M, Mambo E, Ribeiro FR, Varzim G, Oliveira J, Teixeira MR, Lopes C, Sidransky D (2004) A quantitative promoter methylation profile of prostate cancer. *Clin Cancer* **10**: 8472–8478.

Jiang Y, Cui L, Chen W, Shen S, Ding L (2012) The prognostic role of RASSF1A promoter methylation in breast cancer: a meta-analysis of published data. *PLoS One* **7**: e36780.

Jo H, Kim J, Kang G, Park N, Song Y, Kang S, Lee H (2006) Association of promoter hypermethylation of the RASSF1A gene with prognostic parameters in endometrial cancer. *Clin Cancer Res* **16**: 205–209.

Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer. *Nat Rev Genet* **3**: 415–428.

Kanzaki H, Hanafusa H, Yamamoto H (2006) Single nucleotide polymorphism at codon 133 of the RASSF1 gene is preferentially associated with human lung adenocarcinoma risk. *Cancer Lett* **238**: 128–134.

Karray-Chouayekh S, Trifa F, Khabir A, Boujelbane N, Sellami-Boudawara T, Daoud J, Frikha M, Jlidi R, Gargouri A, Mokdad-Gargouri R (2010) Aberrant methylation of RASSF1A is associated with poor survival in Tunisian breast cancer patients. *J Cancer Res Clin Oncol* **136**: 203–210.

Katsanis SH, Katsanis N (2013) Molecular genetic testing and the future of clinical genomics. *Nat Rev Genet* **14**: 415–426.

Kawai Y, Sakano S, Okayama N, Suehiro Y, Matsuyama H, Hinoda Y (2012) Association of RASSF1A genotype and haplotype with the progression of clear cell renal cell carcinoma in Japanese patients. *BJU Int* **110**: 1070–1075.

Kawai Y, Sakano S, Suehiro Y, Okada T, Korenaga Y, Hara T, Naito K, Matsuyama H, Hinoda Y (2010) Methylation level of the RASSF1A

promoter is an independent prognostic factor for clear-cell renal cell carcinoma. *Ann Oncol* 21: 1612–1617.

Kawamoto K, Okino ST, Place RF, Urakami S, Hirata H, Kikuno N, Kawakami T, Tanaka Y, Pookot D, Chen Z, Majid S, Enokida H, Nakagawa M, Dahiya R (2007) Epigenetic modifications of RASSF1A gene through chromatin remodeling in prostate cancer. *Clin Cancer Res* 13: 2541–2548.

Kim D, Kim J, Ji Y, Shim Y, Kim H, Han J, Park J (2003a) Hypermethylation of RASSF1A promoter is associated with the age at starting smoking and a poor prognosis in primary non-small cell lung cancer. *Cancer Res* 63: 3743–3746.

Kim D, Kim J, Park J, Lee S, Ji Y, Kwon Y, Shim Y, Han J, Park J (2003b) Relationship of Ras association domain family 1 methylation and K-ras mutation in primary non-small cell lung cancer. *Cancer Res* 63: 6206–6211.

Kim JS, Chae Y, Ha Y-S, Kim IY, Byun SS, Yun S-J, Kim W-J (2012) Ras association domain family 1A: a promising prognostic marker in recurrent nonmuscle invasive bladder cancer. *Clin Genitourin Cancer* 10: 114–120.

Kim YT, Park J-Y, Jeon YK, Park SJ, Song JY, Kang CH, Sung SW, Kim JH (2009) Aberrant promoter CpG island hypermethylation of the adenomatosis polyposis coli gene can serve as a good prognostic factor by affecting lymph node metastasis in squamous cell carcinoma of the esophagus. *Dis Esophagus* 22: 143–150.

Ko E, Lee B, Bin, Kim Y, Lee EJ, Cho EY, Han J, Shim YM, Park J, Kim D-H (2013) Association of RASSF1A and p63 with poor recurrence-free survival in node-negative stage I-II non-small cell lung cancer. *Clin Cancer Res* 19: 1204–1212.

Kok K, Osinga J, Carritt B, Davis MB, van der Hout AH, van der Veen AY, Landsvater RM, de Leij LF, Berendsen HH, Postmus PE (1987) Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature* 330: 578–581.

Kubo T, Yamamoto H, Ichimura K, Jida M, Hayashi T, Otani H, Tsukuda K, Sano Y, Kiura K, Toyooka S (2009) DNA methylation in small lung adenocarcinoma with bronchioloalveolar carcinoma components. *Lung Cancer* 65: 328–332.

Lee E-S, Issa J-P, Roberts DB, Williams MD, Weber RS, Kies MS, El-Naggar AK (2008) Quantitative promoter hypermethylation analysis of cancer-related genes in salivary gland carcinomas: comparison with methylation-specific PCR technique and clinical significance. *Clin Cancer Res* 14: 2664–2672.

Lee M, Kim H, Byun D, Lee S, Lee C, Kim J, Chang S, Chi S (2001) Frequent epigenetic inactivation of RASSF1A in human bladder carcinoma. *Cancer Res* 61: 6688–6692.

Lee SM, Lee WK, Kim DS, Park JY (2012) Quantitative promoter hypermethylation analysis of RASSF1A in lung cancer: comparison with methylation-specific PCR technique and clinical significance. *Mol Med Rep* 5: 239–244.

Li J, El-Naggar A, Mao L (2005) Promoter methylation of p16INK4a, RASSF1A, and DAPK is frequent in salivary adenoid cystic carcinoma. *Cancer* 104: 771–776.

Li W, Deng J, Jiang P, Zeng X, Hu S, Tang J (2012) Methylation of the RASSF1A and RARβ genes as a candidate biomarker for lung cancer. *Exp Ther Med* 3: 1067–1071.

Li Y-S, Xie Q, Yang D-Y, Zheng Y (2014) Role of RASSF1A promoter methylation in the pathogenesis of hepatocellular carcinoma: a meta-analysis of 21 cohort studies. *Mol Biol Rep* 41: 3925–3933.

Liao X, Siu MK-Y, Chan KY-K, Wong ES-Y, Ngan HY-S, Chan QK-Y, Li AS-M, Khoo U-S, Cheung AN-Y (2008) Hypermethylation of RAS effector related genes and DNA methyltransferase 1 expression in endometrial carcinogenesis. *Int J Cancer* 123: 296–302.

Litovkin K, Joniau S, Lerut E, Laenen A, Gevaert O, Spahn M, Kneitz B, Isebaert S, Haustermans K, Beullens M, Van Eynde A, Bollen M (2014) Methylation of PITX2, HOXD3, RASSF1 and TDRD1 predicts biochemical recurrence in high-risk prostate cancer. *J Cancer Res Clin Oncol* 140(11): 1849–1861.

Liu L, Kron KJ, Pethe VV, Demetrashvili N, Nesbitt ME, Trachtenberg J, Ozcelik H, Fleshner NE, Briollais L, van der Kwast TH, Bapat B (2011) Association of tissue promoter methylation levels of APC, TGFβ2, HOXD3 and RASSF1A with prostate cancer progression. *Int J Cancer* 129: 2454–2462.

Liu L, Yoon J-H, Dammann R, Pfeifer GP (2002) Frequent hypermethylation of the RASSF1A gene in prostate cancer. *Oncogene* 21: 6835–6840.

Liu Y, Gao W, Siegfried JM, Weissfeld JL, Luketich JD, Keohavong P (2007) Promoter methylation of RASSF1A and DAPK and mutations of K-ras, p53, and EGFR in lung tumors from smokers and never-smokers. *BMC Cancer* 7: 74.

Mao W, Li P, Zheng Q, Wang C, Ge M, Hu F, Fang X, Dong Z, Ling Z (2011) Hypermethylation-modulated downregulation of RASSF1A expression is associated with the progression of esophageal cancer. *Arch Med Res* 42: 182–188.

Maruyama R, Toyooka S, Toyooka K, Harada K, Virmani S, Zöchbauer-Müller S, Farinas A, Vakar-Lopes F, Minna J, Sagalowsky A, Czerniak B, Gazdar A (2001) Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res* 61: 8659–8663.

Maruyama R, Toyooka S, Toyooka K, Virmani A, Zöchbauer-Müller S, Farinas A, Minna J, McConnell J, Frenkel E, Gazdar A (2002) Aberrant promoter methylation profile of prostate cancers and its relationship to clinicopathological features. *Clin Cancer* 8: 514–519.

Mehlen P, Puisieux A (2006) Metastasis: a question of life or death. *Nat Rev Cancer* 6: 449–458.

Meng W, Huebner A, Shabsigh A, Chakravarti A, Lautenschlaeger T (2012) Combined RASSF1A and RASSF2A promoter methylation analysis as diagnostic biomarker for bladder cancer. *Mol Biol Int* 2012: 701814.

Miranda E, Destro A, Malesci A, Balladore E, Bianchi P, Baryshnikova E, Franchi G, Morenghi E, Laghi L, Gennari L, Roncalli M (2006) Genetic and epigenetic changes in primary metastatic and nonmetastatic colorectal cancer. *Br J Cancer* 95: 1101–1107.

Mitra S, Mazumder Indra D, Basu PS, Mondal RK, Roy A, Roychoudhury S, Panda CK (2012) Alterations of RASSF1A in premalignant cervical lesions: clinical and prognostic significance. *Mol Carcinog* 51: 723–733.

Montavon C, Gloss BS, Warton K, Barton Ca, Statham AL, Scurry JP, Tabor B, Nguyen TV, Qu W, Samimi G, Hacker NF, Sutherland RL, Clark SJ, O'Brien PM (2012) Prognostic and diagnostic significance of DNA methylation patterns in high grade serous ovarian cancer. *Gynecol Oncol* 124: 582–588.

Neyaz MK, Kumar RS, Hussain S, Naqvi SH, Kohaar I, Thakur N, Kashyap V, Das BC, Husain SA, Bharadwaj M (2008) Effect of aberrant promoter methylation of FHIT and RASSF1A genes on susceptibility to cervical cancer in a North Indian population. *Biomarkers* 13: 597–606.

Niklinska W (2009) Prognostic significance of DAPK and RASSF1A promoter hypermethylation in non-small cell lung cancer (NSCLC). *Folia Histochem Cytobiol* 47: 47–48.

Ohshima J, Haruta M, Fujiwara Y, Watanabe N, Arai Y, Ariga T, Okita H, Koshinaga T, Oue T, Hinotsu S, Nakadate H, Horie H, Fukuzawa M, Kaneko Y (2012) Methylation of the RASSF1A promoter is predictive of poor outcome among patients with Wilms tumor. *Pediatr Blood Cancer* 59: 499–505.

Okamoto Y, Sawaki A, Ito S, Nishida T, Takahashi T, Toyota M, Suzuki H, Shinomura Y, Takeuchi I, Shinjo K, An B, Ito H, Yamao K, Fujii M, Murakami H, Osada H, Kataoka H, Joh T, Sekido Y, Kondo Y (2011) Aberrant DNA methylation associated with aggressiveness of gastrointestinal stromal tumour. *Gut* 61: 392–401.

Pallarés J, Velasco A, Eritja N, Santacana M, Dolcet X, Cuatrecasas M, Palomar-Asenjo V, Catasús L, Prat J, Matias-Guiu X (2008) Promoter hypermethylation and reduced expression of RASSF1A are frequent molecular alterations of endometrial carcinoma. *Mod Pathol* 21: 691–699.

Pan Z, Li J, Pan X, Chen S, Wang Z, Li F, Qu S, Shao R (2009) Methylation of the RASSF1A gene promoter in Uigur women with cervical squamous cell carcinoma. *Tumori* 95: 76–80.

Pijnenborg JMa, Dam-de Veen GC, Kisters N, Delvoux B, van Engeland M, Herman JG, Groothuis PG (2007) RASSF1A methylation and K-ras and B-raf mutations and recurrent endometrial cancer. *Ann Oncol* 18: 491–497.

Ponomaryova Aa, Rykova EY, Cherdyntseva NV, Skvortsova TE, Dobrodeev AY, Zav'yalov Aa, Bryzgalov LO, Tuzikov Sa, Vlassov VV, Laktionov PP (2013) Potentialities of aberrantly methylated circulating DNA for diagnostics and post-treatment follow-up of lung cancer patients. *Lung Cancer* 81: 397–403.

Qian ZR, Sano T, Yoshimoto K, Yamada S, Ishizuka A, Mizusawa N, Horiguchi H, Hirokawa M, Asa SL (2005) Inactivation of RASSF1A tumor suppressor gene by aberrant promoter hypermethylation in human pituitary adenomas. *Lab Invest* 85: 464–473.

Reis-Filho JS, Pusztai L (2011) Gene expression profiling in breast cancer: classification, prognostication, and prediction. *Lancet* 378: 1812–1823.

Safar A, Spencer H, Su X, Coffey M, Cooney C, Ratnasinghe L, Hutchins L, Fan C (2005) Methylation profiling of archived non-small cell lung cancer: a promising prognostic system. *Clin Cancer Res* 11: 4400–4405.

Seidel C, Bartel F, Rastetter M, Bluemke K, Wurl P, Taubert H, Dammann R (2005) Alterations of cancer-related genes in soft tissue sarcomas:

hypermethylation of RASSF1A is frequently detected in leiomyosarcoma and associated with poor prognosis in sarcoma. *Int J Cancer* **114**: 442–447.

Shi D-T, Han M, Gao N, Tian W, Chen W (2014) Association of RASSF1A promoter methylation with gastric cancer risk: a meta-analysis. *Tumour Biol* **35**: 943–948.

Shinozaki M, Hoon D, Giuliano A, Hansen N, Wang H, Turner R, Taback B (2005) Distinct hypermethylation profile of primary breast cancer is associated with sentinel lymph node metastasis. *Clin Cancer Res* **11**: 2156–2162.

Sinha R, Hussain S, Mehrotra R, Kumar RS, Kumar K, Pande P, Doval DC, Basir SF, Bharadwaj M (2013) Kras gene mutation and RASSF1A, FHIT and MGMT gene promoter hypermethylation: indicators of tumor staging and metastasis in adenocarcinomatous sporadic colorectal cancer in Indian population. *PLoS One* **8**: e60142.

Stuopelytė K, Daniūnaitė K, Laurinavičienė A, Ostapenko V, Jarmalaitė S (2013) High-resolution melting-based quantitative analysis of RASSF1 methylation in breast cancer. *Medicina (Kaunas)* **49**: 78–83.

Stutterheim J, Ichou FA, den Ouden E, Versteeg R, Caron HN, Tytgat GaM, van der Schoot CE (2012) Methylated RASSF1a is the first specific DNA marker for minimal residual disease testing in neuroblastoma. *Clin Cancer Res* **18**: 808–814.

Tanemura A, Terando A, Sim M, van Hoesel A, de Maat M, Morton D, Hoon D (2009) CpG island methylator phenotype predicts progression of malignant melanoma. *Clin Cancer Res* **15**: 1801–1807.

Tomizawa Y, Kohno T, Kondo H, Otsuka A, Nishioka M, Niki T, Yamada T, Maeshima A, Yoshimura K, Saito R, Minna J, Yokota J (2002) Clinico-pathological significance of epigenetic inactivation of RASSF1A at 3p21.3 in stage I lung adenocarcinoma. *Clin Cancer* **8**: 2362–2368.

Van der Weyden L, Adams DJ (2007) The Ras-association domain family (RASSF) members and their role in human tumourigenesis. *Biochim Biophys Acta* **1776**: 58–85.

Wang H-L, Liu P, Zhou P-Y, Zhang Y (2014a) Promoter methylation of the RASSF1A gene may contribute to colorectal cancer susceptibility: a meta-analysis of cohort studies. *Ann Hum Genet* **78**: 208–216.

Wang H-L, Zhang Y, Liu P, Zhou P-Y (2014b) Aberrant promoter methylation of RASSF1A gene may be correlated with colorectal carcinogenesis: a meta-analysis. *Mol Biol Rep* **41**: 3991–3999.

Wang J, Lee J, Wang L, Liu D, Lu C (2004) Value of p16 INK4a and RASSF1A promoter hypermethylation in prognosis of patients with resectable non-small cell lung cancer. *Clin Cancer* 6119–6125.

Wang J, Wang B, Chen X, Bi J (2011) The prognostic value of RASSF1A promoter hypermethylation in non-small cell lung carcinoma: a systematic review and meta-analysis. *Carcinogenesis* **32**: 411–416.

Wang S, Dorsey TH, Terunuma A, Kittles Ra, Ambs S, Kwabi-Addo B (2012) Relationship between tumor DNA methylation status and patient characteristics in African-American and European-American women with breast cancer. *PLoS One* **7**: e37928.

Wang Y, Yu Z, Wang T, Zhang J, Hong L, Chen L (2007) Identification of epigenetic aberrant promoter methylation of RASSF1A in serum DNA and its clinicopathological significance in lung cancer. *Lung Cancer* **56**: 289–294.

Xu J, Shetty PB, Feng W, Chenault C, Bast Jr RC, Issa JJ, Hilsenbeck SG, Yu Y (2012) Methylation of HIN-1, RASSF1A, RIL and CDH13 in breast cancer is associated with clinical characteristics, but only RASSF1A methylation is associated with outcome. *BMC Cancer* **12**: 243.

Yanagawa N, Tamura G, Oizumi H, Kanauchi N, Endoh M, Sadahiro M, Motoyama T (2007) Promoter hypermethylation of RASSF1A and RUNX3 genes as an independent prognostic prediction marker in surgically resected non-small cell lung cancers. *Lung Cancer* **58**: 131–138.

Yang Q, Zage P, Kagan D, Tian Y, Seshadri R, Salwen H, Liu S, Chlenski A, Cohn S (2004) Association of epigenetic inactivation of RASSF1A with poor outcome in human neuroblastoma. *Clin Cancer Res* **10**: 8493–8500.

Yang X, Dai W, Kwong DL-W, Szeto CYY, Wong EH-W, Ng WT, Lee AWM, Ngan RKC, Yau CC, Tung SY, Lung ML (2014) Epigenetic markers for noninvasive early detection of nasopharyngeal carcinoma by methylation-sensitive high resolution melting. *Int J Cancer* **136**(4): E127–E135.

Yao D, Shi J, Shi B, Wang N, Liu W, Zhang G, Ji M, Xu L, He N, Hou P (2012) Quantitative assessment of gene methylation and their impact on clinical outcome in gastric cancer. *Clin Chim Acta* **413**: 787–794.

Yee KS, Grochola L, Hamilton G, Grawenda A, Bond EE, Taubert H, Wurl P, Bond GL, O'Neill E (2012) A RASSF1A polymorphism restricts p53/p73 activation and associates with poor survival and accelerated age of onset of soft tissue sarcoma. *Cancer Res* **72**: 2206–2217.

Zhang C-Y, Zhao Y-X, Xia R-H, Han J, Wang B-S, Tian Z, Wang L-Z, Hu Y-H, Li J (2014) RASSF1A promoter hypermethylation is a strong biomarker of poor survival in patients with salivary adenoid cystic carcinoma in a Chinese population. *PLoS One* **9**: e110159.

Zhou SL, Cui J, Fan ZM, Li XM, Li JL, Liu BC, Zhang DY, Liu HY, Zhao XK, Song X, Wang R, Yan ZC, Yi HX, Wang LD (2013) Polymorphism of A133S and promoter hypermethylation in Ras association domain family 1A gene (RASSF1A) is associated with risk of esophageal and gastric cardia cancers in Chinese population from high incidence area in northern China. *BMC Cancer* **13**: 259.