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/P13K/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

Keywords: RASSF1A; hippo pathway; gene methylation; cancer; biomarker
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; Stuopelytę 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 (Fiegé et al, 2005; Avraham et al, 2012).

Oestrogen receptor (ER) status is one of the most important prognostic 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 Stuopelytę 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 RASSF1A 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 cancer 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...
| 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          | •    | •                                | •                                     | •                     |                          |       | Jamalaite et al, 2008     |
|                     | 543*        | •    | •                                | •                                     | •                     |                          |       | 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*       | •    | •                                | •                                     | •                     |                          |       | 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          | •    | •                                | •                                     | •                     |                          |       | Ara 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*       | •    | •                                | •                                     | •                     |                          |       | Li et al, 2014            |
|                     | 1215*       | •    | •                                | •                                     | •                     |                          |       | Shi et al, 2014           |
|                     | 630*        | •    | •                                | •                                     | •                     |                          |       | Wang et al, 2014a         |
|                     | 1505*       | •    | •                                | •                                     | •                     |                          |       | 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                  |
|----------------------|-------------|------|----------------------------------|---------------------------------------|-----------------------|---------------------------|-------|---------------------------|
| Head and neck cancer | 60          | •    | •                                | •                                     | •                     |                           |       | Liao et al, 2008          |
|                      | 60          | •    | •                                | •                                     | •                     |                           |       | Neyaz et al, 2008         |
|                      | 62          | •    | •                                | •                                     | •                     |                           |       | Pallarés et al, 2008      |
|                      | 110         | •    | •                                | •                                     | •                     |                           |       | Mita et al, 2012          |
|                      | 60          | •    | •                                | •                                     | •                     |                           |       | Li et al, 2005            |
|                      | 50          | •    | •                                | •                                     | •                     |                           |       | Ghosh et al, 2008         |
|                      | 69          | •    | •                                | •                                     | •                     | Early age of onset       |       | Lee et al, 2008           |
|                      | 68          | •    | •                                | •                                     | •                     |                           |       | Huang et al, 2009         |
|                      | 482         | •    | •                                | •                                     | •                     |                           |       | Yang et al, 2014          |
|                      | 189         | •    | •                                | •                                     | •                     |                           |       | 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*       | •    | •                                | •                                     | •                     |                           |       | 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*       | •    | •                                | •                                     | •                     |                           |       | 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 promoter hypermethylation | Reference | Cohort size | Risk of cancer | Other information |
|---------------------------------------------------------------|-----------|-------------|----------------|------------------|
| **Other**                                                    |           |             |                |                  |
| Local recurrence or distant metastasis                        |           |             | Poor overall survival |                  |
| Advanced stage and/or high grade                              |           |             | Poor disease-free survival |                  |
| **Cancer type**                                               |           |             |                |                  |
| Renal cancer                                                 | Liu et al., 2011 | 219 |                |                  |
| Renal cancer                                                 | Danusone et al., 2014 | 253 |                |                  |
| Renal cancer                                                 | Ge et al., 2014 | 1123 |                |                  |
| Renal cancer                                                 | Li et al., 2014 | 71 |                |                  |
| Renal cancer                                                 | Kawai et al., 2010 | 105 |                |                  |
| Renal cancer                                                 | Kawai et al., 2012 | 84 |                |                  |
| Renal cancer                                                 | Ohshima et al., 2012 | 84 |                |                  |
| Renal cancer                                                 | Danielsen et al., 2014 | 105 |                |                  |
| Renal cancer                                                 | Ge et al., 2014 | 253 |                |                  |
| Renal cancer                                                 | Liu et al., 2011 | 219 |                |                  |
| Renal cancer                                                 | Danusone et al., 2014 | 253 |                |                  |
| Renal cancer                                                 | Ge et al., 2014 | 1123 |                |                  |
| Renal cancer                                                 | Li et al., 2014 | 71 |                |                  |
| Renal cancer                                                 | Kawai et al., 2010 | 105 |                |                  |
| Renal cancer                                                 | Kawai et al., 2012 | 84 |                |                  |
| Renal cancer                                                 | Ohshima et al., 2012 | 84 |                |                  |
| Renal cancer                                                 | Danielsen et al., 2014 | 105 |                |                  |

**Abbreviations:** ER = oestrogen receptor; HER = progesterone receptor.

*Meta-analysis.*

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).

**GASTROINTESTINAL CANCER**

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; 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.

**OTHER CANCERS**

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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 (Danjumaite *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 (Shimozaki *et al*, 2005; Cho *et al*, 2012), gynaecological (Pan *et al*, 2009; Montavon *et al*, 2012), GI (Kim *et al*, 2009; Okamoto *et al*, 2009) 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          | Gynecological cancer | Head and neck cancer | Lung cancer | Renal cancer | Prostate cancer | Bladder cancer | Brain cancer | Gastrointestinal cancer | Sarcoma |
|----------------------|----------------------|----------------------|-------------|--------------|----------------|----------------|--------------|------------------------|---------|
| Risk                 | ++                   | ++                   | ++          | ++           | ++             | ++             | ++           | ++                     | ++      |
| Advanced stage       | ++                   | ++                   | ++          | ++           | ++             | ++             | ++           | ++                     | ++      |
| Local recurrence/ol | ++                   | ++                   | ++          | ++           | ++             | ++             | ++           | ++                     | ++      |
| Poor overall survival| ++                   | ++                   | ++          | ++           | ++             | ++             | ++           | ++                     | ++      |

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

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 CpG 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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