

RASSF1A hypermethylation in pretreatment serum DNA of neuroblastoma patients: a prognostic marker

A Misawa^{*,1,2}, S Tanaka², S Yagyu¹, K Tsuchiya¹, T Iehara¹, T Sugimoto¹ and H Hosoi¹

¹Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan; ²Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

The tumour suppressor gene *RASSF1A* is known to be frequently silenced by promoter hypermethylation in neuroblastoma tumours. Here we explored the possible prognostic significance of aberrant promoter hypermethylation of *RASSF1A* in serum DNA samples of patients with neuroblastoma as a surrogate marker for circulating tumour cells. We analysed the methylation status of the *RASSF1A* gene in matched tumour and pretreatment serum DNA obtained from 68 neuroblastoma patients. Hypermethylation of *RASSF1A* in tumour samples was found in 64 patients (94%). In contrast, serum methylation of *RASSF1A* was observed in 17 patients (25%). Serum methylation of *RASSF1A* was found to be statistically associated with age ≥ 12 months at diagnosis ($P=0.002$), stage 4 ($P<0.001$) and *MYCN* amplification ($P<0.001$). The influence of serum *RASSF1A* methylation on prognosis was found to be comparable with that of the currently most reliable marker, *MYCN* amplification on univariate analysis (hazard ratio, 9.2; 95% confidence interval (CI), 2.8–30.1; $P<0.001$). In multivariate analysis of survival, methylation of *RASSF1A* in serum had a hazard ratio of 2.4 (95% CI, 0.6–9.2), although this association did not reach statistical significance ($P=0.194$). These findings show that the methylation status of *RASSF1A* in the serum of patients with neuroblastoma has the potential to become a prognostic predictor of outcome.

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Neuroblastoma is the most common extracranial solid tumour in children and is characterised by a wide range of clinical behaviours, from spontaneous regression to rapid progression with a fatal outcome (Maris *et al*, 2007). The clinical outcome is associated with disease stage, age at diagnosis, *MYCN* amplification and histological classification. Although numerous genetic abnormalities, including *MYCN* amplification, are associated with tumour progression and poor outcome, the molecular mechanisms responsible for the pathogenesis of aggressive neuroblastoma remain unclear. Identifying such molecular changes may contribute to improved clinical management and outcome prediction of newly diagnosed neuroblastomas.

In recent years, changes in the status of DNA methylation, known as epigenetic alterations, have turned out to be one of the most common molecular alterations in human neoplasia including neuroblastoma (Misawa *et al*, 2005; Sugino *et al*, 2007). Several potential tumour-suppressor genes have been described as frequently silenced by hypermethylation in neuroblastomas. Methylation of promoter CpG islands is known to inhibit transcriptional initiation and cause permanent silencing of down-

stream genes. Loss of heterozygosity of chromosome 3p21.3 is one of the most frequent alterations in solid tumours. Located within this 3p21.3 locus, the RAS-association domain family 1, isoform A gene (*RASSF1A*) encodes a RAS effector that has been identified as a tumour suppressor of many different cancer types (Dammann *et al*, 2000). *RASSF1A* falls into the category of genes frequently inactivated by methylation rather than mutational events. This gene is silenced and inactivated by promoter region hypermethylation in many adult and childhood cancers, including neuroblastoma (Astuti *et al*, 2001; Harada *et al*, 2002; Wong *et al*, 2004; Yang *et al*, 2004; Banelli *et al*, 2005; Lazcoz *et al*, 2006; Michalowski *et al*, 2008). *RASSF1A* has been shown to play important roles in cell cycle regulation, apoptosis and microtubule stability as a tumour suppressor gene (Agathangelou *et al*, 2005).

It is well known that DNA fragments are frequently and abundantly found in the serum of cancer patients, with significantly higher levels in patients with metastasis (Hesson *et al*, 2007). A number of studies have evaluated the potential of circulating tumour-related methylated DNA in serum for the molecular diagnosis and prognosis of various types of cancer (Müller *et al*, 2003; Ibanez de Caceres *et al*, 2004; Mori *et al*, 2005). Methylation-specific PCR assay is a sensitive and specific assay for tumour-related DNA methylation in serum. Several studies have investigated the prospect of using DNA methylation as a surrogate marker for circulating tumour cells in serum samples from breast cancer or melanoma patients (Fiegl *et al*, 2005; Koyanagi *et al*, 2006). However, no studies of neuroblastoma have assayed serum samples for aberrant DNA methylation. Therefore, this study

*Correspondence: Dr A Misawa, Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan; E-mail: amisawa@kuhp.kyoto-u.ac.jp
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investigated whether it is possible to detect *RASSF1A* epigenetic alterations in the serum of neuroblastoma patients, and aberrant *RASSF1A* methylation in patient pretherapeutic serum is of prognostic significance in neuroblastoma using a series of matched neuroblastoma tumour and serum DNA.

MATERIALS AND METHODS

Patients and sample collection

Clinical data were collected retrospectively by reviewing the medical database at the Hospital of Kyoto Prefectural University of Medicine for the period between 1985 and 2004. After approval by the Institutional Review Board, 68 neuroblastoma patients were identified on the basis of histological examination of tumour specimens that met the following criteria: the patient had an available tumour specimen; a serum specimen was available; and the patient either died or had >1 year of follow-up time. The clinical data included information regarding tumour stage, age at diagnosis, sex, *MYCN* gene status and outcome. Staging was evaluated according to the criteria of the International Neuroblastoma Staging System (Maris *et al*, 2007). Patients of any age who had stage 1 or 2 disease and those younger than 12 months with stage 3 or 4S disease were given either surgery or surgery with chemotherapy (Matsumura and Michon, 2000). Patients aged 12 months or older with stage 3 and any patients with stage 4 disease were treated according to the protocol by the Japanese Neuroblastoma Study Group (Sawaguchi *et al*, 1990; Tsuchida and Kaneko, 2000; Kaneko *et al*, 2002; Suita *et al*, 2007). The patients with stage 4 disease underwent high-dose chemotherapy with autologous stem-cell rescue after the initial chemotherapy. Instead of pre-specified sample size determination, power analysis was conducted after collecting clinical data to guarantee statistical power and to evaluate whether *RASSF1A* methylation is a prognostic marker for survival. In a realistic scenario, a study of 68 patients had power of 96% to detect a single marker with hazard ratio larger than 5.

Tumour samples at the time of diagnosis and before the administration of chemotherapy were frozen immediately and stored at -80°C until DNA extraction. In addition, match-paired serum samples were assessed. Peripheral blood was obtained before any therapy or surgery. To avoid contamination of serum DNA by the DNA from WBCs, serum was prepared exclusively from the liquid fraction of clotted blood after centrifugation at $1000 \times g$ for 10 min and stored it at -20°C until DNA extraction. For the extraction of free DNA, we used 200 μl of stored serum, which supplemented with 1 μg salmon testes DNA (Sigma, St Louis, MO, USA) as a carrier DNA. DNA was extracted from tumour samples using a QIAmp DNA Mini Kit and from serum samples using a QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Analysis of DNA methylation

Treatment of tumour DNA and serum DNA with sodium bisulphite was performed with an EZ DNA methylation kit (Zymo Research, Orange, CA, USA) following the protocol of the manufacturer. Methylation-specific PCR was performed with primers specific for either methylated or unmethylated DNA spanning the region within the *RASSF1A* gene (Figure 1). The primers used were methylation-specific RAM-1 (5'-GTG TTAACGCGTTGCGTATC-3') and RAM-2 (5'-AACCCCGCGAACT AAAACGA-3') and unmethylation-specific RAU-1 (5'-TTTGGT TGGAGTGTGTTAATG-3') and RAU-2 (5'-CAAACCCACAACT AAAACAA-3'), as described earlier (Lo *et al*, 2001). PCR conditions consisted of an initial incubation for 10 min at 95°C followed by 35 cycles for tumour samples or 40 cycles for serum

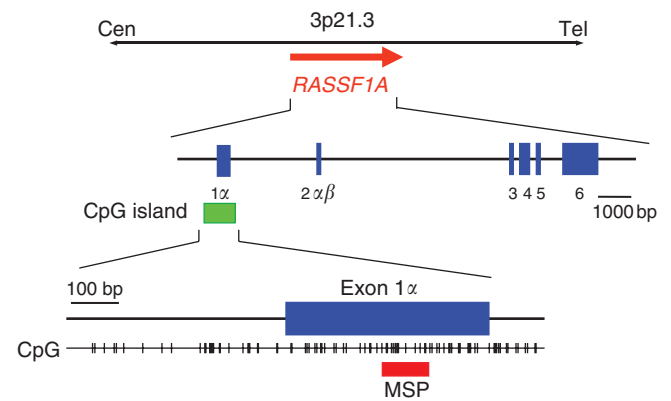


Figure 1 Genomic structure of the *RASSF1A* gene. Vertical tick marks, CpG sites; blue boxes, exons; green box, CpG island in the promoter; red box, region analysed by methylation-specific PCR.

samples of denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 60 s, followed by a final extension step of 72°C for 10 min. Lymphocyte DNA and *in vitro* methylated (using *SssI* CpG methylase; New England Biolabs, Beverly, MA, USA) lymphocyte DNA were used as unmethylated and methylated controls, respectively. The PCR products obtained were analysed by electrophoresis in 2% agarose gels and stained with ethidium bromide. Samples were scored as methylation positive when methylated alleles were visualised as bands in the methylated DNA lane and as methylation negative when bands were seen only in the unmethylated DNA lane. The analysis of the samples in this study was performed by an analyst blinded to the clinical and biological information.

Statistical analysis

The primary end point was overall survival defined by the period from diagnosis of the primary tumour to any cause of death. The relationship between clinicopathological variables and methylation status of the *RASSF1A* gene was shown initially using contingency tables and χ^2 test. Survival curves for *RASSF1A* methylation were derived by the Kaplan–Meier method. Univariate analysis was conducted using Cox's proportional hazard models and log-rank test. Performance of *RASSF1A* methylation as a prognostic marker was also analysed after adjustment for known prognostic factors by (i) subset analysis of stage 3 patients using contingency tables and Fisher's exact test and (ii) multivariate Cox's proportional hazard models including age, sex and tumour stage. Two-sided *P*-values <0.05 were considered as significant. SAS 9.13 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

RESULTS

A total of 124 patients with histologically confirmed neuroblastoma or ganglioneuroblastoma were treated at the Hospital of Kyoto Prefectural University of Medicine between January 1985 and May 2004. Sixty-eight patients met the criteria of this retrospective study. The detailed patient disposition is shown in Figure 2 and the baseline characteristics of patients are presented in Table 1. Of the 68 patients, 24 were classified as stage 1, 11 as stage 2, 11 as stage 3, 18 as stage 4 and 4 as stage 4S. At the time of diagnosis, 42 patients (62%) were younger than 12 months, and 26 (38%) were older. We found no significant differences between included and excluded patients for age or stage statistically. Twelve patients (18%) had tumours with *MYCN* amplification, and *MYCN* amplification was not detected in the tumours from 56 (82%) patients by southern

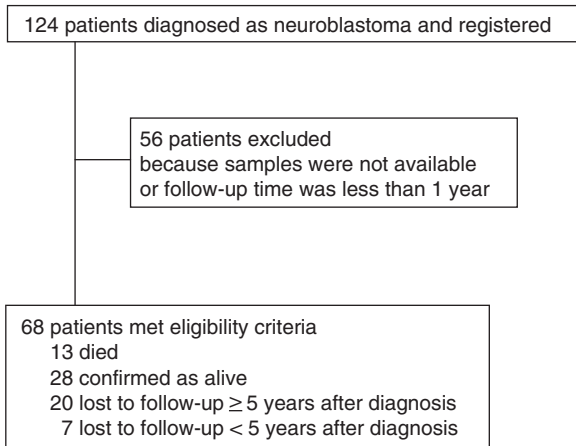


Figure 2 Patient disposition.

Table 1 Characteristics of patients

Characteristic	No. of patients (%)
Sex	
Male	27 (39.7)
Female	41 (60.3)
Age at diagnosis	
< 12 months	42 (61.8)
≥ 12 months	26 (38.2)
Stage	
1	24 (35.3)
2	11 (16.2)
3	11 (16.2)
4	18 (26.5)
4S	4 (5.9)
MYCN	
Non-amplified	56 (82.4)
Amplified	12 (17.6)
Diagnosis	
GNB	7 (10.3)
NB	61 (89.7)
Serum RASSF1A	
Unmethylated	51 (75.0)
Methylated	17 (25.0)

blot analysis or fluorescence *in situ* hybridisation. The median follow-up time was 72 months, with a range from 9 to 248 months.

Detection of RASSF1A promoter methylation in tumours

This study initially investigated the hypermethylation status of the RASSF1A tumour suppressor genes in 68 neuroblastoma tumours. Only four (one each at stage 1, 2, 4S and 3) tumours showed no methylation of RASSF1A (Supplementary Table). All other neuroblastoma tumours (64 of 68; 94%) showed methylated RASSF1A. Hypermethylation in tumours was observed very frequently in all of the stages of neuroblastoma examined, including stage 1, 2 and 4S tumours (Supplementary Table) and no correlation between RASSF1A methylation and known prognostic factors including stage, age and MYCN amplification was detected. No relationship between RASSF1A methylation in tumours and outcome was also observed. RASSF1A methylation was not observed in any of the three benign ganglioneuromas.

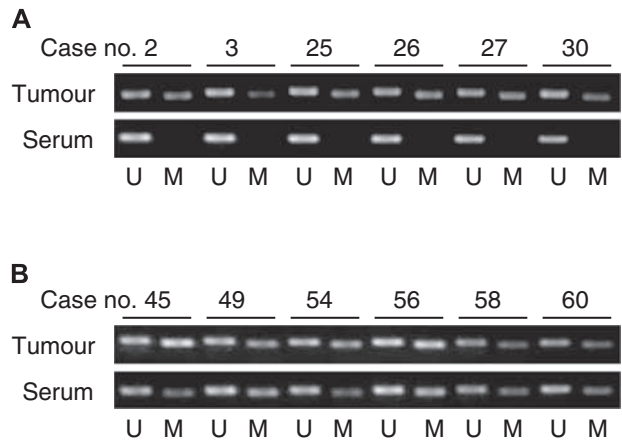


Figure 3 RASSF1A methylation status of tumour and serum DNA in neuroblastoma patients. M, methylated; U, unmethylated. The sizes of the PCR products for methylated and unmethylated primers are 93 and 105 bp, respectively. (A) Cases stage 1 and 2 with good prognosis tumour DNAs are methylated but absent in the serum DNAs. (B) In contrast, in stage 3 and 4 MYCN-amplified cases, methylated DNAs are detected in both tumour and serum samples.

Table 2 Associations between clinical factors and serum RASSF1A methylation status

Characteristic	Methylated no.	Unmethylated no.	Total no.	P-value
Age at diagnosis				0.002
< 12 months	5	37	42	
≥ 12 months	12	14	26	
Stage				<0.001
1/2/4S	3	36	39	
3	3	8	11	
4	11	7	18	
MYCN				<0.001
Non-amplified	5	51	56	
Amplified	12	0	12	

Detection of RASSF1A promoter methylation in serum

The hypermethylation status of RASSF1A in the matched serum DNA samples was then determined and compared with the pattern of hypermethylation found in the corresponding tumour DNA samples (Figure 3). RASSF1A hypermethylation was detected in 17 of 68 (25%) matched serum DNA samples (Table 1). The detailed overview is shown in Supplementary Table.

Correlation of serum RASSF1A methylation status with clinical factors

The methylation status of RASSF1A in the pretherapeutic serum of the 68 patients was analysed for association with known prognostic factors (Table 2). Serum RASSF1A methylation showed a significant statistical association with age ≥ 12 months (P=0.002). RASSF1A methylation in serum was detected more frequently in disseminated stage 4 tumours than local-regional (stage 1, 2 and 3) and 4S tumours (P<0.001). Furthermore, serum-methylated RASSF1A was significantly correlated with MYCN amplification (P<0.001). Notably, all cases with MYCN amplification showed RASSF1A methylation of serum DNA (Supplementary Table).

Table 3 Univariate analysis of survival

Characteristic	Hazard ratio	95% CI	P-value
Age at diagnosis			
< 12 months	Reference		
≥ 12 months	23.6	3.1–181.9	0.002
Sex			
Male	Reference		
Female	1.0	0.3–3.0	0.983
Stage			
1/2/3/4S	Reference		
4	19.8	4.4–89.5	<0.001
MYCN			
Non-amplified	Reference		
Amplified	8.2	2.7–24.7	<0.001
Serum RASSF1A			
Unmethylated	Reference		
Methylated	9.2	2.8–30.1	<0.001

CI = confidence interval.

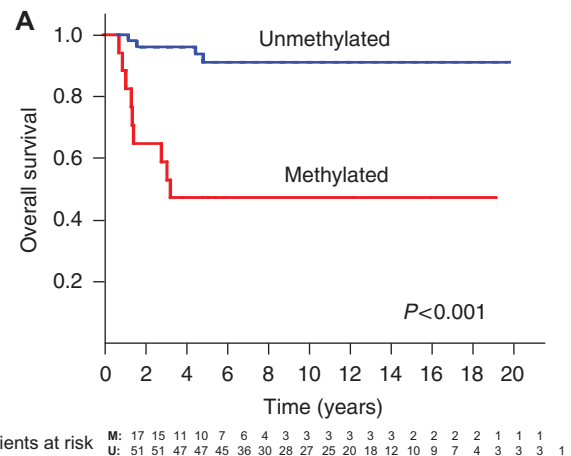
Analysis of prognostic significance of RASSF1A methylation in serum

The association of pretherapeutic serum methylation status of RASSF1A with clinical outcome was analysed in 68 patients with known follow-ups. Univariate analyses revealed prognostic significance for age at diagnosis ≥ 12 months, stage 4 and MYCN amplification ($P=0.002$, $P<0.001$ and $P<0.001$, respectively; Table 3) in this cohort, as expected. Patients with serum-methylated RASSF1A had significantly worse overall survival than patients with serum-unmethylated RASSF1A ($P<0.001$, log-rank test; Figure 4A). The 5-year survival was more than 90% in patients without serum methylation of RASSF1A, whereas lower than 50% in patients with serum methylation of RASSF1A (hazard ratio, 9.2; 95% confidence interval (95% CI), 2.8–30.1; $P<0.001$); Table 3). RASSF1A methylation in serum and the known prognostic factors were also correlated with relapse-free survival as well as with overall survival ($P<0.001$; Table 4; Figure 4B). Furthermore, a subset analysis revealed that stage 3 patients also had a trend towards poorer prognosis when RASSF1A was methylated in serum. When limited to cases in stage 3, two of the three patients with serum-methylated RASSF1A died, whereas all eight patients with serum-unmethylated RASSF1A are alive ($P=0.055$, Fisher's exact test). In a multivariate analysis including age, sex and tumour stage, serum RASSF1A methylation was still associated with poor outcome with a hazard ratio of 2.4 (95% CI, 0.6–9.2), although this did not reach statistical significance ($P=0.194$; Table 5).

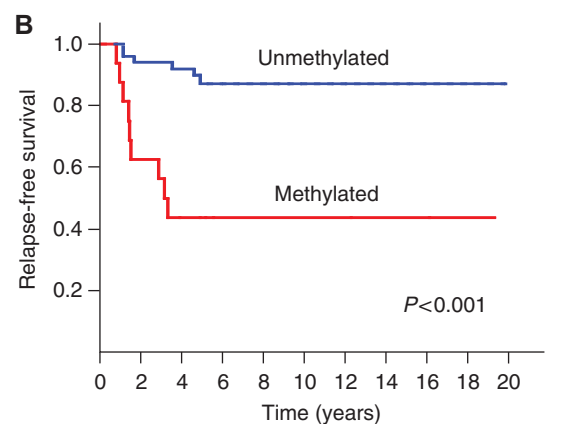
DISCUSSION

In patients with malignancies, aberrant methylation of serum DNA has been reported (Müller *et al*, 2003; Ibanez de Caceres *et al*, 2004; Fiegl *et al*, 2005; Mori *et al*, 2005; Koyanagi *et al*, 2006). We have detected cell-free tumour DNA in serum of neuroblastoma patients (Gotoh *et al*, 2005). Prognosis in stage 4 neuroblastoma patients with metastases is poor despite intensive chemotherapy (Maris *et al*, 2007). Therefore, this study aimed to explore the possible prognostic significance of aberrant promoter hypermethylation of RASSF1A, which has been found frequently in neuroblastoma tumours, using pretherapeutic serum of neuroblastoma patients as a surrogate marker for circulating tumour cells.

We first investigated the RASSF1A methylation status in 68 neuroblastoma tumour DNA samples in comparison with matched



No. of patients at risk M: 17 15 11 10 7 6 4 3 3 3 3 3 3 2 2 2 2 1 1 1 1
U: 51 51 47 47 45 36 30 28 27 25 20 18 12 10 9 7 4 3 3 3 1



No. of patients at risk M: 16 14 10 9 6 5 3 3 3 3 3 3 2 2 2 2 1 1 1 1
U: 51 51 46 46 43 35 29 27 26 25 20 18 12 10 9 7 4 3 3 3 1

Figure 4 Kaplan–Meier survival curves of 68 neuroblastoma patients: correlation of pretherapeutic serum RASSF1A methylation status with overall survival (A) and relapse-free survival (B). M, methylated: patients with serum methylation of RASSF1A. U, unmethylated: patients without serum methylation of RASSF1A. The 5-year overall survival was more than 90% in patients without methylation, whereas lower than 50% in patients with methylation ($P<0.001$).

serum DNA samples. The methylation of RASSF1A was observed in this study in 94% of primary tumours. Our results show that promoter hypermethylation of RASSF1A occurs at a high frequency in primary neuroblastoma tumours and no correlation between RASSF1A methylation and known prognostic factors including stage, age and MYCN amplification, or outcome was seen. The high proportion of RASSF1A promoter methylation in tumours agrees with earlier reports in the literature, which have found RASSF1A to be hypermethylated in 52–94% of tumour DNA samples (Astuti *et al*, 2001; Harada *et al*, 2002; Wong *et al*, 2004; Yang *et al*, 2004, 2007; Banelli *et al*, 2005; Lazcoz *et al*, 2006; Michalowski *et al*, 2008). Several earlier studies with one exception (Yang *et al*, 2004) failed to find a statistical correlation between RASSF1A methylation in tumours and poor outcome (Astuti *et al*, 2001; Harada *et al*, 2002; Banelli *et al*, 2005; Michalowski *et al*, 2008). We also did not observe any relationship between RASSF1A methylation in tumours and prognosis. RASSF1A hypermethylation in tumours can be a relatively early event in neuroblastoma tumorigenesis as it is detectable in non-advanced early-stage tumours with high frequency. Although the prognostic significance of epigenetic changes of single genes in neuroblastoma tumour DNA remain controversial, a few studies have indicated that poor prognosis is associated with the CpG island methylator phenotype

Table 4 Univariate analysis of relapse

Characteristic	Hazard ratio	95% CI	P-value
Age at diagnosis			
< 12 months	Reference		
≥ 12 months	12.5	2.8–55.3	<0.001
Sex			
Male	Reference		
Female	1.0	0.4–2.9	0.972
Stage			
1/2/3/4S	Reference		
4	14.3	4.0–51.0	<0.001
MYCN			
Non-amplified	Reference		
Amplified	7.2	2.6–20.0	<0.001
Serum RASSF1A			
Unmethylated	Reference		
Methylated	6.8	2.4–19.1	<0.001

CI = confidence interval.

Table 5 Multivariate analysis of survival

Characteristic	Hazard ratio	95% CI	P-value
Age at diagnosis			
< 12 months	Reference		
≥ 12 months	1.2	1.0–1.5	0.066
Sex			
Male	Reference		
Female	0.6	0.1–2.5	0.452
Stage			
1/2/3/4S	Reference		
4	8.4	1.5–46.4	0.014
Serum RASSF1A			
Unmethylated	Reference		
Methylated	2.4	0.6–9.2	0.194

CI = confidence interval.

(Abe *et al*, 2005; Banelli *et al*, 2005; Yang *et al*, 2007), suggesting that aberrant methylation of multiple genes is likely to contribute to neuroblastoma pathogenesis.

As a next step, we analysed RASSF1A methylation status in 68 paired serum DNA samples. In contrast to tumours, RASSF1A methylation was detected in neuroblastoma patient serum from only 25% (17 out of 68). To investigate the clinical significance of the serum RASSF1A methylation, associations with established prognostic factors and outcome were evaluated. RASSF1A methylation in serum was found to be statistically associated with established prognostic factors. Serum RASSF1A methylation was more frequently detected in neuroblastoma patients with age ≥ 12

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months at diagnosis ($P=0.002$), stage 4 ($P<0.001$) and MYCN amplification ($P<0.001$). Furthermore, the presence of methylation of RASSF1A in serum was associated with poorer outcome. The influence of serum RASSF1A methylation on prognosis was found to be comparable with that of the currently most reliable marker, MYCN amplification in univariate analysis. A subset analysis of stage 3 patients showed a trend associating poor survival with serum RASSF1A methylation ($P=0.055$), although the data were limited due to the small number of patients in the subgroup. In multivariate analysis of survival, methylation of RASSF1A in serum had a hazard ratio of 2.42, but this association did not reach statistical significance ($P=0.194$). Further validation studies using a larger set of patients are necessary to confirm our findings.

The presence of tumour-derived DNA within the blood stream has been identified earlier (Müller *et al*, 2003; Fiegl *et al*, 2005; Mori *et al*, 2005). Recently, one study showed that the detection of circulating tumour cells was correlated with tumour-related methylated DNA in patients with melanoma (Koyanagi *et al*, 2006), suggesting that circulating tumour cells are a potential source of circulating methylated DNA. Our study suggests that methylated RASSF1A DNA in serum is a surrogate marker for circulating neuroblastoma cells. Another recently published study showed that RASSF1A methylation was also detectable in ovarian cancer patient's serum at a high frequency from methylated tumour cases including several stage I tumours (Ibanez de Caceres *et al*, 2004). In the earlier study, there was no statistical association between tumour stage and positive detection in serum. However, some other studies have shown limited detection of RASSF1A methylation in the serum of patients with other neoplasms (Murray *et al*, 2004; Hesson *et al*, 2007). These differing results may suggest that free neoplastic DNA from ovarian cancer can access the blood stream more readily than that from other neoplasms including neuroblastoma.

In conclusion, this is the first study to examine epigenetic changes in a tumour suppressor gene, RASSF1A, the promoter of which is hypermethylated at a high frequency in neuroblastoma tumours, using serum DNA in a cohort of neuroblastoma patients. This study demonstrates the utility of detecting circulating methylated RASSF1A, which can be measured in serum, as a potentially predictive marker of neuroblastoma outcome. RASSF1A methylation in serum could have useful clinical applications in neuroblastoma management, if our results are confirmed in larger studies. However, we should not forget the limitation when attempting to translate our findings into the clinical fields as highly sensitive methylation analysis could be tricky because of incomplete bisulphite conversion by inexperienced analysts.

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