BIOMARKERS

Novel Circulating Hypermethylated RASSF1A ddPCR for Liquid Biopsies in Patients With Pediatric Solid Tumors

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original reports abstract

PURPOSE Liquid biopsies can be used to investigate tumor-derived DNA, circulating in the cell-free DNA (cfDNA) pool in blood. We aimed to develop a droplet digital polymerase chain reaction (ddPCR) assay detecting hypermethylation of tumor suppressor gene *RASSF1A* as a simple standard test to detect various pediatric tumor types in small volume blood samples and to evaluate this test for monitoring treatment response of patients with high-risk neuroblastoma.

METHODS We developed a ddPCR assay to sensitively detect tumor-derived hypermethylated *RASSF1A* DNA in liquid biopsies. We tested this assay in plasma of 96 patients with neuroblastoma, renal tumors, rhabdomyosarcoma, or Hodgkin lymphoma at diagnosis and in cerebrospinal fluid of four patients with brain tumors. We evaluated the presence of hypermethylated *RASSF1A* in plasma samples during treatment and follow-up in 47 patients with neuroblastoma treated according to high-risk protocol and correlated results with blood mRNA–based and bone marrow mRNA–based minimal residual disease detection and clinical outcomes.

RESULTS The total cfDNA level was significantly higher in patients with metastatic neuroblastoma and nephroblastoma compared with healthy adult and pediatric controls. Hypermethylated *RASSF1A* was present in 41 of 42 patients with metastatic neuroblastoma and in all patients with nephroblastoma, with the median percentage of 69% and 21% of total *RASSF1A*, respectively. Hypermethylated *RASSF1A* levels decreased during therapy and recurred at relapse.

CONCLUSION Our findings demonstrate the value of ddPCR-based detection of hypermethylated *RASSF1A* as a circulating molecular tumor marker in neuroblastoma. Our preliminary investigation of *RASSF1A* hypermethylation detection in circulating cfDNA of other pediatric tumor entities demonstrates potential as a pan-tumor marker, but requires investigation in larger cohorts to evaluate its use and limitations.

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INTRODUCTION

Cancer remains one of the most common causes of childhood death in high-income countries.[1] Although the combination of intensive chemotherapy, surgery, radiation therapy, and immunotherapy has improved outcomes in children with solid tumors, disease still recurs in 50% of patients with neuroblastomas;[2,3] 46% of patients with Ewing sarcomas;[4] and approximately 30% of patients with localized rhabdomyosarcomas,[5] osteosarcomas,[6] and renal tumors.[7] Response to treatment is primarily based on imaging. In patients with neuroblastoma, bone marrow (BM) histology or (immuno)cytology assesses the extent of disease.[8] In neuroblastoma and rhabdomyosarcoma, reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) for the detection of minimal residual disease (MRD) in peripheral blood or BM is shown to be more sensitive[9-13] and predictive of outcomes, but even patients with low or negative MRD results can suffer from recurrent disease,[9,14] or mRNA markers can be down-regulated upon epithelial-to-mesenchymal transition.[15]

Liquid biopsies, for example, peripheral blood, can also be a source for tumor-derived cell-free DNA (cfDNA). As the genomic view is not limited to the boundaries of a tissue biopsy, liquid biopsies better represent spatial and intra-tumor heterogeneity. Liquid biopsies have shown promise in assisting diagnosis and monitoring therapy response in adult oncology.[16-18] Pediatric tumors have lower mutational burdens with few recurrent mutations[19] but a variety of copy number alterations[20] and epigenetic changes.[21] The tumor suppressor gene *RASSF1A* is silenced in nearly all adult cancers and associated with poor prognosis and high-risk disease.[22-24] Promotor hypermethylation[23,25,26] or, less frequently, a combination of hypermethylation and 3p21.3 allelic loss[22,23,27] causes inactivation. *RASSF1A* is hypermethylated in neuroblastoma,[22,28-35]

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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CONTEXT

Key Objective

Molecular testing of circulating tumor DNA (ctDNA) has the potential to improve pediatric solid tumor diagnosis and discrimination of subtypes and monitoring of treatment response. Our aim was to develop a *RASSF1A* hypermethylation droplet digital polymerase chain reaction as a standard test to detect ctDNA in several pediatric tumor types using small blood volumes and as a test to monitor treatment response of patients with neuroblastoma.

Knowledge Generated

We developed a sensitive and quantitative droplet digital polymerase chain reaction–based assay for hypermethylated *RASSF1A* detection. Our findings demonstrate the value of hypermethylated *RASSF1A* as a molecular circulating tumor marker in neuroblastoma. *RASSF1A* was frequently hypermethylated in plasma samples from patients with nephroblastoma, rhabdomyosarcoma, and Hodgkin lymphoma.

Relevance

Our study supports the use of ctDNA in assisting the monitoring of therapy response in patients with neuroblastoma and shows the potential of ctDNA in assisting the diagnosis of other pediatric solid tumor entities.

hepatoblastoma,[29,36] nephroblastoma,[29,37,38] medulloblastoma and primitive neuroectodermal tumors,[29,39] and osteosarcoma and Ewing sarcoma.[29,40-42] These accumulating data suggest *RASSF1A* hypermethylation to be as common in pediatric tumor entities as in adult tumor entities. *RASSF1A* hypermethylation is rare in normal tissues,[23] but present in placenta, and therefore is also suited for fetal DNA detection in maternal plasma.[43,44] We previously investigated hypermethylated *RASSF1A* in cfDNA from patients with neuroblastoma by performing qPCR.[33] We demonstrated the promise of this marker, but observed loss of cfDNA because of bisulfite conversion, and were unable to quantify the low amounts of circulating tumor DNA (ctDNA).[33] In this study, we harnessed the sensitivity and accuracy of droplet digital PCR (ddPCR) and developed a ddPCR method with methylation-sensitive restriction enzymes (MSREs) to overcome these limitations. We furthermore investigated the feasibility of our hypermethylated *RASSF1A* ddPCR assay in detecting different pediatric tumor types in small volume patient plasma samples.

METHODS

Methods on patient inclusion, sample collection, cfDNA isolation, and RT-qPCR for mRNA markers[45] and single nucleotide polymorphism array can be found in the Data Supplement.

Hypermethylated *RASSF1A* ddPCR

To discriminate between methylated and unmethylated *RASSF1A*, every sample was subjected to two different ddPCR reactions (Fig 1): one with MSRE and the other without; all remaining conditions were identical. ACTB-1 primer-probe set was added to control for cfDNA input, and this amplicon is unaffected by the MSRE. ACTB-2 primer-probe set was added to control for MSRE performance since this amplicon is digested by the enzymes. *RASSF1A*, ACTB-1, and ACTB-2 primer and probe sets are listed in the Data Supplement. Primer and probe sequences for *RASSF1A* and ACTB-2 have been described before by O'Brien et al.[44] A detailed protocol can be found in the Data Supplement. To avoid false positivity, a threshold was based on healthy donors for both the single- and double-digest reactions (see the Results) and a minimum of four positive droplets per duplicate. If a sample was scored positive, the percentage of hypermethylated *RASSF1A* was calculated as $(RASSF1A/ACTB^{\text{with MSRE}})/(RASSF1A/ACTB^{\text{without MSRE}}) \times 100\%$. *RASSF1A* ddPCR performance was compared with that of *RASSF1A* qPCR by testing 16 rhabdomyosarcoma and renal tumor cfDNA samples. *RASSF1A* qPCR was performed as described previously.[33]

Statistical Analysis

As cfDNA and ctDNA levels were not normally distributed, they are presented as median (interquartile range) and statistical significance was determined by the Kruskal–Wallis test. Fisher's exact test was used to analyze the correlation between ctDNA and/or mRNA positivity and outcomes. Correlation analysis between cfDNA, ctDNA, and mRNA levels was performed using Spearman's test. Events were defined as relapse, progressive,[8] or refractory disease, when the progression was not according to the International Neuroblastoma Response Criteria but resulted in change of treatment protocol. Receiver operating characteristic analysis was used to identify a cutoff for hypermethylated *RASSF1A* copies/mL. This cutoff was used to identify two subgroups for the comparison of event-free survival using Kaplan-Meier method. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA) software. Results were considered significant if $P \leq .05$.

RESULTS

Limit of Detection and Limit of Blank: Single and Double MSRE Digest

The dilution series of neuroblastoma cell line IMR32 DNA (100% hypermethylated *RASSF1A*) in DNA from blood

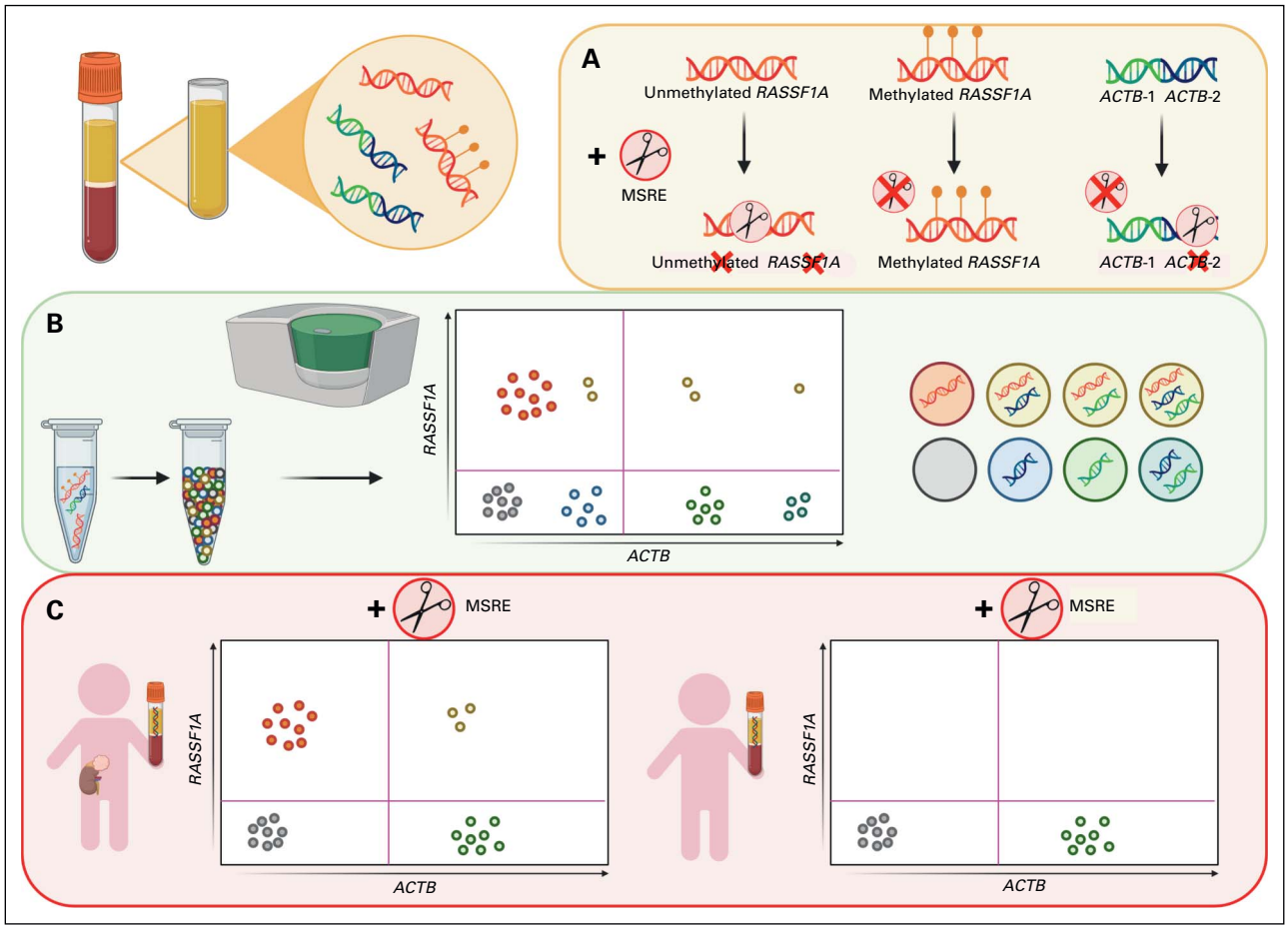

FIG 1. Concept of quantifying methylated *RASSF1A* using MSRE and ddPCR. (A) An MSRE incubation of a cfDNA sample results in the digestion of unmethylated *RASSF1A*, whereas methylated *RASSF1A* remains intact. Two amplicons of *ACTB* are added, and ACTB-1 is unaffected by the MSRE, whereas ACTB-2 is digested by the MSRE, as a control for MSRE performance. Every sample is subjected to two different ddPCR reactions, (B) one without the MSRE and (C) the other with the MSRE. ACTB-2 primers and probe are added in a lower concentration, resulting in a lower amplitude to discriminate between the ACTB-1 and ACTB-2 clusters. (C) Only in cfDNA from patients with circulating tumor DNA present, *RASSF1A* will be detected after digestion with the MSRE, as the absence of *RASSF1A* methylation will result in *RASSF1A* digestion, preventing the detection of this unmethylated *RASSF1A* allele by ddPCR. cfDNA, cell-free DNA; ddPCR, droplet digital polymerase chain reaction; MSRE, methylation-sensitive restriction enzymes.

from a healthy male and in H_2O showed a good linearity (a detailed description is given in the Data Supplement). The limit of detection, however, is defined by the level of positivity in the control samples, also called the limit of blank. For the limit of blank, we evaluated *RASSF1A* positivity in 22 samples stored at room temperature from adult male controls from which plasma was separated after 24, 48, 72, or 144 hours and 18 pediatric control samples (plasma separation within 24 hours). To test the efficacy of single-digest MSRE (BstUI-only), both hypermethylated *RASSF1A* and *ACTB* were measured in these control samples after digestion. We observed a correlation between the number of hypermethylated *RASSF1A* copies and *ACTB* copies in the adult controls (Spearman $r_s = 0.91$, $P < .0001$) and to a lesser extent in the pediatric controls (Spearman $r_s = 0.69$, $P = .002$), with a maximum of 0.039 *RASSF1A* copies per *ACTB* copies/mL plasma (Data Supplement). Although we cannot formally exclude that hypermethylated *RASSF1A* is derived from necrotic cells during storage of the samples, these data suggest that, although the ACTB-2 cluster was not clearly present, BstUI-only was not able to digest all cfDNA in our samples. A threshold on the basis of this ratio would greatly reduce the sensitivity of the assay and result in many inconclusive samples, and therefore, we investigated the use of two MSREs in a double-digest reaction. Double digestion by MSREs HhaI and Bsh1236I instead of BstUI in 43 adult and 18 pediatric control samples resulted in a more efficient digestion of *RASSF1A*. The number of hypermethylated *RASSF1A* copies was no longer dependent on the cfDNA concentration (Data Supplement). A prolonged time to plasma separation did not result in a significant increase in *RASSF1A* copies/mL, neither for the single-digest nor double-digest method (Data Supplement). On the basis of mean + 3 × standard deviation in hypermethylated

RASSF1A copies/mL plasma of these controls, we set the threshold on 14 copies/mL plasma. As a large number of patient samples were already tested using the single-digest method, all patient samples with ≥ 4 positive droplets and a ratio ≤ 0.039 *RASSF1A/ACTB* copies/μL were also tested using the double-digest method and scored according to the new double-digest threshold. To compare *RASSF1A* ddPCR performance with that of *RASSF1A* qPCR,[33] we tested 16 diagnostic rhabdomyosarcoma and renal tumor plasma samples using both techniques. All 11 samples that were positive by qPCR, of which three were positive-not-quantifiable, tested positive by ddPCR, and 1 in 5 qPCR-negative samples were tested positive by ddPCR.

Total cfDNA Is Increased in Patients With Neuroblastoma and Nephroblastoma

We investigated plasma samples from patients with high-risk neuroblastoma (47) at diagnosis and during therapy and diagnostic plasma samples from pediatric patients with non–high-risk neuroblastoma (17), rhabdomyosarcoma (14), renal tumor (13), Hodgkin lymphoma (five), and cerebrospinal fluid (CSF) from CNS tumors (four). For clinical details, see the Data Supplement. We isolated cfDNA from 200 to 1,000 μL plasma or CSF and compared diagnostic plasma cfDNA levels (*ACTB*) with 24 healthy adult and 18 healthy pediatric plasma control samples, processed within 24 hours (Fig 2A, Table 1). Total cfDNA levels were significantly higher in patients with metastatic neuroblastoma and nephroblastoma compared with adult and pediatric controls ($P < .0001$, $P < .0001$, $P < .0001$, and $P = .0117$, respectively). Patients with localized neuroblastoma had significantly lower cfDNA levels compared with metastatic neuroblastoma ($P = .0004$) and were not significantly different from the adult and pediatric controls ($P = .4$ and $P > .99$, respectively). There was a trend to higher cfDNA levels in patients with rhabdomyosarcoma and Hodgkin lymphoma, which was only significant compared with adult controls ($P = .015$ and $P = .013$, respectively; Table 1).

Hypermethylated *RASSF1A* Is Detected in Diagnostic Plasma of Patients With Different Tumor Entities

At diagnosis, *RASSF1A* hypermethylation was detected in 41 of 42 patients with metastatic neuroblastoma (Fig 2B and Table 1). The one negative patient was stage MS and upstaged to stage M because of two new bone lesions. Hypermethylated *RASSF1A* was detected in all diagnostic plasma samples from patients with nephroblastoma and absent in plasma from two patients with Cystic Partially Differentiated Nephroblastoma and bilateral differentiated nephroblastomatosis, providing the possibility that only malignant tumors are detected by this marker. Eight of 14 plasma samples from patients with rhabdomyosarcoma were positive, as were 4 of 5 Hodgkin lymphoma plasma samples. Only one CSF sample from a patient with medulloblastoma was positive, and this was the sample with the highest cfDNA concentration.

Cell-Free Detection of Hypermethylated RASSF1A at Diagnosis and During Therapy

Plasma was available from 47 patients with high-risk neuroblastoma during the course of treatment. Clinical details, time of sampling, and ctDNA and mRNA results per sample can be found in the Data Supplement. Single nucleotide polymorphism array data confirmed 3p loss in 9 of 32 tumor samples, and in all nine patients, hypermethylated *RASSF1A* was detected in plasma, indicating that *RASSF1A* hypermethylation can still be identified in neuroblastoma with only one *RASSF1A* allele. At diagnosis, the absolute and relative levels of hypermethylated RASSF1A were significantly higher in the group of patients who will experience an event, although with a substantial overlap (median 37,243 copies/mL [interquartile range: 6,749-174,727] *v* 8,221 copies/mL [3,951-18,339], $P = .012$, 70.2% [45.0-91.7] *v* 56.5% [17.1-74.5], $P = .030$, respectively; Figs 3A and 3B). Receiver operating characteristic analysis revealed a cutoff of 27,681 hypermethylated *RASSF1A* copies/mL with a sensitivity of 64% and a specificity of 89% (Data Supplement) that identifies a group that has a significantly poorer event-free survival (Data Supplement, log-rank $P = .0007$). As the majority of the total cfDNA was tumor-derived, this led to a significant increase in cfDNA at diagnosis for patients who will experience an event (59,714 copies/mL [27,547-246,149] *v* 21,450 copies/mL [16,107-63,446], $P = .023$; Fig 3C). For other time points, there was no significant difference in total cfDNA levels between the patients with and without an event. At relapse, ctDNA levels were comparable with levels at diagnosis. Hypermethylated *RASSF1A* positivity did not correlate with an event for any of the time points (Fig 3D).

Comparison of ctDNA With the Detection of mRNA in BM and Blood

We previously showed that qPCR-based *RASSF1A* hypermethylation correlated with mRNA marker panel positivity or negativity in BM cells in patients when tumor burden was high or no tumor was detected.[33] Marker discrepancies indicated either low-level BM infiltration (ctDNA$^-$&mRNA panel$^+$) or primary tumor or soft tissue lesions without BM involvement (ctDNA$^+$&mRNA panel$^-$). To confirm these results in the current cohort, we tested cell fractions of corresponding blood (227) and BM (224) samples for mRNA markers[45] and compared them with hypermethylated *RASSF1A* in plasma by ddPCR. We again observe a strong correlation when the tumor load is to be expected high (at time of diagnosis or event) or absent (Fig 4), but see both ctDNA$^-$&mRNA$^+$ and vice versa when the tumor load is expected to be lower, for example, during therapy. In 227 matched blood samples, ctDNA was concordant with blood mRNA in 73% (75 ctDNA$^+$&mRNA$^+$

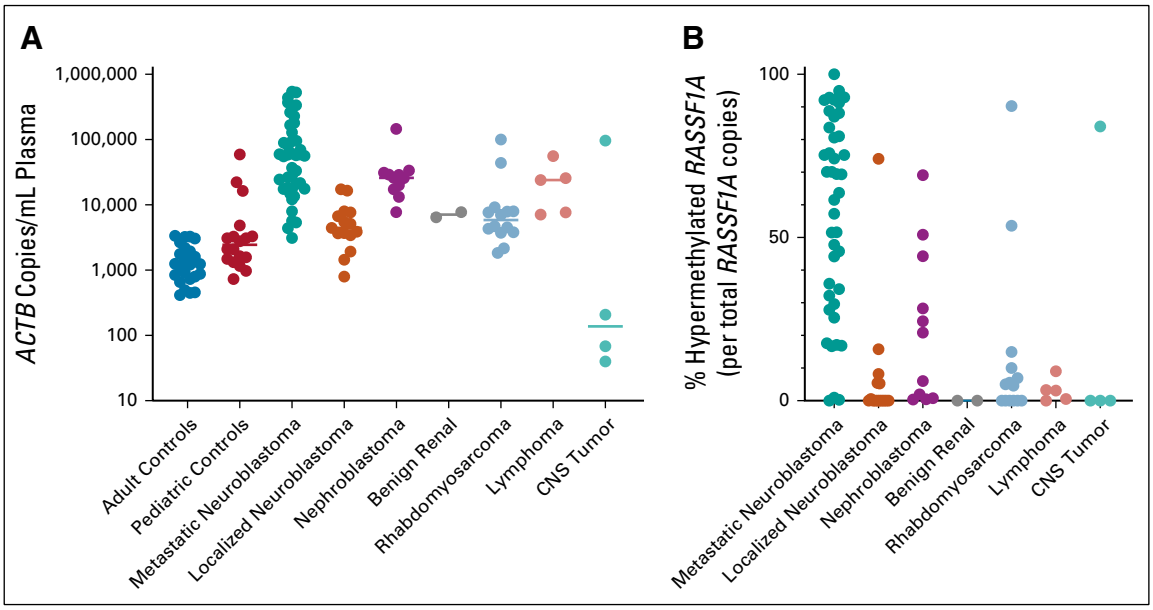

FIG 2. Amount of cfDNA and circulating hypermethylated *RASSF1A*. (A) Level of cfDNA at diagnosis in patients with various pediatric solid tumor entities, compared with healthy adult and pediatric controls. cfDNA was quantified by β-actin (*ACTB*), in copies/mL plasma or CSF (cerebrospinal fluid). Lines indicate the median. (B) The percentage of hypermethylated *RASSF1A* of total *RASSF1A* copies at diagnosis in patients with metastatic neuroblastoma (n = 42), localized neuroblastoma (n = 15), nephroblastoma (n = 11), rhabdomyosarcoma (n = 14), lymphoma (n = 5), and CNS tumors (n = 4). Adult and pediatric controls were used to establish a threshold for positivity. In 41 of 42 patients with metastatic neuroblastoma and 6 of 15 patients with localized neuroblastoma, hypermethylated *RASSF1A* was detected. In all 11 patients with nephroblastoma, 8 of 14 patients with rhabdomyosarcoma, 4 of 5 patients with lymphoma, and 1 of 4 patients with CNS tumor, hypermethylated *RASSF1A* was detected. Two plasma samples of patients with benign renal tumors (a Cystic Partially Differentiated Nephroblastoma and a bilateral differentiated nephroblastomatosis) were negative for hypermethylated *RASSF1A*. cfDNA, cell-free DNA; CFS, cerebrospinal fluid.

and 91 ctDNA$^-$&mRNA$^-$), 47 samples were ctDNA-positive only, and 14 samples mRNA-positive only. Spearman correlation of those 75 ctDNA$^+$&mRNA$^+$ indicated an association between ctDNA and mRNA results (r_s = 0.65, $P > .001$). In 224 matched BM mRNA and ctDNA blood samples, paired positive or negative results were found in 65% (103 and 43 samples, respectively). In contrast to the blood samples, BM mRNA–only identified more positive

TABLE 1. Levels of cfDNA and Circulating Hypermethylated *RASSF1A* in Various Pediatric Solid Tumor Entities and Adult and Pediatric Controls

Tumor	Total cfDNA (copies/mL)[a]	Hypermethylated RASSF1A–Positive Samples (No.)	Hypermethylated RASSF1A of Total RASSF1A[a] (%)	Hypermethylated RASSF1A (copies/mL)[a]
Metastatic neuroblastoma	56,996 (17,694-138,639)[b]	41 of 42	69.4 (34.1-83.7)	19,281 (5,170-55,196)
Localized neuroblastoma	4,431 (3,441-7,669)	6 of 15	6.8 (5.4-13.9)	440 (273-546)
Nephroblastoma	26,023 (17,390-31,177)[b]	11 of 11	20.9 (1.4-36.3)	2,250 (421-6,946)
Benign renal	6,462 and 7,731	0 of 2		
Rhabdomyosarcoma	5,893 (3,791-8,319)	8 of 14	8.5 (5.4-24.6)	263 (172-1,905)
Lymphoma	23,949 (7,406-40,873)	4 of 5	3.2 (2.5-4.7)	926 (220-1,638)
Medulloblastoma	138 (47-72,711)	1 of 4	84.1	89,336
Adult controls	1,232 (748-2,143)			
Pediatric controls	2,445 (1,446-3,694)			

Abbreviations: cfDNA, cell-free DNA; IQR, interquartile range.

[a]Median and IQR are given.

[b]Total cfDNA is significantly increased in patients with metastatic neuroblastoma compared with adult and pediatric controls, with $P < .0001$ and $P < .0001$, respectively, and in patients with nephroblastoma.

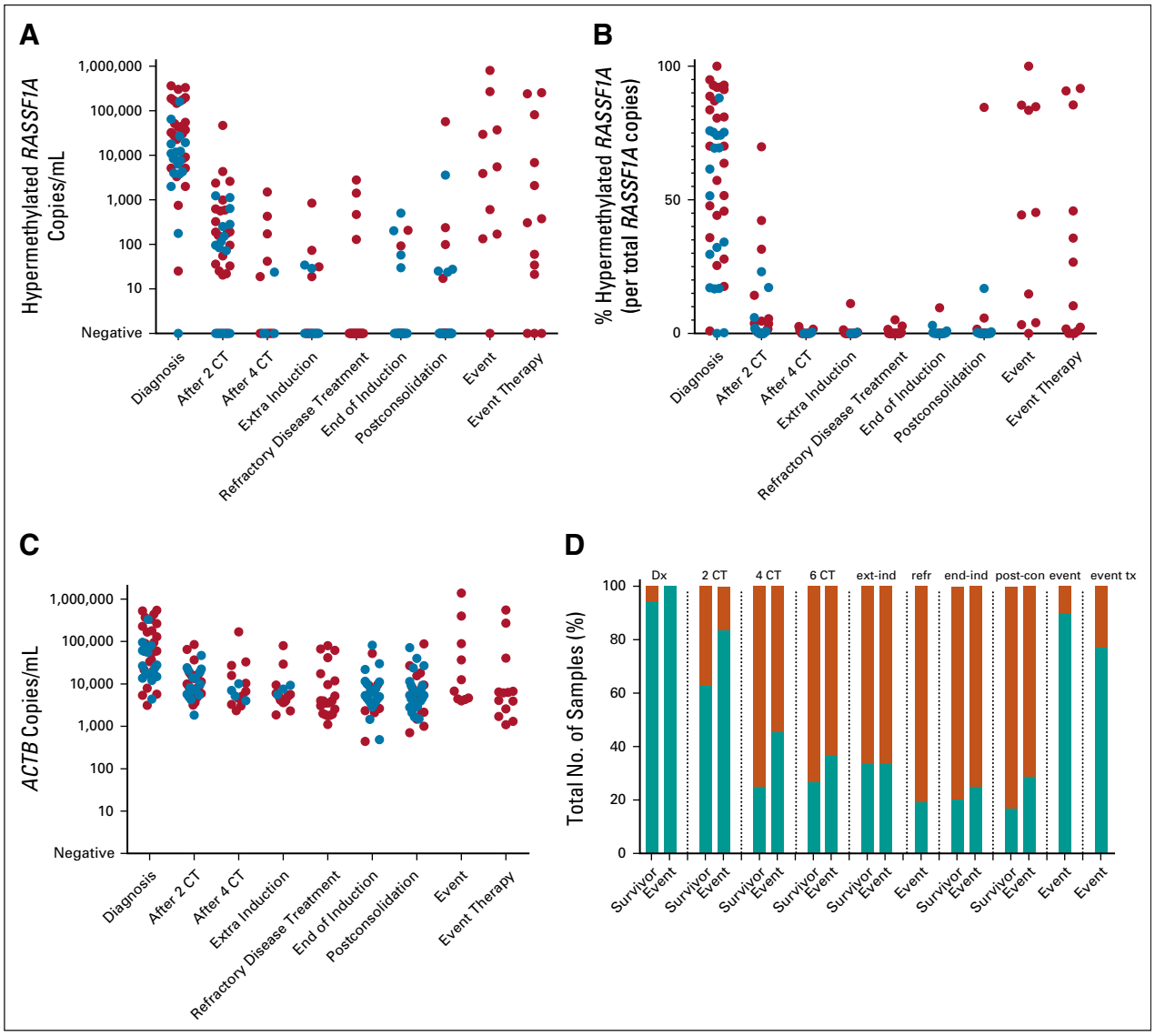

FIG 3. Positivity and levels of circulating hypermethylated *RASSF1A* and total cfDNA during therapy in patients with high-risk neuroblastoma. Red circles indicate samples from a patient who will suffer from an event, and blue circles indicate samples from patients who remain in complete remission (survivor). (A) Amount of hypermethylated *RASSF1A* in copies/mL plasma during therapy. (B) Relative levels of hypermethylated *RASSF1A* per total *RASSF1A* copies during therapy. (C) Levels of cfDNA, measured by β-actin (*ACTB*), in copies/mL plasma during therapy. (D) Fraction of total number of samples tested that were positive for circulating hypermethylated *RASSF1A*. Green bar represents positive samples, and orange bar represents negative samples. cfDNA, cell-free DNA; CT, cycles of chemotherapy; Dx, diagnosis; end-ind, end of induction; event tx, event therapy; ext-ind, extra induction therapy (not for refractory disease); post-con, postconsolidation; refr, refractory disease treatment.

samples (62) compared with ctDNA-only (16). Twenty-seven of those 62 samples were taken during induction chemotherapy. In 103 ctDNA$^+$&mRNA$^+$ samples, Spearman correlation indicated a moderate association between ctDNA and mRNA results (r_s = 0.49, $P > .001$).

Combined ctDNA and mRNA Detection Correlates With Outcomes

We next studied the kinetics of circulating hypermethylated *RASSF1A* and the mRNA markers from the corresponding BM and blood samples. Representative examples from five patients are depicted in Figures 5A-5E, and the combined outcome of circulating hypermethylated *RASSF1A* and BM mRNA for different time points is shown in Figure 5F. We showed that during therapy, the presence of hypermethylated *RASSF1A* in plasma was not associated with poorer prognosis at any of the time points in this patient cohort (Fig 3D). However, when circulating hypermethylated *RASSF1A* results were combined with BM mRNA, positivity with both techniques after two cycles of chemotherapy was associated with unfavorable clinical outcomes of these patients (P = .046; Fig 5F), with the sensitivity and specificity of the ctDNA$^+$&mRNA$^+$ profile being 74% and 63%, respectively. BM mRNA positivity

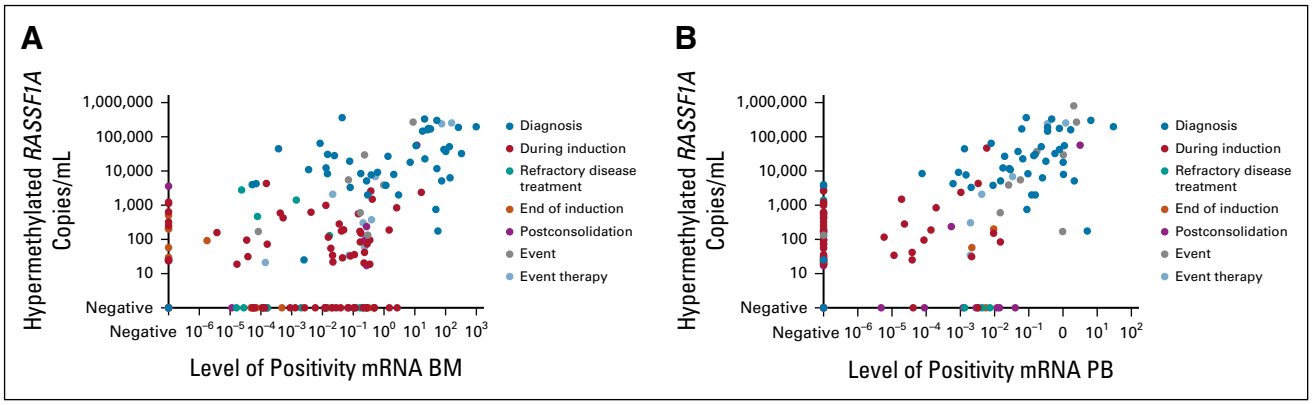

FIG 4. (A) Association between mRNA in BM samples and circulating hypermethylated *RASSF1A* and (B) association between mRNA in blood samples and circulating hypermethylated *RASSF1A*. BM, bone marrow; PB, peripheral blood.

alone at this time point was not predictive of the outcome in this cohort (P = .12). The trend that ctDNA$^+$&mRNA$^+$ positivity at other time points also correlates with an event was not significant in this small cohort. Remarkably, BM mRNA positivity alone during postconsolidation was associated with unfavorable outcomes (P = .077). In summary, the level of hypermethylated *RASSF1A* at diagnosis was correlated with unfavorable outcomes. Moreover, the combination of ctDNA with BM mRNA improved the predictive value after two cycles of chemotherapy in this cohort.

DISCUSSION

Molecular testing of cfDNA has the potential to improve pediatric solid tumor diagnosis, discrimination of subtypes, and MRD monitoring. Our aim was to complete a first step in this evolution of diagnostic modalities by evaluating our *RASSF1A* hypermethylation ddPCR as a standard test to detect ctDNA in several pediatric tumor types using small blood volumes and as a test to monitor treatment response of patients with neuroblastoma.

We previously described qPCR-based detection of circulating hypermethylated *RASSF1A* in patients with neuroblastoma.[33] In our previous study, the majority of positive samples could not be quantified reliably by qPCR, whereas ddPCR technology is adept for precise quantification of low abundant targets.[46] Furthermore, like in many other widely used methods to analyze DNA methylation, cfDNA samples in the qPCR study were bisulfite converted, which is known to degrade the majority of DNA.[47] As cfDNA is often present in low quantities, we investigated the use of an MSRE, previously described by Chan et al and O'Brien et al, as an alternative to bisulfite conversion.[43,44] We noticed higher hypermethylated *RASSF1A* levels in control samples with high total cfDNA levels, also reported by O'Brien et al.[44] We successfully introduced a combination of two MSREs, which resulted in better digestion of unmethylated *RASSF1A*. cfDNA may not always be present as double-stranded DNA, but can also appear as (partially) single-stranded DNA fragments.[48,49] Although the enzyme BstUI performed well in genomic DNA experiments, it is reported to be less active on single-stranded DNA.[50] The addition of HhaI overcomes this, as this enzyme is capable of digesting single-stranded DNA. The use of two different MSRE, and thus an increase in digestion sites, may result in digestion of DNA that is only partially methylated,[51] potentially underestimating present hypermethylated *RASSF1A*. However, as BstUI-only was clearly unable to digest all unmethylated *RASSF1A*, we proceeded with the use of two MSREs. The frequency of low-level positive results detected in healthy adult and pediatric controls defined the limit of detection. Since lack of remnants precluded the retesting of our qPCR study samples,[33] we showed in 16 rhabdomyosarcoma and renal tumor samples the slight superiority of the ddPCR method. In summary, the ddPCR is our preferred method to use for hypermethylated *RASSF1A* detection in plasma samples because the MSRE-ddPCR can reliably quantify ctDNA and saves time and sample.

We corroborate the potential of hypermethylated *RASSF1A* as a ctDNA marker for neuroblastoma, for monitoring treatment response and early relapse detection. This study confirms that cell-free hypermethylated *RASSF1A* correlates with mRNA marker panel positivity in BM and blood in patients at the opposite ends of the disease spectrum, when tumor burden was high or no tumor was detected.[32,33] The difference in kinetics of ctDNA and BM mRNA is illustrated by the prolonged presence of BM mRNA during induction therapy, whereas ctDNA rapidly declines during therapy, but is present again at relapse. The results of this study further support the finding, in an independent cohort, that both ctDNA and mRNA complement each other for the detection of MRD, with the combination showing a correlation with the outcome after two cycles of chemotherapy. Although the detection of ctDNA was shown to be very promising for future MRD studies in neuroblastoma, no definitive conclusions can be made as samples for this study were not prospectively collected, resulting in missing samples. Future research

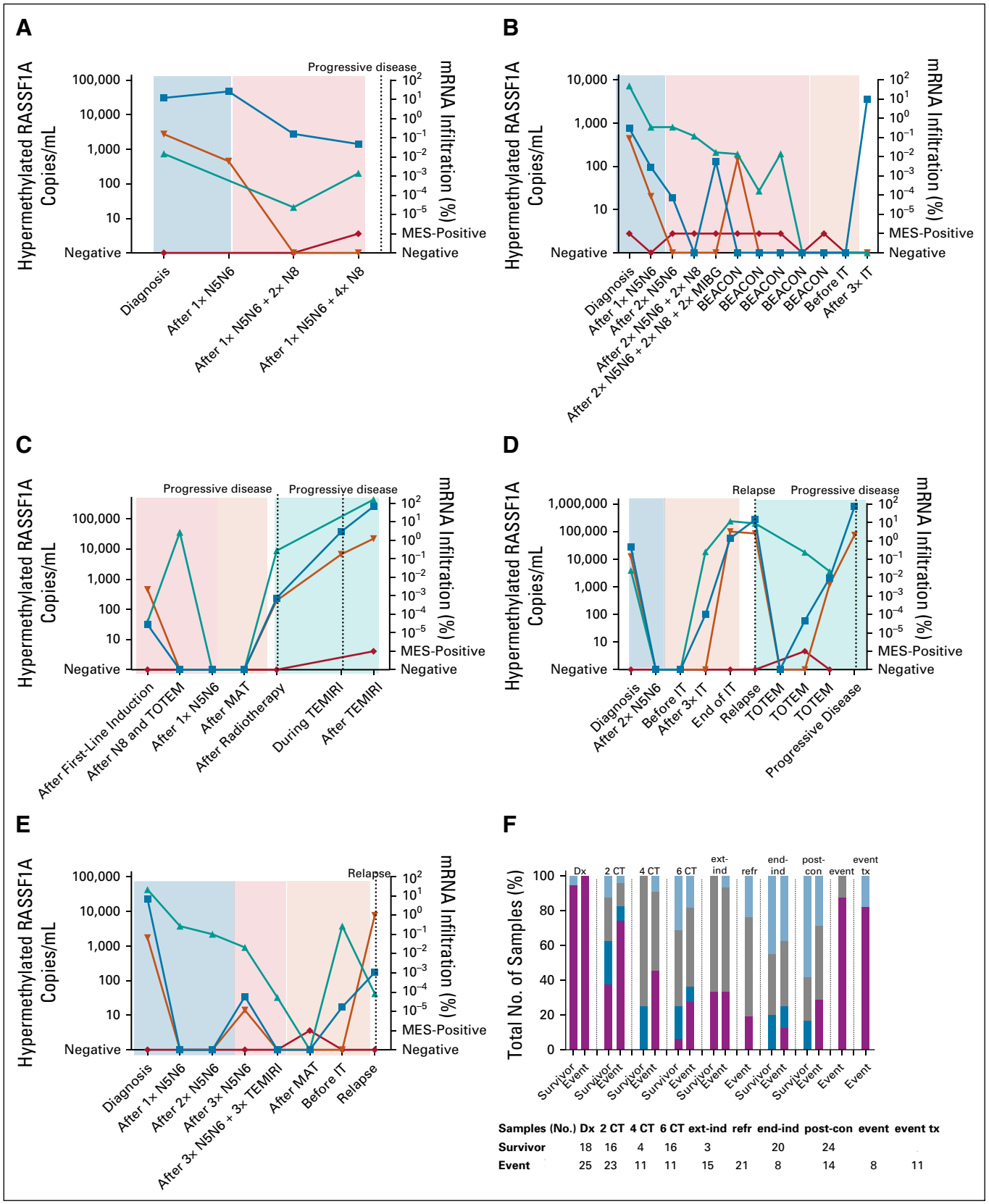

FIG 5. (A-E) For patients with refractory, relapse, or progressive disease, all sequential samples, if available, were analyzed for hypermethylated RASSF1A (blue squares; N2063, N2071, N2099, N2101, and N2123, respectively). Corresponding blood (orange triangles) and BM (green triangles for adrenergic markers and red diamonds for MES markers) samples were tested for mRNA. Colored blocks indicate the treatment: light blue, (continued on following page)

FIG 5. (continued) induction therapy; light red, extra induction therapy; light orange, postconsolidation therapy; light green, relapse or progressive disease treatment. (F) Fraction of total number of tested samples, which were positive for circulating hypermethylated *RASSF1A* and/or BM mRNA, of patients who will suffer an event compared with those who remain in complete remission (survivor). Purple bar represents hypermethylated *RASSF1A*$^+$ and mRNA panel$^+$ samples, dark blue bar represents hypermethylated *RASSF1A* ctDNA$^+$ and mRNA panel$^-$ samples, gray bar represents hypermethylated *RASSF1A*$^-$ and mRNA panel$^+$ samples, and light blue bar represents hypermethylated *RASSF1A* ctDNA$^-$/mRNA panel$^-$ samples. BEACON, TEMIRI, and TOTEM are treatment for refractory or relapsed disease. BEACON, BEACON-Neuroblastoma Trial: bevacizumab, temozolomide ± irinotecan; BM, bone marrow; CT, cycles of chemotherapy; ctDNA; circulating tumor DNA; Dx, diagnosis; end-ind, end of induction; event tx, event therapy; ext-ind, extra induction therapy (not for refractory disease); IT, immunotherapy; MAT, myeloablative therapy; MES, mesenchymal; MIBG, iodine-131-meta-iodobenzylguanidine; N5, N6, and N8; courses of induction chemotherapy; post-con, postconsolidation; refr, refractory disease treatment; TEMIRI, temozolomide and irinotecan; TOTEM, temozolomide and topotecan.

should be undertaken to investigate whether hypermethylated *RASSF1A* can be used as a marker during follow-up for early relapse detection and whether a cutoff can be used to predict event-free survival. As inactivation of *RASSF1A*, for example, by hypermethylation, is advantageous for many tumor entities, in melanoma, demethylation agents lead to apoptosis and cell death[52]; we think that this marker is not lost in time. We will test this hypothesis in prospective collaborative studies on the use of ctDNA in the new SIOPEN HR-2 (NCT04221035) patient cohort, which are being initiated within the SIOPEN liquid biopsy group.

Comparison of the total cfDNA levels in pediatric solid tumors with those of other studies confirms higher levels in patients with neuroblastoma and nephroblastoma tumors.[53-57] Consistent with literature, a high tumor-derived fraction of total cfDNA was found in patients with neuroblastoma and nephroblastoma, demonstrating the potential of liquid biopsies in these tumor entities.[54,56,58] Plasma samples from patients with other tumor entities in this study were less conclusive, which may indicate differences in the extent that different tumor types shed tumor DNA into circulation, a lower frequency of *RASSF1A* hypermethylation in other tumor entities,[29] or may just be artifacts of low sample numbers in the preliminary sample collection evaluated.

In this study, we developed a sensitive and quantitative ddPCR-based assay for hypermethylated *RASSF1A* detection and determined threshold values for positive results. Our findings demonstrate the value of hypermethylated *RASSF1A* as a molecular circulating tumor marker in neuroblastoma. Furthermore, our preliminary investigation of *RASSF1A* hypermethylation detection in circulating cfDNA demonstrates potential as a pan-tumor marker, but requires further investigation to evaluate its use and limitations.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2019. CA Cancer J Clin 69:7-34, 2019
2. Park JR, Kreissman SG, London WB, et al: Effect of tandem autologous stem cell transplant vs single transplant on event-free survival in patients with high-risk neuroblastoma. JAMA 322:746, 2019
3. Van Wezel EM, Zwijnenburg D, Zappeij-Kannegieter L, et al: Whole-genome sequencing identifies patient-specific DNA minimal residual disease markers in neuroblastoma. J Mol Diagn 17:43-52, 2015
4. Stahl M, Ranft A, Paulussen M, et al: Risk of recurrence and survival after relapse in patients with Ewing sarcoma. Pediatr Blood Cancer 57:549-553, 2011
5. Malempati S, Hawkins DS: Rhabdomyosarcoma: review of the Children's Oncology Group (COG) Soft-Tissue Sarcoma Committee experience and rationale for current COG studies. Pediatr Blood Cancer 59:5-10, 2012
6. Whelan JS, Davis LE: Osteosarcoma, chondrosarcoma, and chordoma. J Clin Oncol 36:188-193, 2018
7. Brok J, Lopez-Yurda M, Tinteren HV, et al: Relapse of Wilms' tumour and detection methods: A retrospective analysis of the 2001 Renal Tumour Study Group–International Society of Paediatric Oncology Wilms' tumour protocol database. Lancet Oncol 19:1072-1081, 2018
8. Park JR, Bagatell R, Cohn SL, et al: Revisions to the International Neuroblastoma Response Criteria: A consensus statement from the National Cancer Institute clinical trials planning meeting. J Clin Oncol 35:2580-2587, 2017
9. Viprey VF, Gregory WM, Corrias MV, et al: Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: A European HR-NBL1/SIOPEN study. J Clin Oncol 32:1074-1083, 2014
10. Cheung NK, Ostrovnaya I, Kuk D, et al: Bone marrow minimal residual disease was an early response marker and a consistent independent predictor of survival after anti-GD2 immunotherapy. J Clin Oncol 33:755-763, 2015
11. Kreissman SG, Seeger RC, Matthay KK, et al: Purged versus non-purged peripheral blood stem-cell transplantation for high-risk neuroblastoma (COG A3973): A randomised phase 3 trial. Lancet Oncol 14:999-1008, 2013
12. Burchill SA, Beiske K, Shimada H, et al: Recommendations for the standardization of bone marrow disease assessment and reporting in children with neuroblastoma on behalf of the International Neuroblastoma Response Criteria Bone Marrow Working Group. Cancer 123:1095-1105, 2017
13. Lak NS, Voormanns TL, Zappeij-Kannegieter L, et al: Improving risk stratification for pediatric patients with rhabdomyosarcoma by molecular detection of disseminated disease. Clin Cancer Res 10.1158/1078-0432.CCR-21-1083 [epub ahead of print on July 20, 2021]
14. Stutterheim J, Zappeij-Kannegieter L, Versteeg R, et al: The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma. Eur J Cancer 47:1193-1202, 2011
15. van Wezel EM, van Zogchel LM, van Wijk J, et al: Mesenchymal neuroblastoma cells are undetected by current mRNA marker panels: The development of a specific neuroblastoma mesenchymal minimal residual disease panel. JCO Precis Oncol 3:1-11, 2019
16. Wan JCM, Massie C, Garcia-Corbacho J, et al: Liquid biopsies come of age: Towards implementation of circulating tumour DNA. Nat Rev Cancer 17:223-238, 2017
17. Siravegna G, Marsoni S, Siena S, et al: Integrating liquid biopsies into the management of cancer. Nat Rev Clin Oncol 14:531-548, 2017
18. Rolfo C, Cardona AF, Cristofanilli M, et al: Challenges and opportunities of cfDNA analysis implementation in clinical practice: Perspective of the International Society of Liquid Biopsy (ISLB). Crit Rev Oncol Hematol 151:102978, 2020
19. Lawrence MS, Stojanov P, Polak P, et al: Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 499:214-218, 2013
20. Klega K, Imamovic-Tuco A, Ha G, et al: Detection of somatic structural variants enables quantification and characterization of circulating tumor DNA in children with solid tumors. JCO Precis Oncol 10.1200/PO.17.00285
21. Lawlor ER, Thiele CJ: Epigenetic changes in pediatric solid tumors: Promising new targets. Clin Cancer Res 18:2768-2779, 2012
22. Hesson LB, Cooper WN, Latif F: The role of RASSF1A methylation in cancer. Dis Markers 23:73-87, 2007
23. Donninger H, Vos MD, Clark GJ: The RASSF1A tumor suppressor. J Cell Sci 120:3163-3172, 2007
24. Grawenda AM, O'Neill E: Clinical utility of RASSF1A methylation in human malignancies. Br J Cancer 113:372-381, 2015
25. Dubois F, Bergot E, Zalcman G, et al: RASSF1A, puppeteer of cellular homeostasis, fights tumorigenesis, and metastasis—An updated review. Cell Death Dis 10:928, 2019
26. Malpeli G, Innamorati G, Decimo I, et al: Methylation dynamics of RASSF1A and its impact on cancer. Cancers (Basel) 11:959, 2019
27. Hogg RP, Honorio S, Martinez A, et al: Frequent 3p allele loss and epigenetic inactivation of the RASSF1A tumour suppressor gene from region 3p21.3 in head and neck squamous cell carcinoma. Eur J Cancer 38:1585-1592, 2002
28. Abe M, Ohira M, Kaneda A, et al: CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. Cancer Res 65:828-834, 2005
29. Harada K, Toyooka S, Maitra A, et al: Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. Oncogene 21:4345-4349, 2002
30. Hoebeeck J, Michels E, Pattyn F, et al: Aberrant methylation of candidate tumor suppressor genes in neuroblastoma. Cancer Lett 273:336-346, 2009
31. Misawa a, Tanaka S, Yagyu S, et al: RASSF1A hypermethylation in pretreatment serum DNA of neuroblastoma patients: A prognostic marker. Br J Cancer 100:399-404, 2009
32. Stutterheim J, Ichou FA, Den Ouden E, et al: Methylated RASSF1a is the first specific DNA marker for minimal residual disease testing in neuroblastoma. Clin Cancer Res 18:808-814, 2012

33. Van Zogchel LMJ, Van Wezel EM, Van Wijk J, et al: Hypermethylated RASSF1A as circulating tumor DNA marker for disease monitoring in neuroblastoma. JCO Precis Oncol 4:291-306, 2020
34. Wong IHN, Chan J, Wong J, et al: Ubiquitous aberrant RASSF1A promoter methylation in childhood neoplasia. Clin Cancer Res 10:994-1002, 2004
35. Kiss NB, Kogner P, Johnsen JI, et al: Quantitative global and gene-specific promoter methylation in relation to biological properties of neuroblastomas. BMC Med Genet 13:1-12, 2012
36. Honda S, Miyagi H, Suzuki H, et al: RASSF1A methylation indicates a poor prognosis in hepatoblastoma patients. Pediatr Surg Int 29:1147-1152, 2013
37. Wagner KJ, Cooper WN, Grundy RG, et al: Frequent RASSF1A tumour suppressor gene promoter methylation in Wilms' tumour and colorectal cancer. Oncogene 21:7277-7282, 2002
38. Ehrlich M, Jiang G, Fiala E, et al: Hypomethylation and hypermethylation of DNA in Wilms tumors. Oncogene 21:6694-6702, 2002
39. Mühlisch J, Schwering A, Grotzer M, et al: Epigenetic repression of RASSF1A but not CASP8 in supratentorial PNET (sPNET) and atypical teratoid/rhabdoid tumors (AT/RT) of childhood. Oncogene 25:1111-1117, 2006
40. Lim S, Yang MH, Park JH, et al: Inactivation of the RASSF1A in osteosarcoma. Oncol Rep 10:897-901, 2003
41. Wang WG, Chen SJ, He JS, et al: The tumor suppressive role of RASSF1A in osteosarcoma through the Wnt signaling pathway. Tumour Biol 37:8869-8877, 2016
42. Avigad S, Shukla S, Naumov I, et al: Aberrant methylation and reduced expression of RASSF1A in Ewing sarcoma. Pediatr Blood Cancer 53:1023-1028, 2009
43. Chan KC, Ding C, Gerovassili A, et al: Hypermethylated RASSF1A in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. Clin Chem 52:2211-2218, 2006
44. O'Brien H, Hyland C, Schoeman E, et al: Non-invasive prenatal testing (NIPT) for fetal Kell, Duffy and Rh blood group antigen prediction in alloimmunised pregnant women: Power of droplet digital PCR. Br J Haematol 189:e90-e94, 2020
45. van Zogchel LM, Zappeij-Kannegieter L, Javadi A, et al: Specific and sensitive detection of neuroblastoma mRNA markers by multiplex RT-qPCR. Cancers (Basel) 13:150, 2021
46. Taylor SC, Laperriere G, Germain H: Droplet digital PCR versus qPCR for gene expression analysis with low abundant targets: From variable nonsense to publication quality data. Scientific Rep 7:1-8, 2017
47. Grunau C, Clark S, Rosenthal A: Bisulfite genomic sequencing: Systematic investigation of critical experimental parameters. Nucleic Acids Res 29:e65, 2001
48. Sanchez C, Snyder MW, Tanos R, et al: New insights into structural features and optimal detection of circulating tumor DNA determined by single-strand DNA analysis. NPJ Genom Med 3:31, 2018
49. Burnham P, Kim MS, Agbor-Enoh S, et al: Single-stranded DNA library preparation uncovers the origin and diversity of ultrashort cell-free DNA in plasma. Scientific Rep 6:1-9, 2016
50. The Restriction Enzyme Database (REBASE). http://rebase.neb.com/rebase/rebase.html
51. Mikeska T, Candiloro IL, Dobrovic A: The implications of heterogeneous DNA methylation for the accurate quantification of methylation. Epigenomics 2:561-573, 2010
52. McKenna S, García-Gutiérrez L: Resistance to targeted therapy and RASSF1A loss in melanoma: What are we missing? Int J Mol Sci 22:5115, 2021
53. Bettegowda C, Sausen M, Leary RJ, et al: Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 6:224ra24, 2014
54. Chicard M, Boyault S, Daage LC, et al: Genomic copy number profiling using circulating free tumor DNA highlights heterogeneity in neuroblastoma. Clin Cancer Res 22:5564-5573, 2016
55. Chicard M, Colmet-Daage L, Clement N, et al: Whole-exome sequencing of cell-free DNA reveals temporo-spatial heterogeneity and identifies treatment-resistant clones in neuroblastoma. Clin Cancer Res 24:939-949, 2018
56. Jiménez I, Chicard M, Colmet-Daage L, et al: Circulating tumor DNA analysis enables molecular characterization of pediatric renal tumors at diagnosis. Int J Cancer 144:68-79, 2019
57. Su Y, Wang L, Jiang C, et al: Increased plasma concentration of cell-free DNA precedes disease recurrence in children with high-risk neuroblastoma. BMC Cancer 20:102, 2020
58. Paemel RV, Koker AD, Vandeputte C, et al: Minimally invasive classification of paediatric solid tumours using reduced representation bisulphite sequencing of cell-free DNA: A proof-of-principle study. Epigenetics 16:196-208, 2020

