BIOMARKERS

original reports abstract

Cell-Free DNA as a Diagnostic and Prognostic Biomarker in Pediatric Rhabdomyosarcoma

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PURPOSE Total cell-free DNA (cfDNA) and tumor-derived cfDNA (ctDNA) can be used to study tumor-derived genetic aberrations. We analyzed the diagnostic and prognostic potential of cfDNA and ctDNA, obtained from pediatric patients with rhabdomyosarcoma.

METHODS cfDNA was isolated from diagnostic plasma samples from 57 patients enrolled in the EpSSG RMS2005 study. To study the diagnostic potential, shallow whole genome sequencing (shWGS) and cell-free reduced representation bisulphite sequencing (cfRRBS) were performed in a subset of samples and all samples were tested using droplet digital polymerase chain reaction to detect methylated *RASSF1A* (*RASSF1A*-M). Correlation with outcome was studied by combining cfDNA *RASSF1A*-M detection with analysis of our rhabdomyosarcoma-specific RNA panel in paired cellular blood and bone marrow fractions and survival analysis in 56 patients.

RESULTS At diagnosis, ctDNA was detected in 16 of 30 and 24 of 26 patients using shallow whole genome sequencing and cfRRBS, respectively. Furthermore, 21 of 25 samples were correctly classified as embryonal by cfRRBS. *RASSF1A*-M was detected in 21 of 57 patients. The presence of *RASSF1A*-M was significantly correlated with poor outcome (the 5-year event-free survival [EFS] rate was 46.2% for 21 *RASSF1A*-M–positive patients, compared with 84.9% for 36 *RASSF1A*-M–negative patients [$P < .001$]). *RASSF1A*-M positivity had the highest prognostic effect among patients with metastatic disease. Patients both negative for *RASSF1A*-M and the rhabdomyosarcoma-specific RNA panel (28 of 56 patients) had excellent outcome (5-year EFS 92.9%), while double-positive patients (11/56) had poor outcome (5-year EFS 13.6%, $P < .001$).

CONCLUSION Analyzing ctDNA at diagnosis using various techniques is feasible in pediatric rhabdomyosarcoma and has potential for clinical use. Measuring *RASSF1A*-M in plasma at initial diagnosis correlated significantly with outcome, particularly when combined with paired analysis of blood and bone marrow using a rhabdomyosarcoma-specific RNA panel.

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INTRODUCTION

Rhabdomyosarcoma, the most common sarcoma among children and adolescents, accounts for approximately 3% of pediatric tumors.[1] Despite considerable research regarding treatment and risk stratification, 1 of 3 patients will experience relapse.[2-6] The use of liquid biopsies in pediatric patients is drawing growing interest.[7,8] Our group reported that the presence of rhabdomyosarcoma-derived mRNA in the cellular fraction of peripheral blood (PB) and bone marrow (BM) at initial diagnosis is correlated with poor outcome and could potentially improve current risk stratification.[9] Studies on other pediatric solid tumors demonstrated cell-free DNA (cfDNA) analysis from plasma to provide added value for diagnostics, prognostics, and response monitoring.[10-16] In rhabdomyosarcoma, the presence of tumor-derived cfDNA (ctDNA) has been shown to correlate to tumor burden throughout treatment in a few small case series.[17,18] ctDNA can be studied using various techniques, using genetic aberrations present in rhabdomyosarcoma. The alveolar subtype has a tumor-driving fusion between the *PAX3* or *PAX7* gene and the *FOXO1* gene. Epigenetic analyses revealed distinct methylation profiles in alveolar and embryonal rhabdomyosarcoma, allowing for the classification of cases into fusion-positive versus fusion-negative tumors.[19,20] Van Paemel et al[21] showed that these distinct methylation patterns can be detected in ctDNA from diagnostic plasma, using cell-free reduced representation bisulphite sequencing (cfRRBS) to correctly classify rhabdomyosarcoma as either the embryonal or alveolar subtype. Copy number aberrations (CNAs) have been

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

In pediatric rhabdomyosarcoma, the use of liquid biopsies can assist in generating a more comprehensive view of the molecular landscape of the tumor. We explore different methods for analysis of cell-free DNA (cfDNA) from plasma by cell-free reduced representation bisulphite sequencing, shallow whole genome sequencing, and droplet digital polymerase chain reaction for *RASSF1A* methylation (*RASSF1*-M). Furthermore, we study whether combining cfDNA analyses with detection of rhabdomyosarcoma-specific RNA in the cellular fraction of blood and bone marrow (BM) has a complementary value.

Knowledge Generated

Both cell-free reduced representation bisulphite sequencing and shallow whole genome sequencing have diagnostic potential, whereas the presence of *RASSF1A*-M at diagnosis correlates to poor survival, especially in patients testing positive for rhabdomyosarcoma-specific RNA in cells from blood and BM.

Relevance

Analysis of cfDNA through different molecular approaches can be of additional value to current clinical risk stratification, especially the detection of *RASSF1A*-M in cfDNA and rhabdomyosarcoma-specific RNA in paired blood and BM.

found to occur in several chromosomes.[3,22] These can be analyzed in cfDNA by shallow whole genome sequencing (shWGS).[23] Recently, Van Paemel et al[16] showed that shWGS data from cfDNA can be complementary to CNA analysis on the primary tumor.

However, cfDNA typically contains a relatively small amount of ctDNA; the remaining cfDNA is derived from healthy cells, which can cause high background noise and limit the ability to detect a tumor-derived signal.[24] To overcome this, a tumor-specific assay can be used, such as droplet digital polymerase chain reaction (ddPCR) which is highly sensitive and less expensive.[25] A target suited for analysis by ddPCR is methylation of the tumor-suppressor gene *RASSF1A*; this gene has been shown to be silenced by methylation in several adult[26] and pediatric[27-30] tumors. Moreover, methylated *RASSF1A* (*RASSF1A*-M) has been detected in cfDNA in patients with neuroblastoma.[10,31] Recently, we developed a methylation-specific enzyme-based approach involving ddPCR to detect *RASSF1A*-M in several pediatric solid tumors, including rhabdomyosarcoma.[14]

Here, we report the detection of ctDNA in plasma of patients with rhabdomyosarcoma for diagnostic purposes, such as cfRBBS and shWGS. Furthermore, we study the prognostic potential of *RASSF1A*-M detection in cfDNA and measure the added value of combining *RASSF1A*-M ctDNA detection with our rhabdomyosarcoma-specific mRNA panel in paired BM and PB samples.

METHODS

Patients and Sample Collection

Plasma samples were collected prospectively from the same cohort described in our previous article,[9] consisting of all patients included in the Dutch Minimal Residual Disease add-on study within the EpSSG RMS2005 trial (EudraCT number: 2005-000217-35) from 2013 through July 2019. Informed consent was given via the EpSSG RMS2005 trial until 2017. From 2017, consent was provided if the patients/caretakers consented to the collection of samples for biobanking. PB was collected in EDTA tubes (Becton-Dickinson, Franklin Lakes, NJ) and processed within 24 hours. Plasma was obtained by centrifuging the blood samples at 1,375×*g* for 10 minutes and stored at −20°C until further processing. Matched tumor material was not available.

CfRRBS and shWGS

We performed cfRRBS[21] and shWGS[16,32] on cfDNA as described and validated previously. In brief, cfDNA was isolated from 200 μL of plasma as described previously.[16,21,32] For shWGS, the modified copy number profile abnormality score was calculated in order to quantify the copy number tumor burden present in the cfDNA.[16] On the basis of 80 healthy volunteers, the level corresponding to a 1% false discovery rate was set at 0.355 for shWGS.

ddPCR Assay for Measuring *RASSF1A*-M

For ddPCR, cfDNA was isolated from plasma samples using the Quick-cfDNA Serum & Plasma kit (Zymo Research, Irvine, CA). The *RASSF1A*-M ddPCR assay was performed using double digestion with the methylation-sensitive restriction enzymes HhaI and Bsh1236I BstUI; Thermo Fisher Scientific, Waltham, MA) using a thermocycler T100 and QX200 reader (Bio-Rad, Hercules, CA) as described previously.[14] The sequences and concentrations of the primers and probes, cycling conditions, and analyses were performed as described previously, with the threshold for *RASSF1A*-M positivity per sample set at ≥ 14 copies/ml and ≥ 4 *RASSF1A*-M–positive droplets, as determined in 18 healthy pediatric and 22 adult control plasmas.[14] The percentage of *RASSF1A*-M was calculated relative to total *RASSF1A*. On the basis of the plasma volume available

(ranging from 150 μL to 1 mL), different amounts of plasma were used to isolate cfDNA. To correct for variations in the amount of input plasma, cfDNA is reported in ng/mL plasma. In all ddPCR assays, total cfDNA was determined using the reference gene *ACTB*. Since there was no matched tumor material available, we used data on *RASSF1A*-M in rhabdomyosarcoma tumors from published data sets from Clay et al,[33] Koelsche et al,[34] and specifically requested data from Seki et al.[20] Data from Clay and Koelsche were analyzed in R2.[35] We focused on hypermethylation of the promotor region of *RASSF1A* as this is typically hypermethylated in cancer.[36] We calculated the mean beta value and report the range of the beta values.[37,38]

Detection of Rhabdomyosarcoma-Specific mRNA Using an RNA Panel

Rhabdomyosarcoma-specific mRNA was detected in the cellular fractions of matched diagnostic patient PB and BM samples using our previously reported 11-marker RNA panel.[9] The RNA panel was considered positive if either PB or BM was positive.

Statistical Analysis

Statistical analyses were performed using SPSS version 23. Figures were generated using GraphPad Prism version 8. The correlation between continuous variables was determined using Pearson's test. Continuous variables were analyzed using the nonparametric Mann-Whitney *U* test, and two or more groups were analyzed using the Kruskal-Wallis test. Independence between two categorical variables was determined using the nonparametric Pearson chi-square test. Event-free survival (EFS) and overall survival (OS) were estimated using the Kaplan-Meier approach, and differences in survival were analyzed using the log-rank test. Differences were considered significant at $P < .05$.

RESULTS

Patient and Sample Characteristics

We collected a total of 152 plasma samples from 65 patients, treated according to the EpSSG RMS2005 protocol; diagnostic plasma samples were available for 57 patients. The patient characteristics, assigned risk group and tumor histology, are summarized in Table 1. The median follow-up was 4.21 years (range, 0.34-10.60 years).

Diagnostic Potential of Various Molecular Techniques for Detecting ctDNA

First, total cfDNA levels at diagnosis were determined by measuring *ACTB* using ddPCR for all samples. No significant differences in total cfDNA levels were observed between patients with respect to tumor histology, risk group, localized versus metastatic disease, tumor size, or event-free survival (Data Supplement). Next, we examined the feasibility to detect ctDNA using cfRRBS, shWGS, and ddPCR (Table 2, Data Supplement). Overall, in 39 of 57 patients (68.4%), at least one of these techniques detected ctDNA in diagnostic plasma samples. Please note that cfRRBS and shWGS were tested on a subset of samples.

TABLE 1. Patient Characteristics of the Patients With Rhabdomyosarcoma (N = 65)

Characteristic	N (%)
Age at diagnosis, years	
< 1	1 (1.5)
1-10	38 (58.5)
> 10	26 (40.0)
Sex	
Female	23 (35.4)
Male	42 (64.6)
Histology	
Alveolar rhabdomyosarcoma	22 (33.8)
Embryonal rhabdomyosarcoma	40 (61.5)
Botryoid rhabdomyosarcoma	1 (1.5)
Spindle cell/leiomyomatous rhabdomyosarcoma	1 (1.5)
Rhabdomyosarcoma not otherwise specified	1 (1.5)
Postsurgical tumor staging (IRS grouping)	
I	4 (6.3)
II	11 (16.9)
III	27 (41.5)
IV	23 (35.4)
Tumor size, cm	
≤ 5	29 (44.6)
> 5	36 (55.4)
Regional lymph node involvement	
No evidence of lymph node involvement	42 (64.6)
Evidence of regional lymph node involvement	22 (33.8)
No information about lymph node involvement	1 (1.5)
Risk group	
Low risk	1 (1.5)
Standard risk	24 (36.9)
High risk	14 (21.5)
Very high risk	3 (4.6)
Metastatic	23 (35.4)
Site of origin of primary tumor	
Orbit	10 (15.4)
Head neck nonparameningeal	5 (7.7)
Parameningeal	10 (15.4)
Bladder prostate	8 (12.3)
Genitourinary nonbladder prostate	10 (15.4)
Extremities	14 (21.5)
Other sites	8 (12.3)

Abbreviation: IRS, intergroup rhabdomyosarcoma studies.

Methylation profiling for diagnostic classification. As negative control, cfRRBS was performed on 31 samples from

TABLE 2. Overview of the Results of Different Approaches on Cell-Free DNA of n = 57 Diagnostic Plasma Samples

Technique	Result	N (%)
RASSF1A-M ddPCR (n = 57)	Positive	21 (36.8)
	Negative	36 (63.2)
cfRRBS (n = 26)	Embryonal subtype	21 (80.8)[a]
	Alveolar subtype	3 (11.5)[b]
	No tumor DNA	2 (7.7)
shWGS (n = 30)	CNA present	16 (53.3)
	Flat	11 (36.7)
	Fail	3 (10.0)

Abbreviations: cfRRBS, cell-free reduced representation bisulphite sequencing; CNA, copy number aberration; ddPCR, droplet digital polymerase chain reaction; *RASSF1A*-M, methylated *RASSF1A*; shWGS, shallow whole genome sequencing.

[a]One case was originally classified as botryoid on the basis of the clinical diagnosis.

[b]All three of these cases were originally classified as embryonal on the basis of the clinical diagnosis.

healthy controls, all classified correctly as normal (Data Supplement). We applied cfRRBS to diagnostic samples from 24 patients with the embryonal subtype, 1 with botryoid subtype, and 1 with alveolar subtype, successfully detecting rhabdomyosarcoma DNA in 24 of these 26 samples (92.3% of cases). Twenty of these samples were correctly identified as embryonal tumors. Three cases with embryonal histology were classified as alveolar, one case of botryoid rhabdomyosarcoma was classified as embryonal, and no tumor DNA was detected in two samples (one alveolar and one embryonal).

CNAs. We performed shWGS on 30 plasma samples and obtained a median copy number profile abnormality score of 0.35 (range, 0.27-3.94; Data Supplement). In three cases (two embryonal and one alveolar), the analysis failed (Table 2, Data Supplement). Twelve embryonal cases (7 of 12 metastatic) and four alveolar (all metastatic) cases had CNAs, while 10 embryonal cases and one botryoid case had no CNA. Most CNAs were detected in patients with metastatic disease, and 7 of 16 (43.8%) patients with detectable CNAs suffered from an event.

Methylated RASSF1A. Using data from Clay et al,[33] Koelsche et al,[34] and Seki et al,[20] the mean beta value of *RASSF1A*-M was 0.550 (range, 0.032-0.933; Data Supplement). We next examined the presence of RASSF1A-M in plasma using ddPCR. Methylated RASSF1A was detectable in 21 of 57 diagnostic plasma samples; 9 of 37 embryonal cases, 10 of 17 alveolar cases, 1 of 1 spindle case, 0 botryoid case, and one not-otherwise-specified case, with a median *RASSF1A*-M concentration of 2.46 ng/mL (range, 0.22-273.11 ng/mL). In patients with alveolar tumors and metastatic disease, methylated RASSF1A was more frequently detected, compared with embryonal histology ($P = .014$) and localized disease (*RASSF1A*-M–positive in 9 of 37 patients with localized, 12 of 20 patients with metastatic disease $P = .008$). The total level of *RASSF1A*-M varied widely within the *RASSF1A*-M–positive samples and was correlated with tumor histology (Data Supplement). To correct for variations in total cfDNA, we calculated the percentage of *RASSF1A*-M relative to total *RASSF1A* for each patient, yielding a median percentage of 15.1% (range, 2.0%-92.7%) for the *RASSF1A*-M–positive samples. Although metastatic and alveolar tumors more often show the presence of *RASSF1A*-M in cfDNA, the *RASSF1A*-M percentage in positive samples was similar in alveolar and embryonal tumors ($P = .55$) and in localized and metastatic cases ($P = .35$; Figs 1A and 1B). We found no correlation between tumor size at diagnosis and either total *RASSF1A*-M ($r = 0.132$ and $P = .64$; Data Supplement) or the percentage of *RASSF1A*-M ($r = -.229$ and $P = .41$; Fig 1C). Finally, we found no difference in total cfDNA levels (*ACTB*) between *RASSF1A*-M–positive and *RASSF1A*-M–negative cases ($P = .96$; Fig 1D).

Cell-Free *RASSF1A*-M Correlates With Poor Outcome

We examined whether the detection of ctDNA in 57 diagnostic plasma samples was associated with patient outcome. Eleven of 21 (52.3%) *RASSF1A*-M–positive patients suffered from an event.

The 5-year EFS rate was 46.2% for the *RASSF1A*-M–positive patients, compared with 84.9% for the *RASSF1A*-M–negative patients ($P = .001$; Fig 2A), and the 5-year OS rate was 55.7% for the *RASSF1A*-M–positive patients, compared with 100% for the *RASSF1A*-M–negative patients ($P < .001$; Fig 2B). The prognostic value of detecting *RASSF1A*-M at diagnosis was attributed almost exclusively to patients with metastasized disease (Figs 2C and 2D, Data Supplement).

In 27 samples, both shWGS and *RASSF1A*-M were performed (Data Supplement). In six patients, shWGS was positive while *RASSF1A*-M was negative, and only one patient suffered from an event, while 6 of 10 double-positive patients suffered from an event, suggesting that the presence of both *RASSF1A*-M and ctDNA by shWGS may be more prognostic than detection of ctDNA by shWGS alone.

We next examined whether combining *RASSF1A*-M detection with detection of rhabdomyosarcoma-specific mRNA (on the basis of our previously published mRNA panel[9]) tested in 56 matched diagnostic PB and BM samples could improve the predictive value. Rhabdomyosarcoma-specific mRNA was detected in 18 of 56 PB and/or BM samples (8 of 18 tested positive on conventional BM histology, Data Supplement). Five-year EFS ranged from 92.9% to 13.6% for *RASSF1A*-M^{neg}/mRNA panel^{neg} and *RASSF1A*-M^{pos}/mRNA panel^{pos} ($P = .006$) and 5 years OS from 100% to 36.4% for *RASSF1A*-M^{neg}/mRNA panel^{neg} and *RASSF1A*-M^{pos}/mRNA panel^{pos}, respectively ($P < .001$; Figs 3A and 3B).

To validate the association of *RASSF1A*-M to clinical outcome, we performed univariate and multivariable Cox regression analyses for EFS (Data Supplement and Table 3,

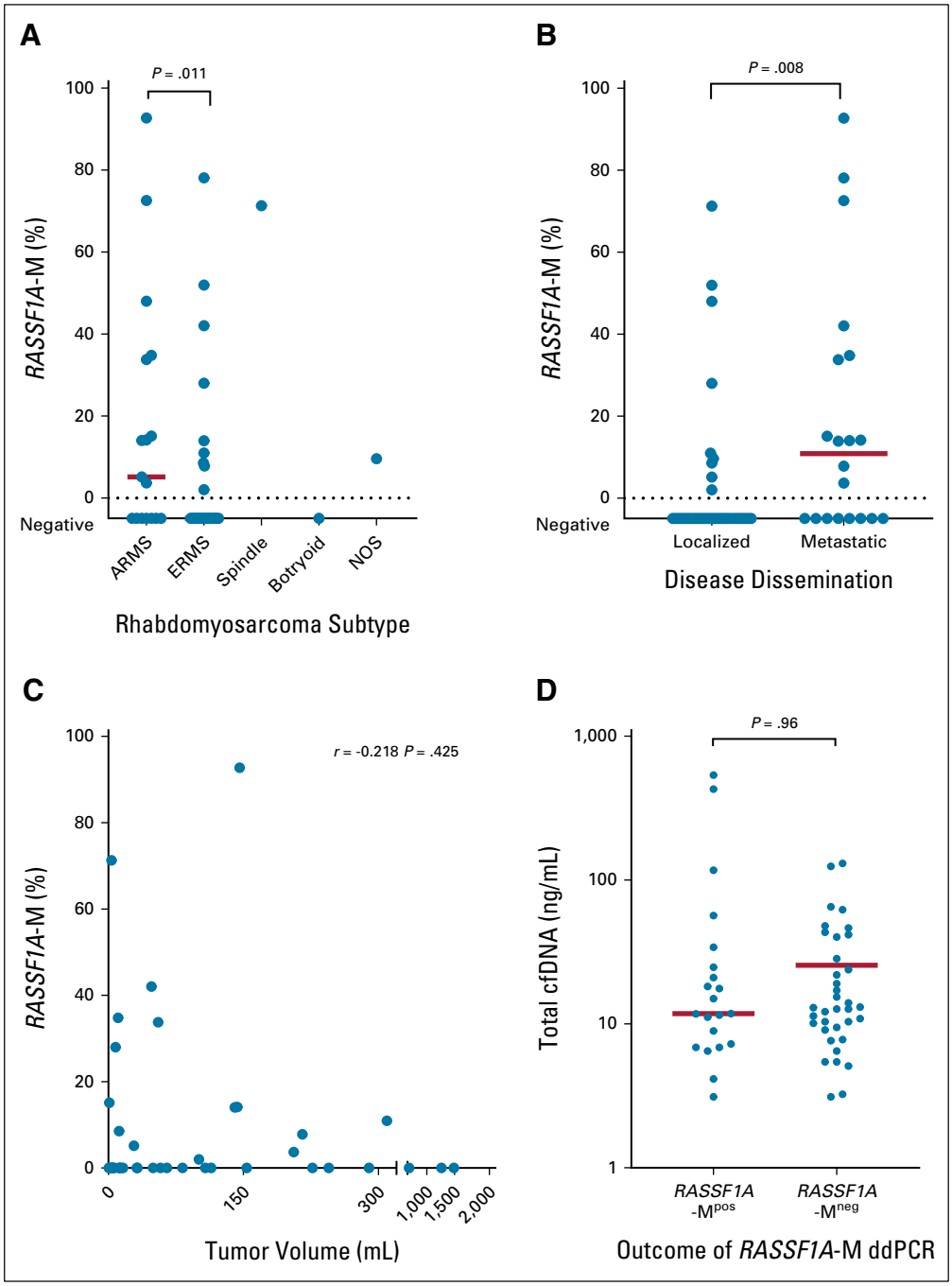


FIG 1. Methylated *RASSF1A* (*RASSF1A*-M) in diagnostic plasma samples of patients with rhabdomyosarcoma. The percentage of cell-free methylated RASSF1A (*RASSF1A*-M) is calculated according to total *RASSF1A* copies at diagnosis in patients: (A) with different subtypes, (B) with localized and metastatic disease, and (C) plotted against tumor volume at diagnosis. (D) Level of cfDNA (quantified by *beta-actin* [*ACTB*]) at diagnosis in plasma samples with detectable *RASSF1A*-M and with no detectable *RASSF1A*-M; note that the *y*-axis is plotted on a log scale. In this figure, each symbol represents an individual patient, and the red horizontal lines represent the median values. Tumor size was determined by MRI, CT scan, or ultrasonography. ARMS, alveolar rhabdomyosarcoma; cfDNA, cell-free DNA; CT, computed tomography; ddPCR, droplet digital polymerase chain reaction; ERMS, embryonal rhabdomyosarcoma; MRI, magnetic resonance imaging; NOS, not otherwise specified.

respectively). In the multivariable model, only *RASSF1A*-M, RNA panel, and tumor size larger than 5 cm had a significant effect on outcome. The known EpSSG RMS2005 risk group classification, metastatic disease, alveolar subtype, over 10 years of age, and lymph node involvement were not significantly associated with outcome in our multivariable model. Finally, OS could not be analyzed due to the low number of events in this cohort.

RASSF1A-M During Treatment and Clinical Follow-Up

For 33 patients, a total of 95 samples drawn during primary treatment and/or subsequent clinical follow-up were available. *RASSF1A*-M was measured in the follow-up samples only if the patient was *RASSF1A*-M–positive at diagnosis or—if a diagnostic sample was not available—at relapse. Among the 23 patients for whom samples were collected during primary treatment, only two patients (Data Supplement) were *RASSF1A*-M–positive after two cycles of chemotherapy, but *RASSF1A*-M–negative in all subsequent samples. In eight patients, *RASSF1A*-M was measured in a sample taken during a clinical event (five at first relapse, two at second relapse, and one at progressive disease during primary treatment). Five of these eight samples were *RASSF1A*-M–positive (three at first relapse

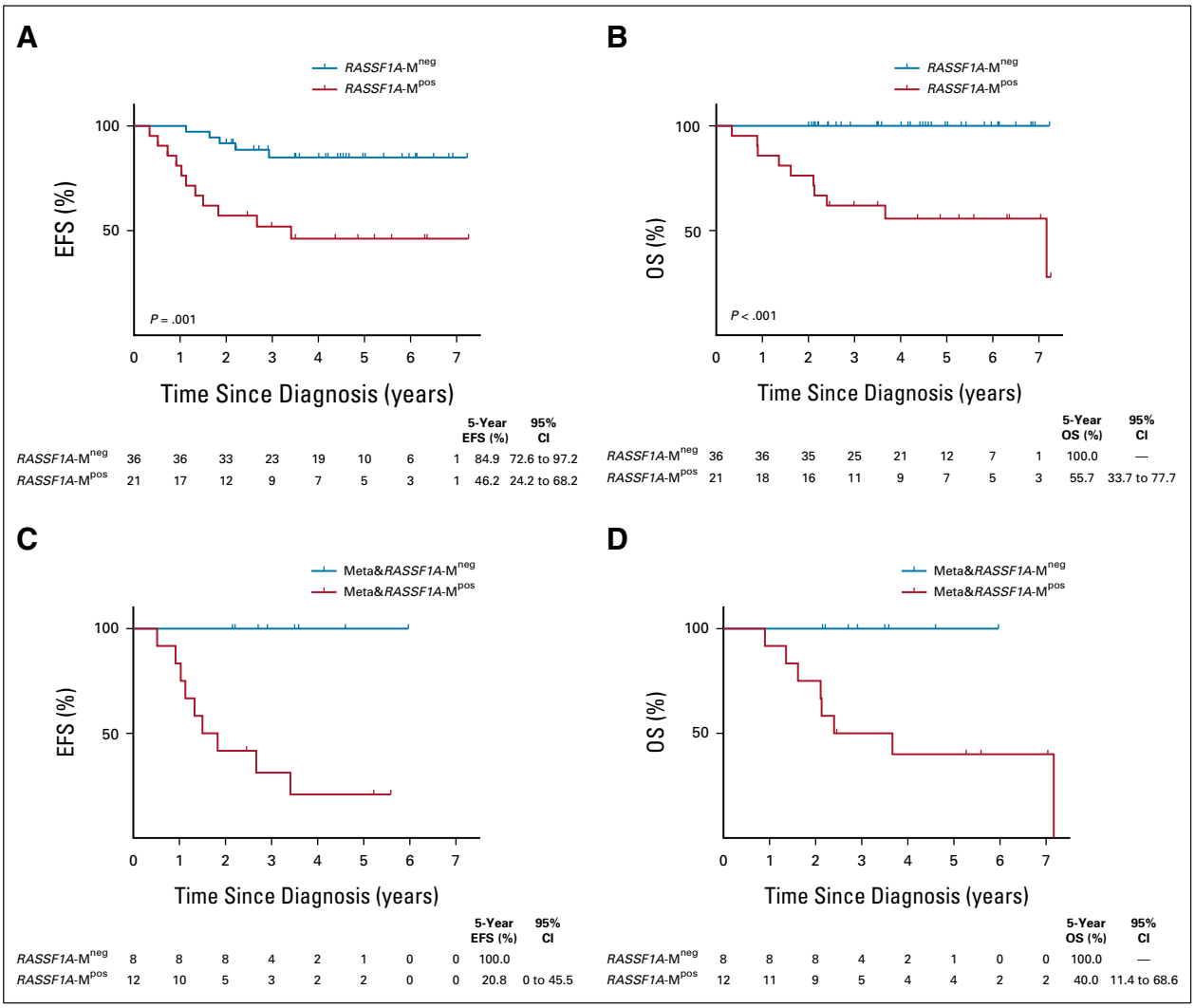


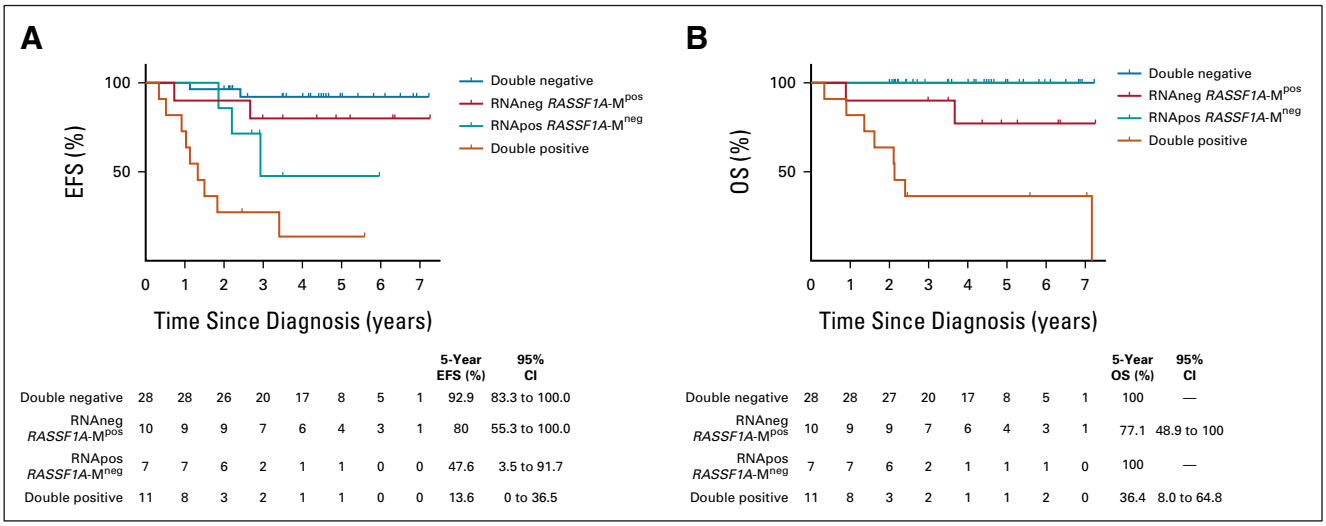
FIG 2. Survival outcome defined by detection of cell-free methylated RASSF1A (*RASSF1A*-M) at diagnosis. (A and B) EFS and OS, respectively, of patients with no detectable methylated *RASSF1A* in the diagnostic plasma (*RASSF1A*-Mneg; n = 36) and patients with detectable methylated *RASSF1A* in the diagnostic plasma (*RASSF1A*-Mpos; n = 21). (C and D) EFS and OS of *RASSF1A*-M–negative patients (n = 8) and *RASSF1A*-M–positive patients (n = 12) with metastatic disease. Shown below each plot is the number of patients at each time point and 5-year survival with the 95% CI. cfDNA, cell-free DNA; EFS, event-free survival; OS, overall survival.

and two at second relapse); no samples at initial diagnosis were available for these five patients. After initiating relapse therapy, all subsequent samples from these patients were *RASSF1A*-M–negative. The sample taken from the patient at progressive disease (patient RMS133) was *RASSF1A*-M–negative, and no previous plasma samples were available for this patient.

DISCUSSION

On the basis of our findings, we propose that each cfDNA-based technique can address a specific clinical need, ranging from assisting at initial tumor diagnosis to fine-tuning of risk stratification. In our cohort, cfRRBS proved its potential as a highly sensitive method for identifying rhabdomyosarcoma-derived cfDNA at initial diagnosis, and the majority was classified correctly as embryonal. Van Paemel et al[21] found that cfRRBS was also able to correctly identify alveolar ctDNA. Thus, cfRRBS can provide added value at initial diagnosis, particularly if the ability to perform a tumor biopsy is restricted by clinical features such as tumor location or the patient's condition and when the ability to distinguish between other types of pediatric solid tumors is important.[21]

We detected CNAs in 53.3% of samples analyzed by shWGS, mostly metastatic cases. On the basis of the literature, CNAs are present in nearly all fusion-negative rhabdomyosarcomas[20,39] and in approximately one third of all fusion-positive rhabdomyosarcomas.[39,40] We detected CNAs in the cfDNA of only half of the patients with fusion-negative tumors. This relatively low rate may have been due in part to contamination of the cfDNA with genomic DNA, as the protocol for drawing and storing blood was not



									5-Year EFS (%)	95% CI
Double negative	28	28	26	20	17	8	5	1	92.9	83.3 to 100.0
RNAneg *RASSF1A*-M^{pos}	10	9	9	7	6	4	3	1	80	55.3 to 100.0
RNApos *RASSF1A*-M^{neg}	7	7	6	2	1	1	0	0	47.6	3.5 to 91.7
Double positive	11	8	3	2	1	1	0	0	13.6	0 to 36.5

									5-Year OS (%)	95% CI
Double negative	28	28	27	20	17	8	5	1	100	—
RNAneg *RASSF1A*-M^{pos}	10	9	9	7	6	4	3	1	77.1	48.9 to 100
RNApos *RASSF1A*-M^{neg}	7	7	6	2	1	1	1	0	100	—
Double positive	11	8	3	2	1	1	2	0	36.4	8.0 to 64.8

FIG 3. Survival outcome defined by detection of cell-free methylated RASSF1A (*RASSF1A*-M) from plasma and rhabdomyosarcoma-specific RNA in blood and bone marrow at diagnosis. (A and B) EFS and OS of 56 patients on the basis of the absence or presence of rhabdomyosarcoma-specific RNA (RNA-negative and RNA-positive, respectively) combined with *RASSF1A*-M status. Shown below each plot is the number of patients at each time point and 5-year survival with the 95% CI. OS, overall survival; EFS, event-free survival.

standardized, which can lower the sensitivity to detect CNA.[16] Van Paemel et al noted that performing shWGS on cfDNA can provide additional value with respect to analyzing CNAs in the primary tumor, resulting in a more complete overview of the patient's genetic landscape and bypassing any potential heterogeneity within the tumor and/or metastatic lesions. This is important to consider when designing further studies.

TABLE 3. HRs With 95% CI Estimated With a Multivariable Cox Proportional Hazard Regression Model for Event-Free Survival

Variables	HR (95% CI)
RASSF1A-M–positive	4.52 (1.34 to 15.27)[a]
Standard risk	1
High risk	1.29 (0.22 to 7.74)
Metastatic disease	2.69 (0.69 to 10.47)
RASSF1A-M–positive	4.15 (1.38 to 12.49)[a]
Localized *v* metastatic disease	1.99 (0.70 to 5.61)
RASSF1A-M–positive	3.38 (1.14 to 9.97)[a]
RNA panel	7.60 (2.37 to 24.36)[a]
RASSF1A-M–positive	4.82 (1.60 to 14.51)[a]
Alveolar rhabdomyosarcoma	1.16 (0.42 to 3.25)
RASSF1A-M–positive	5.72 (1.96 to 16.69)[a]
Age at diagnosis > 10 years	2.14 (0.99 to 7.44)
RASSF1A-M–positive	5.87 (2.02 to 17.07)[a]
Tumor size > 5 cm	8.05 (1.81 to 35.81)[a]
RASSF1A-M–positive	4.27 (1.39 to 13.13)[a]
Lymph node involvement	1.34 (0.46 to 3.88)

Abbreviation: HR, hazard ratio.
[a]Indicates significance at $P < .05$.

On the basis of the previous reports, demonstrating feasibility to use *RASSF1A*-M ddPCR as a tumor-specific marker with a high specificity due to extremely low background in plasma from healthy controls,[14,15] we studied *RASSF1A*-M ddPCR in cfDNA of patients with rhabdomyosarcoma. One of the limitations of this study was the absence of paired primary tumor samples. However, the presence of *RASSF1A*-M, as extracted from data published by several groups,[20,33,34] indicated the potential to detect *RASSF1A*-M in primary tumors, with admittedly a large variation in the level of *RASSF1A*-M. Still, for the patients in our cohort who were *RASSF1A*-M–negative, on the basis of cfDNA obtained at diagnosis, we were unable to determine whether this was due to absence of *RASSF1A* methylation or no detectable ctDNA. This is underlined by the 18 samples testing negative for *RASSF1A*-M, in which ctDNA was detected by cfRRBS and/or shWGS. Future studies should include matching tumor material to establish the contribution of different approaches for cfDNA analysis. Nonetheless, we were able to detect *RASSF1A*-M in cfDNA in 36% of diagnostic samples and found a strong correlation between *RASSF1A*-M positivity and event-free survival and OS. Importantly, this predictive value was obtained almost exclusively in the group of patients with metastatic disease. This finding might suggest that more aggressive tumors contain methylated *RASSF1A* and deserves further investigations in a follow-up study, including matching primary tumor material. Interestingly, in the samples that were tested by both shWGS and *RASSF1A*-M, the results suggest that detection of ctDNA by both

methods may be more prognostic than detection of ctDNA by shWGS alone. This should be studied further in a larger cohort.

As we previously showed rhabdomyosarcoma-specific RNA detection in PB and/or BM at diagnosis to detect additional disseminated disease and to correlate with the outcome,[9] we now showed that combining mRNA and ctDNA (*RASSF1A*-M) in paired diagnostic samples identifies patients with very good and very poor outcome. Our multivariable analysis revealed that combining the cfDNA *RASSF1A*-M assay with rhabdomyosarcoma-specific RNA detection in PB and BM samples provides an even better tool for discriminating between low-risk patients and patients with a poor prognosis. Given the relatively small number of patients in our cohort, however, we were unable to investigate the effect of adding both *RASSF1A*-M and the RNA panel to established prognostic factors, particularly in the EpSSG RMS2005 risk group; nevertheless, our results can form a starting point for future studies involving a prospective cohort.

An interesting finding from our study is the dynamics of ctDNA. Before our study, we hypothesized that ctDNA would still be present during primary treatment and decrease slowly, tracking the decrease in tumor burden. However, in our rhabdomyosarcoma cohort, we found that most of the samples were negative for ctDNA after the first course of chemotherapy. This rapid transition to a ctDNA-negative state is consistent with the results reported by Klega et al[18] who found that most samples were negative for ctDNA before the second course of chemotherapy. Thus, an interesting question is whether performing earlier sampling and obtaining multiple samples during the first 2 weeks after the start of treatment would reveal the presence of ctDNA and—if so—would lead to the development of a prognostic marker, similar to the marker for minimal residual disease developed for use in leukemia.[41,42]

In conclusion, we demonstrate the feasibility to study ctDNA in pediatric rhabdomyosarcoma by different approaches. The choice of a given technique will depend on whether the underlying question is diagnostic or prognostic. We show that the presence of methylated *RASSF1A* in cfDNA is associated with poor outcome and can be used to improve risk stratification at diagnosis. Furthermore, we show that combining detection of methylated *RASSF1A* in plasma with analysis of tumor-specific RNA in blood and bone marrow identified patients with good versus poor outcome.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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