

Circulating Tumor DNA Is Prognostic in Intermediate-Risk Rhabdomyosarcoma: A Report From the Children's Oncology Group

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PURPOSE Novel biomarkers are needed to differentiate outcomes in intermediate-risk rhabdomyosarcoma (IR RMS). We sought to evaluate strategies for identifying circulating tumor DNA (ctDNA) in IR RMS and to determine whether ctDNA detection before therapy is associated with outcome.

PATIENTS AND METHODS Pretreatment serum and tumor samples were available from 124 patients with newly diagnosed IR RMS from the Children's Oncology Group biorepository, including 75 patients with fusion-negative rhabdomyosarcoma (FN-RMS) and 49 with fusion-positive rhabdomyosarcoma (FP-RMS) disease. We used ultralow passage whole-genome sequencing to detect copy number alterations and a new custom sequencing assay, Rhabdo-Seq, to detect rearrangements and single-nucleotide variants.

RESULTS We found that ultralow passage whole-genome sequencing was a method applicable to ctDNA detection in all patients with FN-RMS and that ctDNA was detectable in 13 of 75 serum samples (17%). However, the use of Rhabdo-Seq in FN-RMS samples also identified single-nucleotide variants, such as *MYOD1*^{L122R}, previously associated with prognosis. Identification of pathognomonic translocations between *PAX3* or *PAX7* and *FOXO1* by Rhabdo-Seq was the best method for measuring ctDNA in FP-RMS and detected ctDNA in 27 of 49 cases (55%). Patients with FN-RMS with detectable ctDNA at diagnosis had significantly worse outcomes than patients without detectable ctDNA (event-free survival, 33.3% v 68.9%; $P = .0028$; overall survival, 33.3% v 83.2%; $P < .0001$) as did patients with FP-RMS (event-free survival, 37% v 70%; $P = .045$; overall survival, 39.2% v 75%; $P = .023$). In multivariable analysis, ctDNA was independently associated with worse prognosis in FN-RMS but not in the smaller FP-RMS cohort.

CONCLUSION Our study demonstrates that baseline ctDNA detection is feasible and is prognostic in IR RMS.

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INTRODUCTION

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children and adolescents with approximately 350 new cases per year in the United States.¹ RMS is subdivided into two genetic subtypes. Fusion-positive RMS (FP-RMS) is characterized by a somatic translocation between the *FOXO1* gene and either the *PAX3* or *PAX7* gene (*PAXFOXO1*). Fusion-negative RMS (FN-RMS) is characterized by aneuploidy and recurrent single-nucleotide variants (SNVs) often in RAS pathway genes.²

Clinical and histologic features are used for risk stratification and treatment selection in RMS. Intermediate-risk (IR) disease is associated with a 3-year event-free survival (EFS) ranging from 50% to 75% and accounts for the largest portion of newly diagnosed patients.³ Optimal treatment selection at diagnosis is important in RMS as survival after relapse is unusual. Additional biomarkers of prognosis would more precisely identify

patients who will be cured with standard IR RMS treatment and patients who should enroll on clinical trials for high-risk disease.

Circulating tumor DNA (ctDNA) has recently emerged as a new prognostic biomarker in pediatric and adult cancers, including other sarcomas.⁴⁻⁶ Next-generation sequencing (NGS) strategies for the detection of ctDNA allows for quantification of ctDNA levels and identification of somatic variants that may provide additional prognostic information.⁷⁻⁹ Previous work from our group and others has shown that detection, quantification, and profiling of ctDNA in the blood of patients with RMS are feasible.¹⁰⁻¹² In this study, we apply two NGS approaches to serum samples collected through a Children's Oncology Group (COG) banking study of 124 patients with IR RMS. We determine which somatic features are most useful for identifying ctDNA, whether molecular prognostic features can be identified by ctDNA profiling, and whether the presence of detectable ctDNA is prognostic in IR RMS.

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

Is circulating tumor DNA (ctDNA) detectable in the blood of patients with intermediate-risk rhabdomyosarcoma (RMS) and is ctDNA detection associated with outcome?

Knowledge Generated

In this retrospective cohort, detection of ctDNA was feasible and relied on the identification of known somatic variants, such as aneuploidy and translocations, that define the genetic subtypes of RMS. Detection of ctDNA was significantly associated with a shorter event-free and overall survival, refining prognosis for patients with intermediate-risk RMS. Multivariable analysis demonstrated that ctDNA was independently associated with outcome in patients with fusion-negative RMS but did not reach significance in fusion-positive RMS.

Relevance (S. Bhatia)

ctDNA could serve as a prognostic tool and could inform future risk-stratified treatment in patients with intermediate risk RMS, when confirmed in prospective trials.*

*Relevance section written by JCO Associate Editor Smita Bhatia, MD, MPH, FASCO.

PATIENTS AND METHODS

Patient Selection

All patients were enrolled on the COG biology study D9902, had pretreatment serum banked, and were coenrolled on a COG IR RMS trial (D9803 or ARST0531) or met criteria for IR RMS as defined in ARST0531. Matched germline samples and tumor tissue were obtained from a subset of cases. All patients signed written informed consent for D9902 at the time of enrollment. Separate approval for this retrospective use of patient samples and clinical data was obtained from the COG and the Dana-Farber/Harvard Cancer Center institutional review board. Additional details are provided in the Data Supplement (online only).

Design of an RMS-Specific Hybrid Capture Assay

We used two methods to profile tumor and serum samples. Copy number alterations (CNAs) were detected using ultralow passage whole-genome sequencing (ULP-WGS) as previously described (Data Supplement).¹³ To identify translocations and SNVs, we designed an RMS-specific hybrid capture assay. This Rhabdo-Seq panel targets intronic regions of *FOXO1*, *PAX3*, *PAX7*, *VGLL2*, *CITED2*, *NCOA1*, and *NCOA2*, which are translocated in RMS (Data Supplement).¹⁰ In the same hybrid capture panel, we targeted coding regions of 24 genes recurrently mutated in RMS (Data Supplement).¹⁴ Sequencing and analytical methods are presented in the Data Supplement.

RESULTS

Patients

Our study included pretreatment diagnostic serum samples from 124 patients with IR RMS, including 75 with FN-RMS and 49 with FP-RMS. The clinical characteristics of our analytical cohort were similar to those of patients treated on the prior IR RMS COG trial, ARST0531 (Table 1).¹⁵

Matched tumor tissue and germline DNA were available for 69 and 67 patients with FN-RMS, respectively. Matched tumor tissue was available for 35 patients with FP-RMS. Among the FN-RMS, histologies included nine botryoid, two spindle cell, 61 embryonal, and three for whom histology was not specified.

Rhabdo-Seq for Tumor and ctDNA Profiling

To validate the performance of Rhabdo-Seq, we first sequenced cell lines and tumors from patients with FP-RMS. We identified the known complex *PAX3/FOXO1* rearrangement in Rh4 and *PAX7/FOXO1* in CW9019 cell lines. We also performed a serial dilution of DNA extracted from Rh4 into normal DNA to determine the limit of detection (LOD). We found that the fusion could be detected when Rh4 DNA comprised as little as 0.4% of the total DNA sample (Data Supplement). We detected the translocation and patient-specific breakpoints in 28 of 30 tumors with Rhabdo-Seq data. Two patients without an identifiable translocation were reported to have a *FOXO1* fusion by fluorescence in situ hybridization, but we were unable to confirm these primary clinical data. We also analyzed Rhabdo-Seq data from 57 FN-RMS tumors and, when available, matched normal DNA. SNVs were detected in genes frequently mutated in RMS at rates similar to those reported in previous studies (Data Supplement).¹⁴

ctDNA Detection in FN-RMS

To determine which cases of FN-RMS had detectable CNAs and SNVs, we profiled available tumor tissue from 69 patients by both ULP-WGS and Rhabdo-Seq. Matched germline DNA was also profiled by Rhabdo-Seq. All 69 cases had detectable CNAs (Data Supplement), confirming that detection of ctDNA by ULP-WGS would be expected in all patients with ctDNA levels above the LOD (3% of total cell-free DNA; Fig 1A). There was sufficient sequencing

TABLE 1. Study Cohort Demographics

FN-RMS		
Characteristic (n = 75)	ctDNA Cohort, No. (%)	ARST0531, No. (%)
Sex		
Male	46 (61.3)	146 (57.9)
Female	29 (38.7)	106 (42.1)
Age, years		
< 1	5 (6.7)	17 (6.7)
1-9	53 (70.7)	183 (72.6)
10+	17 (22.7)	52 (20.6)
Site		
Bladder/prostate	14 (18.7)	100 (39.7)
Nonbladder/prostate	61 (81.3)	152 (60.3)
Size of primary tumor, cm		
≤ 5	26 (34.7)	195 (77.4)
> 5	49 (65.3)	57 (22.6)
T-stage		
T1	31 (41.3)	101 (40.1)
T2	44 (58.7)	151 (59.9)
Regional nodal status		
N0	66 (88.0)	219 (86.9)
N1	7 (9.3)	29 (11.5)
Unknown	2 (2.7)	4 (1.6)
FP-RMS		
Characteristic (n = 49)	ctDNA Cohort, No. (%)	ARST0531, No. (%)
Sex		
Male	25 (51.0)	62 (44.6)
Female	24 (49.0)	77 (55.4)
Age, years		
< 1	5 (10.2)	9 (6.5)
1-9	16 (32.7)	58 (41.7)
10+	28 (57.1)	72 (51.8)
Stage		
1	7 (14.3)	27 (19.4)
2	10 (20.4)	37 (26.6)
3	32 (65.3)	75 (54.0)
Group		
I	2 (4.1)	8 (5.8)
II	9 (18.3)	34 (24.4)
III	38 (77.6)	97 (69.8)
Size of primary tumor, cm		
≤ 5	28 (57.1)	79 (56.8)
> 5	21 (42.9)	60 (43.2)

(continued in next column)

TABLE 1. Study Cohort Demographics (continued)

FP-RMS		
Characteristic (n = 49)	ctDNA Cohort, No. (%)	ARST0531, No. (%)
T-stage		
T1	27 (55.1)	76 (54.7)
T2	22 (44.9)	63 (45.3)
Regional nodal status		
N0	31 (63.3)	89 (64.0)
N1	18 (36.7)	49 (35.3)
Unknown	0 (0.0)	1 (0.7)
<i>FOXO1</i> partner		
<i>PAX3</i>	36 (73.5)	93 (66.9)
<i>PAX7</i>	8 (16.3)	21 (15.1)
Unknown	5 (10.2)	25 (18.0)

NOTE. ARST0531 trial demographics presented as a larger intermediate-risk rhabdomyosarcoma comparison cohort. *FOXO1* fusion partner data in this table are based on clinic annotations for each cohort.

Abbreviations: ctDNA, circulating tumor DNA; FN-RMS, fusion-negative rhabdomyosarcoma; FP-RMS, fusion-positive rhabdomyosarcoma.

library remaining for Rhabdo-Seq profiling of 57 tumors. We detected a somatic SNV in one or more targeted genes in only 40 of 57 tumor samples (70%), indicating that for a third of patients, we would not be able to detect ctDNA by Rhabdo-Seq regardless of ctDNA content (Fig 1B).

We applied both assays to serum samples from 75 patients with FN-RMS. ctDNA could be detected in 13 patients (17%) by ULP-WGS (Fig 1C and Data Supplement) and in 18 patients (24%) by Rhabdo-Seq (Fig 1D). In total, ctDNA was detected in 23 patients (31%) by either assay (Fig 1E). In 5 cases where ctDNA was detectable by ULP-WGS but negative by Rhabdo-Seq, no targeted SNVs had been detected in the tumor. In all cases for which ctDNA was detected by Rhabdo-Seq but not by ULP-WGS, ctDNA levels were below the LOD for ULP-WGS (Fig 1F). In cases with ctDNA detected by both methods, estimates of ctDNA content were concordant (Data Supplement)

To determine whether ctDNA detection was associated with clinical features, we used the ULP-WGS data applicable to all FN-RMS cases. Detection of ctDNA was associated with disease stage and tumor size (Table 2). We also examined the specific SNVs detected in the tumor and serum samples by Rhabdo-Seq (Fig 1G). SNVs were always detected in serum in cases where SNVs were identified in tumor tissue and for which ctDNA levels were > 3% by ULP-WGS. In two patients, we detected a somatic SNV in the serum that was not detectable in the tumor (an *ARID1A* mutation in PANEP and a *CTNNB1* mutation in PAUPKX), suggesting that these variants were present in subclonal tumor cell populations.

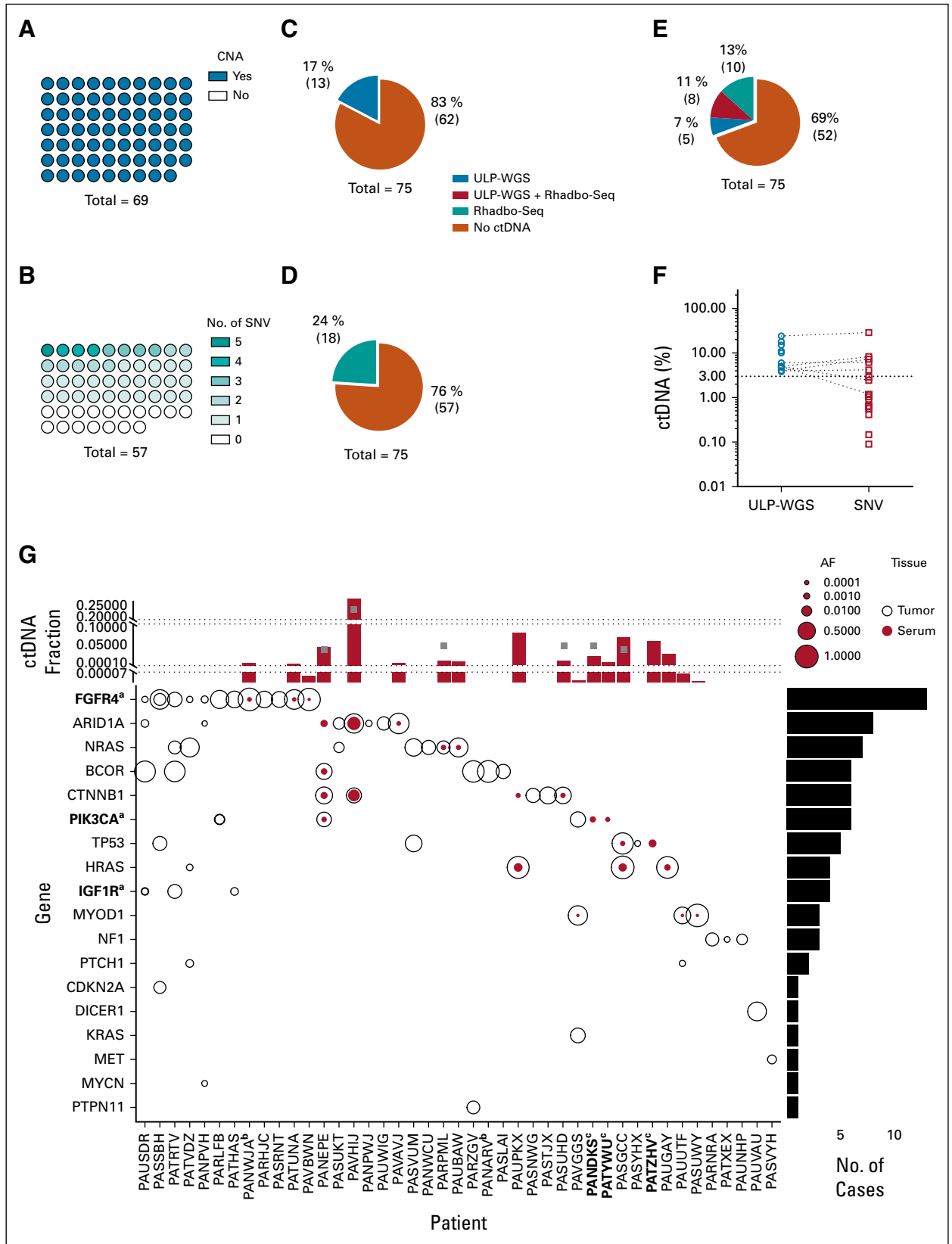


FIG 1. Characterization of FN-RMS genomics and ctDNA detection and quantification. (A) CNAs were identified in all 69 tumor samples by ULP-WGS. (B) SNVs were identified by the Rhabdo-Seq panel in 40 of 57 tumor samples with adequate sequencing coverage. ctDNA was detected in the serum in (C) 17% of the patients by ULP-WGS and (D) 24% of the patients by SNV (continued on following page)

FIG 1. (Continued). identification with Rhabdo-Seq. (E) In total, 31% of patients had detectable ctDNA using a combination of ULP-WGS for CNA detection and Rhabdo-Seq panel for SNV detection. (F) Plotted are the percent ctDNA values for FN-RMS samples with detectable ctDNA by ULP-WGS (blue circles) and by Rhabdo-Seq (red circles). ctDNA content estimates are similar in cases where ctDNA is detected by both methods. However, the lack of SNVs in some cases results in ctDNA being unmeasurable by Rhabdo-Seq. In other cases, the lower limit of detection in Rhabdo-Seq allows for identification of very low content of ctDNA in some samples. (G) The plot depicts SNVs in genes (*y*-axis) targeted by the Rhabdo-Seq panel in patients with FN-RMS (*x*-axis) who have at least one variant found in either a tumor or serum sample. Open black circles represent variants found in the tumor, and solid red circles represent variants in serum. Circle size reflects allelic fraction of the identified variant. The histogram on top depicts ctDNA levels in serum samples from the patient (indicated on the *x*-axis) by ULP-WGS (gray squares) or SNV allelic fraction (red bar). When there are two SNVs, only the highest allelic fraction is represented by the red bar. The histogram is partitioned into three sections to help visualize values in cases with very low content of ctDNA. The histogram to the right represents the number of variants identified in the cohort. ^aGenes in bold indicate that a case had evidence of two mutations in that gene (*FGFR4* in PASSBH, *PIK3CA* in PARLFB, and *IGF1R* in PAUSDR). ^bPatient IDs that are in bold indicate cases without available tumor data. ^cPatient IDs indicate cases without available germline DNA. The five cases with SNVs detected in the serum and not the tumor (PANEPE, PAUPKX, PANDKS, PATYWU, and PATZHV) were analyzed with a patient-matched normal DNA sample. AF, allelic fraction; CNA, copy number alteration; ctDNA, circulating tumor DNA; FN-RMS, fusion-negative rhabdomyosarcoma; SNV, single-nucleotide variant; ULP-WGS, ultralow passage whole-genome sequencing.

Moreover, we detected SNVs in three patients (PANDKS, PATYWU, and PATZHV) without available tumor data (Fig 1G). All five cases were analyzed with matched germline

DNA to verify that these events were somatic. Three patients with *MYOD1*^{L122R} mutations in the tumor had ctDNA in the serum detectable by identification of the *MYOD1*^{L122R} variant by Rhabdo-Seq but below the LOD for ULP-WGS.

TABLE 2. Fusion-Negative Rhabdomyosarcoma Clinical Data According to ctDNA Detection

Characteristic (n = 75)	ctDNA Detected, No. (%)	ctDNA Not Detected, No. (%)	P
Sex			
Male	8 (61.5)	38 (61.3)	
Female	5 (38.5)	24 (38.7)	
Age, years			.370
< 1	2 (15.4)	3 (4.8)	
1-9	9 (69.2)	44 (71.0)	
10+	2 (15.4)	15 (24.2)	
Stage			.010
2	0 (0.0)	23 (37.1)	
3	12 (92.3)	38 (61.3)	
4 ^a	1 (7.7)	1 (1.6)	
Site			.440
Bladder/prostate	1 (7.7)	13 (21.0)	
Nonbladder/prostate	12 (92.3)	49 (79.0)	
Size of primary tumor, cm			.003
≤ 5	0 (0.0)	26 (41.9)	
> 5	13 (100.0)	36 (58.1)	
T-stage			.060
T1	2 (15.4)	29 (46.8)	
T2	11 (84.6)	33 (53.2)	
Regional nodal status			.270
N0	12 (92.3)	54 (87.1)	
N1	0 (0.0)	7 (11.3)	
Unknown	1 (7.7)	1 (1.6)	

Abbreviation: ctDNA, circulating tumor DNA.

^aStage 4 embryonal RMS in patients 10 years old or younger was considered intermediate-risk rhabdomyosarcoma, on the D9803 clinical trial.

ctDNA Detection in FP-RMS

FP-RMS tumors are characterized by *PAX/FOXO1* translocations, whereas CNAs are present in only a portion of cases.² Tumors from 35 FP-RMS cases were profiled with ULP-WGS and Rhabdo-Seq. ULP-WGS detected segmental CNAs in 25 of 35 tumors (71%; Fig 2A). There was sufficient sequencing library remaining for Rhabdo-Seq from 30 tumors, which detected a *PAX/FOXO1* translocation in 28 samples (93%; Fig 2B). All translocations had a unique patient-specific genomic breakpoint (Data Supplement).¹⁰ Two samples did not have a detectable translocation despite adequate sequencing coverage and evidence of tumor content by ULP-WGS, making translocation detection impossible in liquid biopsies.

We next profiled cell-free DNA with ULP-WGS and Rhabdo-Seq from 49 FP-RMS serum samples. We detected ctDNA with ULP-WGS in eight (16%) samples (Fig 2C) and with Rhabdo-Seq in 27 (55%; Fig 2D). The translocation breakpoint was identical in all patient-matched tumor and serum samples. In total, we identified ctDNA in 28 (57%) FP-RMS cases (Fig 2E). Rhabdo-Seq (with a lower LOD) identified a translocation in all cases where ctDNA was detectable by ULP-WGS, except where no rearrangement was found in the tumor. ULP-WGS detected the presence of ctDNA only in cases where ctDNA was estimated to be 3% or greater by Rhabdo-Seq and for which the tumor sample demonstrated CNAs (Fig 2F). ctDNA detection by Rhabdo-Seq was associated with the disease group and local T2 disease (Table 3).

Serum samples for these studies were collected before the development of optimized liquid biopsy collection procedures. Evidence of cellular DNA contamination was found in 22 of 49 FP-RMS serum samples (Data Supplement), preventing accurate estimation of ctDNA content. However, in two cases (PASFIN and PARITH), the ctDNA content was estimated to be > 100%, which is impossible. Trans-Seq analysis of these samples yielded the same results

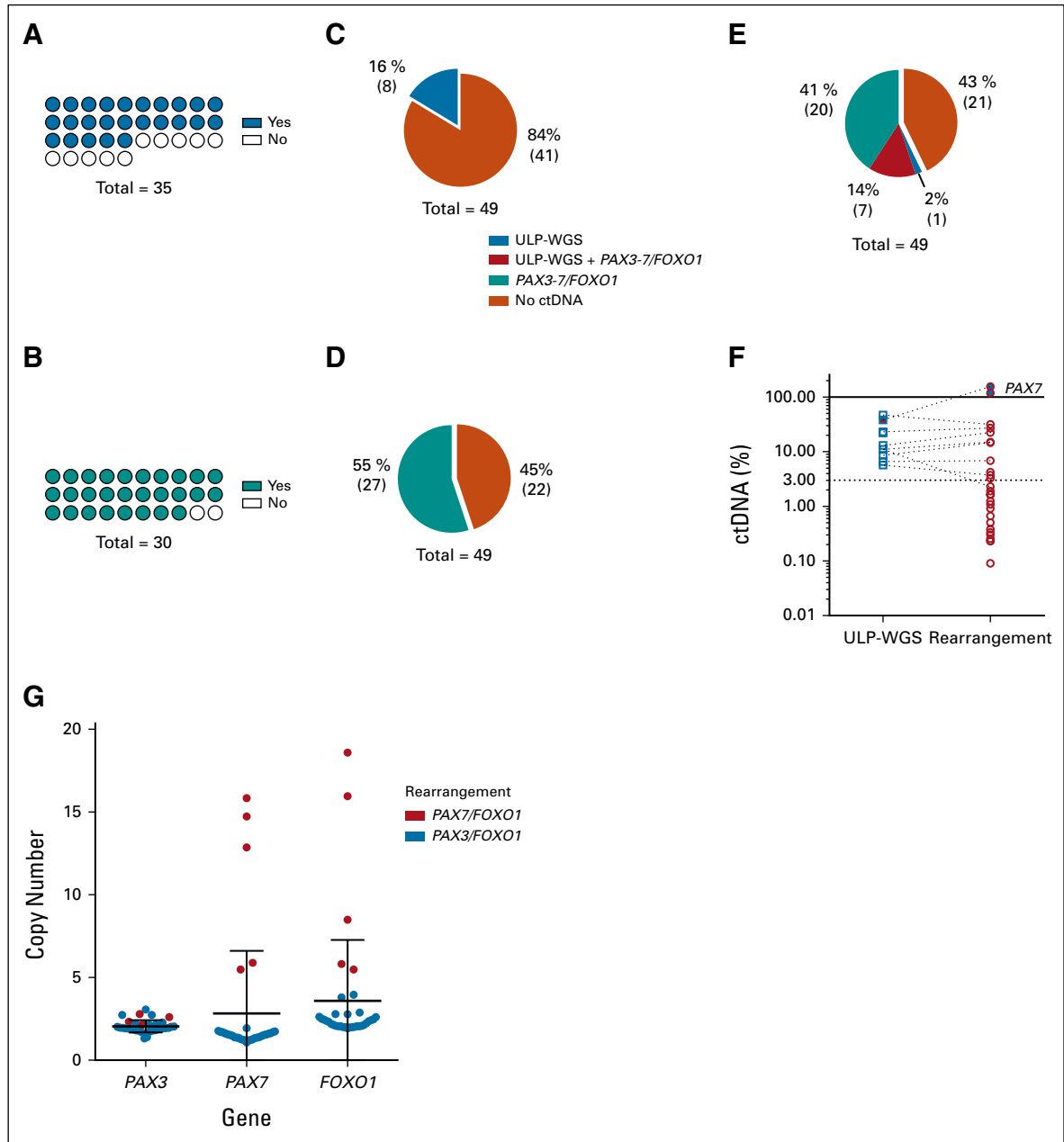


FIG 2. Characterization of fusion-positive rhabdomyosarcoma genomics and ctDNA detection and quantification. (A) CNAs were identified in 25 of 35 samples (71%) by ULP-WGS. (B) Translocations (rearrangement) were detected in 28 of 30 samples (93%) with sufficient sequencing library available for sequencing by Rhabdo-Seq. Of 35 tumors, five did not have sufficient DNA material quality for Rhabdo-Seq panel sequencing. (C) Sixteen percent of serum samples had detectable ctDNA by ULP-WGS. (D) ctDNA was detected by identifying the *PAX/FOXO1* translocation in 55% of serum samples. (E) In total, 57% of patients had detectable ctDNA using both ULP-WGS for CNA detection and Rhabdo-Seq panel for gene rearrangement detection. (F) Plotted are the percent ctDNA values for fusion-negative rhabdomyosarcoma samples with detectable ctDNA by ULP-WGS (blue circles) and by Rhabdo-Seq (red circles). ctDNA content estimates are similar in most cases where ctDNA is detected by both methods. However, the lack of aneuploidy in some cases results in ctDNA being unmeasurable by ULP-WGS. (G) Absolute copy numbers of *PAX3*, *PAX7*, and *FOXO1* locus were inferred from the gene locus using ULP-WGS data from tumor samples. In all the *PAX7/FOXO1* tumors available, both partner genes were amplified from 5 to 18 times explaining the overestimate of ctDNA content in three cases with detectable ctDNA by *PAX7/FOXO1* detection. CNA, copy number alteration; ctDNA, circulating tumor DNA; ULP-WGS, ultralow passage whole-genome sequencing.

TABLE 3. Fusion-Positive Rhabdomyosarcoma Clinical Data According to ctDNA Detection

Characteristic (n = 47 ^a)	ctDNA Detected, No. (%)	ctDNA Not Detected, No. (%)	P
Sex			
Male	16 (59.3)	8 (40)	
Female	11 (40.7)	12 (60)	
Age, years			.130
< 1	1 (3.7)	3 (15)	
1-9	7 (25.9)	8 (40)	
10+	19 (70.4)	9 (45)	
Stage			.190
1	2 (7.4)	5 (25)	
2	5 (18.5)	4 (20)	
3	20 (74.1)	11 (55)	
Group			.008
I	0 (0.0)	2 (10)	
II	1 (3.7)	6 (30)	
III	26 (96.3)	12 (60)	
Size of primary tumor, cm			.140
≤ 5	12 (44.4)	14 (70)	
> 5	15 (55.6)	6 (30)	
T-stage			.037
T1	11 (40.7)	15 (75)	
T2	16 (59.3)	5 (25)	
Regional nodal status			.550
N0	16 (59.3)	14 (70)	
N1	11 (40.7)	6 (30)	
FOXO1 partner			.310
PAX3	23 (85.2)	13 (65)	
PAX7	3 (11.1)	4 (20)	
Unknown	1 (3.7)	3 (15)	

NOTE. FOXO1 fusion partner data in this table are based on clinic annotations. Abbreviation: ctDNA, circulating tumor DNA.

^aTwo fusion-positive patients had no fusion identified in the tumor despite sufficient material for sequencing and adequate coverage and were excluded from this table.

(Data Supplement).¹⁰ Both cases involved PAX7/FOXO1 translocations, known to be amplified in RMS.^{16,17} ULP-WGS data from PAX7/FOXO1 cases in our cohort demonstrated copy gains in both PAX7 and FOXO1 loci (Fig 2G), accounting for the overestimate of ctDNA content. For case PASFIN, the ctDNA content was recalculated to 14.5% when adjusting for the measured amplification from tumor data (tumor data from PARITH were not available). For another PAX7/FOXO1 case, PATGUL, ctDNA content estimates were adjusted from 0.7% to 0.1%. No other cases with PAX7/FOXO1 translocations had detectable ctDNA.

ctDNA Detection Before Therapy Is Prognostic in IR RMS

Previous studies in pediatric and adult sarcomas have shown that detection of ctDNA, before the start of therapy, is associated with a worse outcome.¹⁸ In IR RMS, we found that detection of ctDNA (using ULP-WGS for FN-RMS and translocation for FP-RMS) was associated with a significantly lower 5-year EFS (35.9%; 95% CI, 21.4 to 50.7) and overall survival (OS, 37.3%; 95% CI, 22.3 to 52.3) compared with patients without detectable ctDNA by ULP-WGS (EFS, 69.3%; 95% CI, 58 to 78.1%; $P = .0001$; OS, 81.2%; 95% CI, 70.7 to 88.2; $P = .0001$; Figs 3A and 3B). MYOD1^{L122R} mutations are associated with extremely poor outcomes in FN-RMS, and patients found to have these mutations are receiving intensified therapy on the current COG ARST2032 trial.¹⁴ We expected this mutation to be a stronger biomarker of outcome than detection of ctDNA. In fact, although the three patients found to have MYOD1^{L122R} mutations did not have detectable levels of ctDNA by ULP-WGS, outcomes were significantly worse than for the rest of the patients with FN-RMS (Data Supplement). After excluding these three cases a priori, detection of pretreatment ctDNA was associated with a 5-year EFS of 35.9% (95% CI, 21.4 to 50.7) compared with 83.2% (95% CI, 72.8 to 89.9) for patients without detectable ctDNA ($P = .0001$) and a 5-year OS of 33.3% (95% CI, 10.3 to 58.8) compared with 86% (95% CI, 73.9 to 2.7; $P = .0001$; Figs 3C and 3D). Univariable analysis also showed that local T2 disease (invasion) was associated with outcome, although with a smaller hazard ratio (HR) than ctDNA (Data Supplement). Multivariable analysis of the whole cohort, including patients with a MYOD1^{L122R} mutation, showed that ctDNA was significantly associated with EFS (HR, 2.8; 95% CI, 1.6 to 5.1; $P = .0005$) and OS (HR, 3.9; 95% CI, 2.1 to 7.5; $P < .0001$) as was local T2 disease (EFS HR, 2.0; 95% CI, 1.1 to 3.8; $P = .02$; OS HR, 2.5; 95% CI, 1.2 to 5; $P = .01$) and MYOD1^{L122R} mutation (EFS HR, 9.2; 95% CI, 2.6 to 32.3; $P = .0005$; OS HR, 5.2; 95% CI, 1.1 to 23.5; $P = .03$). These associations were confirmed using additional methods for multivariable modeling (Data Supplement).

To exclude the possibility that differences in the LOD between ULP-WGS and Rhabdo-Seq contributed to our findings, we studied the association between ctDNA detection and outcome using each method. When using only ULP-WGS, detection of ctDNA was associated with a significantly lower 5-year EFS (35.1%; 95% CI, 15.7 to 55.3) and OS (34.3%; 95% CI, 14.9 to 54.8) compared with patients without detectable ctDNA (EFS, 69.5%; 95% CI, 57.7 to 78.6; $P = .0007$; OS, 79.8%; 95% CI, 68.7 to 87.3; $P = .0001$; Data Supplement). Using only Rhabdo-Seq, detection of ctDNA was again associated with a significantly lower 5-year EFS (38.7%; 95% CI, 24.5 to 52.6) and OS (45.1%; 95% CI, 29.6 to 59.4) than for patients without detectable ctDNA (EFS, 71.0%; 95% CI, 55.4 to 82.0; $P = .001$; OS, 71%; 95% CI, 55.4 to 82; $P = .001$; Data Supplement).

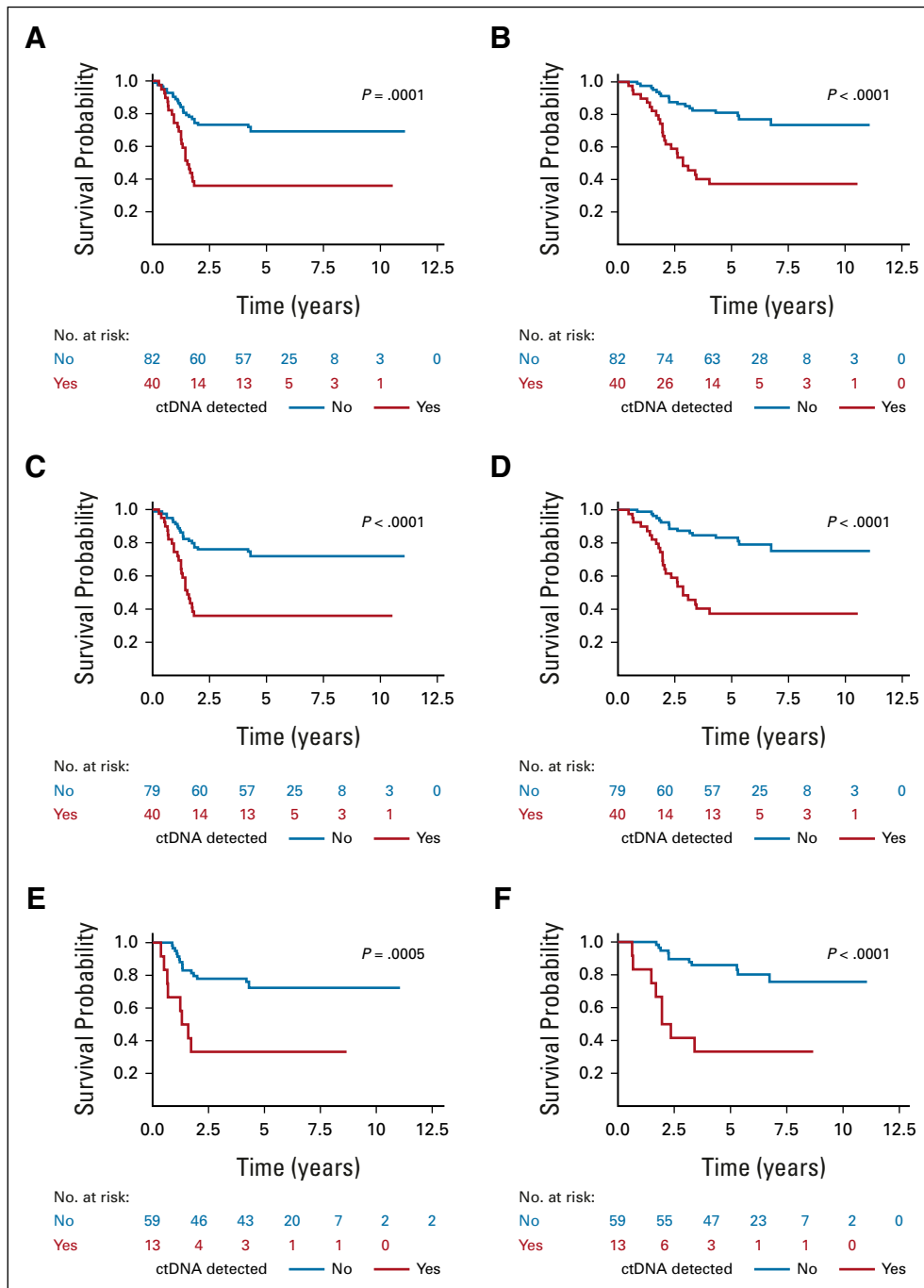


FIG 3. (A) EFS and (B) OS by ctDNA detection status, by ULP-WGS for FN-RMS, and translocation detection for FP-RMS of the entire cohort. (C) EFS and (D) OS by ctDNA detection status, determined by ULP-WGS for FN-RMS and translocation detection for FP-RMS, of the entire cohort excluding cases with *MYOD1*^{L122R} mutations. (E) EFS and (F) OS by ctDNA status, determined by ULP-WGS, in patients with FN-RMS after excluding cases with *MYOD1*^{L122R} mutations. (G) EFS and (H) OS by ctDNA status, determined by translocation detection, in patients with FP-RMS. *P* value from the log-rank test. ctDNA, circulating tumor DNA; EFS, event-free survival; FN-RMS, fusion-negative rhabdomyosarcoma; FP-RMS, fusion-positive rhabdomyosarcoma; OS, overall survival; RMS, rhabdomyosarcoma; ULP-WGS, ultralow passage whole-genome sequencing. (continued on following page)

We also examined the association between ctDNA and outcome for each of the genomic subtypes of RMS. For FN-RMS, we used ULP-WGS to detect ctDNA (Rhabdo-Seq analysis

would be limited to only cases with an SNV in the tumor). Detection of ctDNA was associated with a worse EFS and OS for FN-RMS (Data Supplement). After excluding patients with

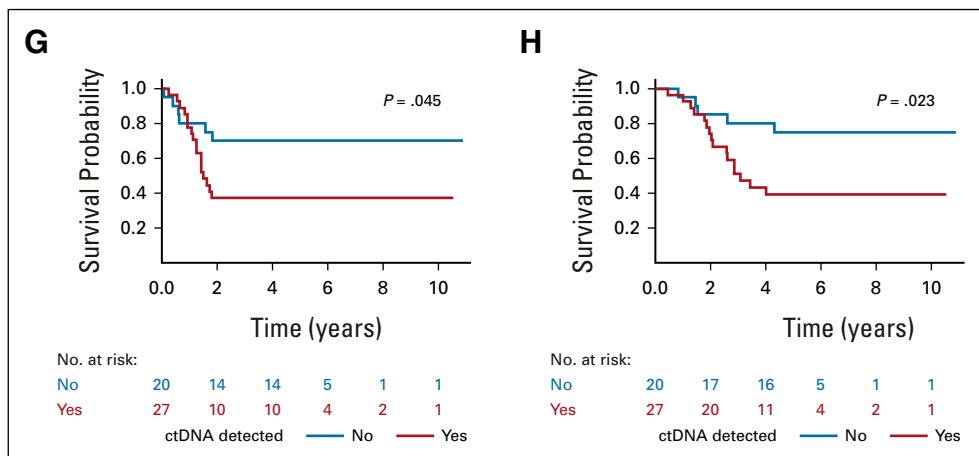


FIG 3. (Continued).

MYOD1^{L122R} mutations, detection of ctDNA was associated with a 5-year EFS of 33.3% (95% CI, 10.3 to 58.8) compared with 72.4% (95% CI, 59 to 82.1) for patients without detectable ctDNA ($P = .0005$) and a 5-year OS of 33.3% (95% CI, 10.3 to 58.8) compared with 86% (95% CI, 73.9 to 92.7), respectively ($P < .0001$; Figs 3E and 3F). Univariable analysis showed that local T2 disease was also associated with outcome (Data Supplement). Stepwise multivariable analysis, including patients with *MYOD1*^{L122R}, showed that ctDNA was significantly associated with EFS (HR, 4; 95% CI, 1.7 to 9.4; $P = .0016$) and OS (HR, 6.5; 95% CI, 2.5 to 16.6; $P < .0001$) as was *MYOD1*^{L122R} (EFS HR, 14.2; 95% CI, 4.0 to 51.3; $P < .0001$; OS HR, 9.3; 95% CI, 2.0 to 44.4; $P = .005$). This was confirmed by additional multivariable models (Data Supplement). Although limited to a subset of cases in FN-RMS ($n = 41$), ctDNA detection by Rhabdo-Seq was also associated with a worse EFS and OS (with a 5-year EFS of 33.5% [95% CI, 12.2 to 56.6]) compared with 75.8% (95% CI, 53.8 to 88.3) for patients without detectable ctDNA ($P = .0018$) and a 5-year OS of 49.1% (95% CI, 21.4 to 72) compared with 83.6% (95% CI, 62.4 to 93.6), respectively ($P = .0086$; Data Supplement).

In FP-RMS, ctDNA detection by translocation (applicable to the largest portion of cases) had a 5-year EFS of 37% (95% CI, 19.6 to 54.6) compared with 70% (95% CI, 45.1 to 85.3) for patients without detectable ctDNA ($P = .045$) and a 5-year OS of 39.2% (95% CI, 21 to 57) and 75% (95% CI, 50 to 88.7), respectively ($P = .023$; Figs 3G and 3H). Univariable analysis also showed that T2 was associated with outcome (Data Supplement). Stepwise multivariable analysis showed that ctDNA was significantly associated with EFS (HR, 2.5; 95% CI, 1 to 6.4; $P = .05$) and OS (HR, 3.1; 95% CI, 1.1 to 8.4; $P = .03$), whereas other multivariable models, including the full model with all variables, showed that T2 stage was associated with outcome (Data Supplement). Finally, in the small cohort of FP-RMS confirmed to have CNAs in their tumors ($n = 25$), ctDNA detection by ULP-WGS was not significantly associated with outcome (Data Supplement).

DISCUSSION

The mainstay of therapy for RMS in North America includes chemotherapy cycles of vincristine, dactinomycin, and cyclophosphamide, often alternating with cycles of vincristine and irinotecan.¹⁵ Local control includes surgery, radiation, or both. Patients with low-risk RMS do well with less intense chemotherapy, whereas few patients with high-risk disease are cured.¹⁹⁻²¹ Patients with IR RMS are defined by a combination of biologic and clinical features, but as many as 25%-50% will relapse and die.^{15,22} More precise risk stratification for patients with IR RMS could better identify patients who will be cured with vincristine, dactinomycin, and cyclophosphamide/vincristine and irinotecan and those who will not but could be made eligible for high-risk trials.

Studies in other pediatric sarcomas demonstrate that detection of pretreatment ctDNA is associated with a worse outcome.^{18,23} In this study, we hypothesized that detection of ctDNA in patients with IR RMS would be associated with a worse outcome and therefore useful for further risk stratification. However, identification of ctDNA requires the detection of somatic variants that define the patient's cancer, differentiating ctDNA from cell-free DNA originating from normal cells.⁴ FP-RMS and FN-RMS are defined by distinct classes of somatic variants.² FP-RMS is defined by a *PAX/FOXO1* fusion and less frequent CNAs. FN-RMS is defined by aneuploidy in nearly every tumor, and SNVs in a small number of genes in 60% of cases.^{2,17} To identify ctDNA in the serum of patients with both genetic subtypes, we applied ULP-WGS that identifies aneuploidy in cell-free DNA and Rhabdo-Seq, designed to detect *PAX/FOXO1*, *VGLL2/CITED2*, *VGLL2/NCOA2*, and other translocations and SNVs in 22 RMS-relevant genes.

ULP-WGS analysis was applicable for ctDNA analysis in all cases of FN-RMS, classifying ctDNA as detectable in 17%. Although Rhabdo-Seq was unsuitable for ctDNA analysis in 30% of FN-RMS cases, this assay provided

additional information, including the detection of prognostic *MYOD1*^{L122R} variants and SNVs in potentially targetable genes such as *FGFR4* and the *RAS* pathway.^{14,24-27} In FP-RMS, identification of *PAX/FOXO1* rearrangements was applicable for the majority of cases and detected ctDNA in 55%. Although ULP-WGS was unsuitable for ctDNA detection in 29% of copy-neutral FP-RMS cases, this assay identified *PAX7/FOXO1* amplifications (allowing for correction of ctDNA content estimates) and prognostic CNAs such as *MYCN* and *CDK4*.¹⁴

The detection of ctDNA before the start of therapy in RMS was associated with aggressive disease features such as tumor size, stage, and invasiveness, which may reflect mechanisms of ctDNA shed. Detection of ctDNA was also associated with a worse outcome in IR RMS with an estimated 5-year EFS and OS consistent with high-risk RMS.^{28,29} Multivariable analyses, using a model that includes all clinical variables, demonstrated that ctDNA is an independent prognostic biomarker of outcome in the full IR RMS cohort and the FN-RMS group but was not independently prognostic in the smaller FP-RMS cohort. Local disease invasion (local T2 disease) and *MYOD1*^{L122R} were also associated with outcome in multivariable analyses. Although these results indicate that ctDNA analysis is a new prognostic factor in IR RMS, they also indicate that more sophisticated risk stratification approaches, such as those using recursive partitioning, may be able to best refine prognosis by integrating ctDNA detection, clinical features, and specific genomic events.

Our study has some limitations. The use of serum samples, banked before the development of ctDNA-specific blood

collection protocols, prevented ctDNA quantification and might have decreased the proportion of positive samples. Another limitation was that serial samples were not available. Numerous studies now demonstrate the prognostic value of tracking changes in ctDNA levels over time.^{7,8,10,18,30-34} One recent report in Ewing sarcoma, another translocation-driven sarcoma, demonstrated that detectable levels of ctDNA present after two cycles of therapy were associated with relapse.³⁵ Future studies will be needed to determine whether changes in ctDNA during therapy are prognostic in RMS or whether measurements of ctDNA after completion of initial chemotherapy could help guide the use and duration of maintenance therapy.^{36,37} Furthermore, additional studies of FP-RMS are warranted to demonstrate the prognostic value of ctDNA in multivariable analyses compared with clinical factors. Finally, this was a retrospective study and patients were not treated as uniformly as they would be on a prospective clinical trial.

Future efforts, such as the ongoing study of ctDNA in patients enrolled on the COG ARST1431 trial for IR RMS, will be needed to build a comprehensive prognostic score that includes ctDNA together with other prognostic variables. We plan to apply both NGS approaches presented in this study, which will allow for subtype-specific detection of ctDNA and detection of additional clinically relevant CNAs and SNVs.

In summary, the findings presented here strongly suggest that ctDNA will be an important tool for future prognostication and risk-stratified treatment strategies in RMS. Collection of high-quality pretreatment, serial on-treatment, and surveillance liquid biopsy samples on large prospective trials will be needed to refine these results.

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DISCLAIMER

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**Circulating Tumor DNA Is Prognostic in Intermediate-Risk Rhabdomyosarcoma: A Report From The Children's Oncology Group**

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