NF1-Driven Rhabdomyosarcoma Phenotypes: A Comparative Clinical and Molecular Study of *NF1*-Mutant Rhabdomyosarcoma and *NF1*-Associated Malignant Triton Tumor

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ABSTRACT

PURPOSE Alterations of the *NF1* tumor suppressor gene is the second most frequent genetic event in embryonal rhabdomyosarcoma (ERMS), but its associations with clinicopathologic features, outcome, or coexisting molecular events are not well defined. Additionally, *NF1* alterations, mostly in the setting of neurofibromatosis type I (NF1), drive the pathogenesis of most malignant peripheral nerve sheath tumor with divergent RMS differentiation (also known as malignant triton tumor [MTT]). Distinguishing between these entities can be challenging because of their pathologic overlap. This study aims to comprehensively analyze the clinicopathologic and molecular spectrum of *NF1*-mutant RMS compared with NF1-associated MTT for a better understanding of their pathogenesis.

METHODS We investigated the clinicopathologic and molecular landscape of a cohort of 22 *NF1*-mutant RMS and a control group of 13 *NF1*-associated MTT. Cases were tested on a matched tumor-normal hybridization capture-based targeted DNA next-generation sequencing.

RESULTS Among the RMS group, all except one were ERMS, with a median age of 17 years while for MTT the mean age was 39 years. Three MTTs were misdiagnosed as ERMS, having clinical impact in one. The most frequent coexisting alteration in ERMS was *TP53* abnormality (36%), being mutually exclusive from *NRAS* mutations (14%). MTT showed coexisting *CDKN2A/B* and PRC2 complex alterations in 38% cases and loss of H3K27me3 expression. Patients with *NF1*-mutant RMS exhibited a 70% 5-year survival rate, in contrast to MTT with a 33% 5-year survival. All metastatic *NF1*-mutant ERMS were associated with *TP53* alterations.

CONCLUSION Patients with NF1-mutant ERMS lacking *TP53* alterations may benefit from dose-reduction chemotherapy. On the basis of the diagnostic challenges and significant treatment and prognostic differences, molecular profiling of challenging tumors with rhabdomyoblastic differentiation is recommended.

ACCOMPANYING CONTENT

Appendix

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INTRODUCTION

Rhabdomyosarcomas (RMSs) comprise a heterogeneous clinical and molecular group of sarcomas showing various degrees of myogenic differentiation. Despite an increased application of next-generation sequencing (NGS), most clinical decisions are not informed by specific molecular alterations. Only recently, the therapeutic strategies have been guided by certain genomic landscapes in patients with embryonal RMS (ERMS). Specifically, Children's Oncology Group (COG) trial ARST2032 excludes patients with high-risk genomic features (*TP53* and *MYOD1*) from dose reduction. In addition, NGS studies have shown that alterations of *NF1* tumor suppressor gene is the second most frequent genetic event in ERMS, after *RAS* isoform mutations.[1-4] To our knowledge, as no dedicated study to date focused on this alteration in RMS, our investigation evaluated a molecularly homogeneous subset of *NF1*-mutant RMS in comparison with another sarcoma with RMS differentiation driven by similar *NF1* alterations, that is, malignant triton tumor (MTT).

CONTEXT

Key Objective

To contrast the main clinical and genomic findings between *NF1*-mutant rhabdomyosarcoma (RMS) and NF1-related malignant triton tumor (MTT; malignant peripheral nerve sheath tumor with RMS differentiation).

Knowledge Generated

Despite the morphologic and clinical overlap between these two groups of tumors, their genomic landscape is quite distinct and can be used in diagnostically challenging cases as an adjunct molecular tool. Moreover, these two entities are associated with vastly different outcomes, showing that patients with MTTs followed a highly aggressive clinical course, with a dismal 33% 5-year overall survival (OS) and 46% risk of metastasis. By contrast, patients with *NF1*-mutant RMS demonstrate a 70% 5-year OS.

Relevance

Our findings highlight the critical impact of precision oncology in further molecular subclassification of rare sarcoma entities that display shared RMS phenotypes.

METHODS

Patient Selection

The files of the Department of Pathology and cBioPortal[5] were searched for RMS harboring *NF1* gene alterations, managed at our institution between 2011 and 2022 with available targeted DNA sequencing (MSK-IMPACT) data. The study was approved by the institutional review board (IRB) committee (IRB 02-060). All study patients provided written informed consent to the use of their genomic data for research (IRB 12-245). Patient and tumor characteristics, treatment modalities, and follow-up were collected from the charts. In all patients the diagnosis was confirmed (C.R.A.) by incorporating histomorphology, immunohistochemistry, and molecular findings.

Similar criteria were applied for the control group of MTT, in which either germline or somatic *NF1* genetic alterations were demonstrated by NGS or a documented clinical and/or family history of neurofibromatosis type 1 (NF1). In four patients with MTT, matched normal blood/DNA and appropriate consent was available to evaluate for germline *NF1* mutations. The presence of rhabdomyoblastic divergent differentiation was identified by morphology and confirmed by desmin and myogenin positivity, whereas the diagnosis of malignant peripheral nerve sheath tumor (MPNST) was further confirmed by loss of H3K27me3 expression in all 11 cases tested.[6] For patients with RMS, risk group assignment was based on current guidelines.[4] For MTT, all patients were considered high risk.

Next-Generation Targeted Sequencing

All cases were tested on the MSK-IMPACT, a targeted DNA-based sequencing panel (410-505 genes)[6] to assess mutational landscape and copy number alterations. *FOXO1* gene rearrangements were excluded in most ERMS cases by fluorescence in situ hybridization or Archer FusionPlex.[7] The germline analysis included 76 genes on the MSK-IMPACT panel associated with hereditary cancer predisposition.[8,9] For copy number calling, a set of normal FFPE and blood control samples were used for reference diploid genome comparison. Coverage of targeted regions was computed using the GATK DepthofCoverage tool.[10] For each tumor sample, the matched patient-derived normal sample was also subjected to the same copy number variant calling algorithm. Log-ratio coverage values were subsequently segmented by circular binary segmentation, and segmented values were input into the ASCETS algorithm[11] to yield whole chromosomal arm-level calls.

Therapeutic Modalities

For patients with RMS, the initial treatment included multidrug chemotherapy regimens and radiotherapy, according to their risk group and COG clinical trials. For patients with MTT with localized disease, the initial management was upfront surgical resection with wide margins. Adjuvant radiotherapy was considered in selective cases on the basis of the location and margin status. Patients presenting with locally advanced/unresectable primary tumor or metastatic disease were treated with radiotherapy and/or doxorubicin-based chemotherapy regimens. Recurrent disease was managed with a combination of local control (palliative surgery, radiotherapy) and systemic chemotherapy.

Reverse Transcription-Quantitative Polymerase Chain Reaction Analysis

RNA was extracted, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was done as previously described,[12] with primers listed in Appendix Table A1.

Statistical Analysis

Survival analysis by comparison of hazard ratios using log-rank P testing, and visualization of Kaplan-Meier curves was performed using R packages survminer version 0.4.9 and survival version 3.2.13 (CRAN[13]). Mutations and gene-level copy number alterations were visualized using the OncoPrint function in the R package ComplexHeatmap version 2.8.0.[14]

RESULTS

Clinical Features and Outcome of *NF1*-Mutant RMS Cohort

Twenty two patients with RMS (seven female, 15 male) were identified, with an age range of 2-70 years (median, 17). All except one were ERMS, with a single spindle cell RMS (SRMS) harboring a hotspot *MYOD1* L122R mutation. The most common (91%) locations were head and neck (n = 8), extremity (n = 6), and paratesticular (n = 4; Table 1; Appendix Table A2). Most patients were classified as low (41%) or intermediate risk (50%). Two patients were classified as high risk. Six patients died of disease, including five intermediate-risk (one SRMS, four ERMS) and one high-risk ERMS, after experiencing local (n = 4) or metastatic (n = 2) relapse (median time to relapse 12.4 months).

Most patients with RMS (68%) were treated by pediatric oncology while few adult age patients were managed by adult sarcoma oncology (32%). All patients with low-risk pediatric ERMS were treated as per ARST-0331-A (ClinicalTrials.gov identifier: NCT00075582) while intermediate risk per D9803 (ClinicalTrials.gov identifier: NCT00003958; n = 4), ARST-1431-A (ClinicalTrials.gov identifier: NCT02567435), or ARST-0531-A (ClinicalTrials.gov identifier: NCT00354835). By contrast, patients with RMS treated by adult medical oncology received neoadjuvant vincristine, dactinomycin, and cyclophosphamide (VAC) chemotherapy, followed by surgical resection and adjuvant radiotherapy.

Among the pediatric cohort, 8 of 15 (53%) experienced disease recurrence (six local, one regional lymph nodes) or progression on primary treatment (one patient; median time to relapse 22 months; Fig 1). Four pediatric patients died of disease after developing relapse (27%; one SRMS, three ERMS), and one is alive with disease.

Among the adult cohort, 2 of 7 (29%) experienced metastases (median time to relapse 5 months) and died of disease. Both patients were treated with second-line chemotherapy, and one received additional radiotherapy and surgery (Fig 1).

Clinical Features and Outcome of *NF1*-Mutant MTT

Thirteen MTTs harboring germline and/or somatic *NF1* alterations and/or clinical findings in keeping with NF1 syndrome were selected (Table 1; Appendix Table A3). There were eight female and five male patients, with an age range of 2-78 years (median, 37 years). Chest wall and thigh location accounted for 77% of cases (five each). Two patients with MTT presented with distant metastatic disease. Only one patient developed MTT in prior radiation field, 8 years postradiation for breast cancer. Disease relapse occurred in 9 of 13 patients (69%) and was more frequently metastatic (6 of 9, 67%) than locoregional (3 of 9, 33%). The median time to recurrence was 10.6 months (range, 0.5-40.7). All metastases occurred in the lungs. Two patients progressed while on primary treatment. Ten patients (77%) died of disease.

Two thirds (9 of 13) of patients were managed by the adult sarcoma oncologists. Seven patients with localized disease underwent up-front gross resection, followed by radiotherapy in 5 of 7 cases and adjuvant chemotherapy in 3 of 7 cases (one VAC, two ifosfamide and doxorubicine). Six patients experienced relapse, four metastatic and two locoregional (Appendix Table A3). Two patients progressed during primary or second-line treatment. The median follow-up time was 6.2 years, and the median survival was 2.4 years (7 of 9 died of disease). Four patients were managed by pediatric oncology (age range, 2-27 years). Disease recurrence occurred in 3 of 4 cases, two metastatic (lungs) and one regional. All three patients succumbed to disease. The median follow-up time was 7.3 years, with a median survival of 2.7 years.

Three patients were initially misdiagnosed as RMS, including one 2-year-old with a paratesticular tumor (case 29) and two adult patients with lower extremity tumors (cases 26 and 27); however, they were subsequently reclassified as MTT on the basis of the NGS showing deep deletions in either *SUZ12* or *EED* genes for cases 26 and 27 (Appendix Fig A1).

Spectrum of *NF1* Gene Alterations in RMS and MTT

Germline NF1 *Alterations*

Among the 14 ERMSs tested, none exhibited germline *NF1* alterations. In the MTT group, four cases had germline DNA sequencing: three *NF1* loss and one wild-type. Overall, 7 of 13 (54%) had NF1 syndrome confirmed either by germline DNA sequencing (n = 3) and/or clinical features (n = 4). All three patients with confirmed germline also displayed clinical features of NF1 syndrome. In the remaining four patients lacking germline sequencing, the confirmatory clinical findings included café au lait spots and axillary freckling, cutaneous, and plexiform neurofibromas. In three of these tumors, no somatic *NF1* alterations were detected by MSK IMPACT testing (Appendix Table A4).

Somatic NF1 *Alterations*

In the *NF1*-mutant RMS cohort, all *NF1* alterations were deemed somatic, mostly truncating mutations (17 of 22; deletions, nonsense mutations), insertions (2 of 22), and splicing variants (2 of 22). The hotspot R134* truncating SNV

TABLE 1. Clinicopathologic Findings for NF1-Altered RMS and MTT Patient Cohorts

Variable	RMS (n = 22), No. (%)	MTT (n = 13), No. (%)
Sex		
Female	7 (32)	8 (62)
Male	15 (68)	5 (38)
Age at diagnosis, years		
<10	8 (36)	2 (15)
≥10	14 (64)	11 (85)
Primary tumor site		
Head and neck	8 (37)	1 (8)
Paratesticular	4 (18)	1 (8)
Pelvis	2 (9)	0 (0)
Extremity	6 (27)	5 (38)
Chest wall	0 (0)	5 (38)
Other	2 (9)	1 lung (8)
Histology		
ERMS	21 (95)	0 (0)
SRMS	1 (5)	0 (0)
MTT	0 (0)	13 (100)
Lymph node involvement		
N0	18 (82)	12 (92)
N1	4 (18)	1 (8)
Metastatic status		
M0	21 (90)	11 (85)
M1	2 (10)	2 (15)
Risk group		
Low	9 (9)	0 (0)
Intermediate	11 (82)	0 (0)
High	2 (9)	13 (100)
Radiation to primary site		
Yes	13 (59)	9 (69)
No	9 (41)	3 (23)
Unknown	0 (0)	1 (8)
Relapse/progression		
Yes	10 (45)	11 (85)
No	11 (50)	1 (8)
Unknown	1 (5)	1 (8)
Type of first relapse/progression		
Local	6 (60)	1 (9)
Regional	1 (10)	2 (18)
Metastatic	1 (10)	6 (55)
Progression under primary treatment	2 (20)	2 (18)
Vital status		
NED	15 (68)	3 (23)
DOD	6 (27)	10 (77)
AWD	1 (5)	0 (0)

Abbreviations: AWD, alive with disease; DOD, died of disease; ERMS, embryonal RMS; MTT, malignant triton tumor; NED, no evidence of disease; RMS, rhabdomyosarcoma; SRMS, spindle cell RMS.

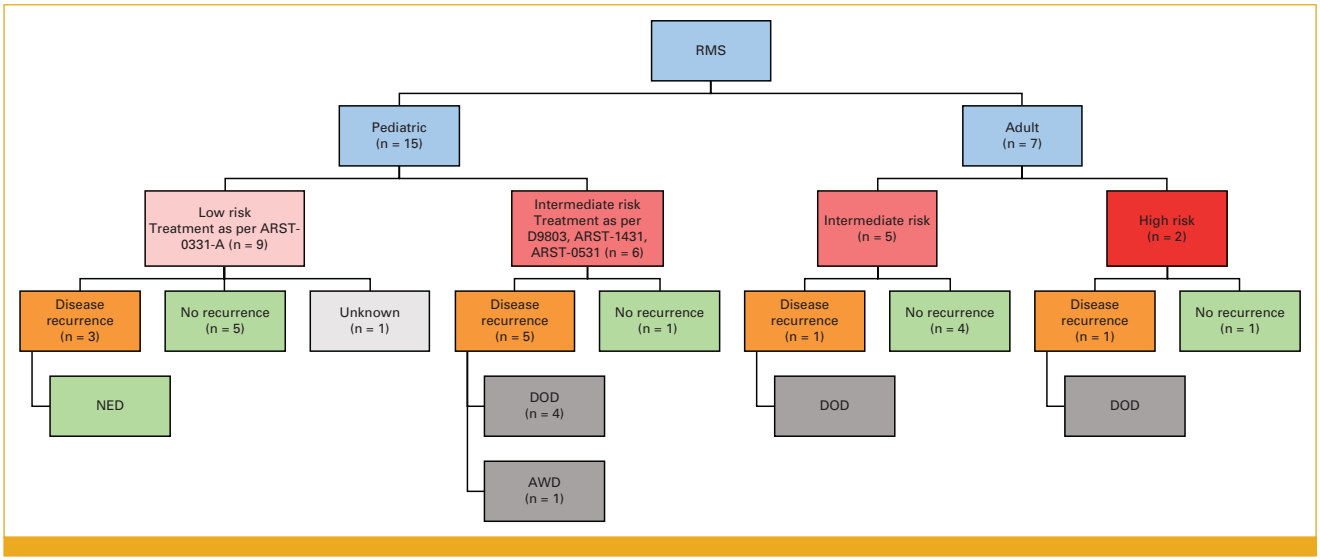

FIG 1. Diagrammatic breakdown of patients with NF1-RMS on the basis of age, risk stratification, and outcome. AWD, alive with disease; DOD, dead of disease; NED, no evidence of disease; RMS, rhabdomyosarcoma.

was the most common alteration (n = 3; Fig 2). The single *NF1*-altered SRMS had a *MYOD1* gene amplification, *MYOD1* L122R mutation, and a deep *NF1* deletion.

In the MTT group, 10 (77%) patients had somatic *NF1* mutations (four truncating SNVs, two deep deletions, two splicing variants, two frameshift deletions). Among them, three had in addition a germline mutation, one case had a clinical history of NF1 syndrome, and two lacked confirmation of NF1 syndrome through either germline DNA sequencing or clinical records while for the remaining four cases, there were insufficient clinical data and lack of germline sequencing for a conclusive assessment. However, these cases displayed somatic variant allelic frequencies ranging between 74% and 100% (Appendix Table A4).

Three (23%) patients had no evidence of somatic *NF1* alteration by MSK-IMAPCT; however, they harbored clinically documented NF1 syndrome.

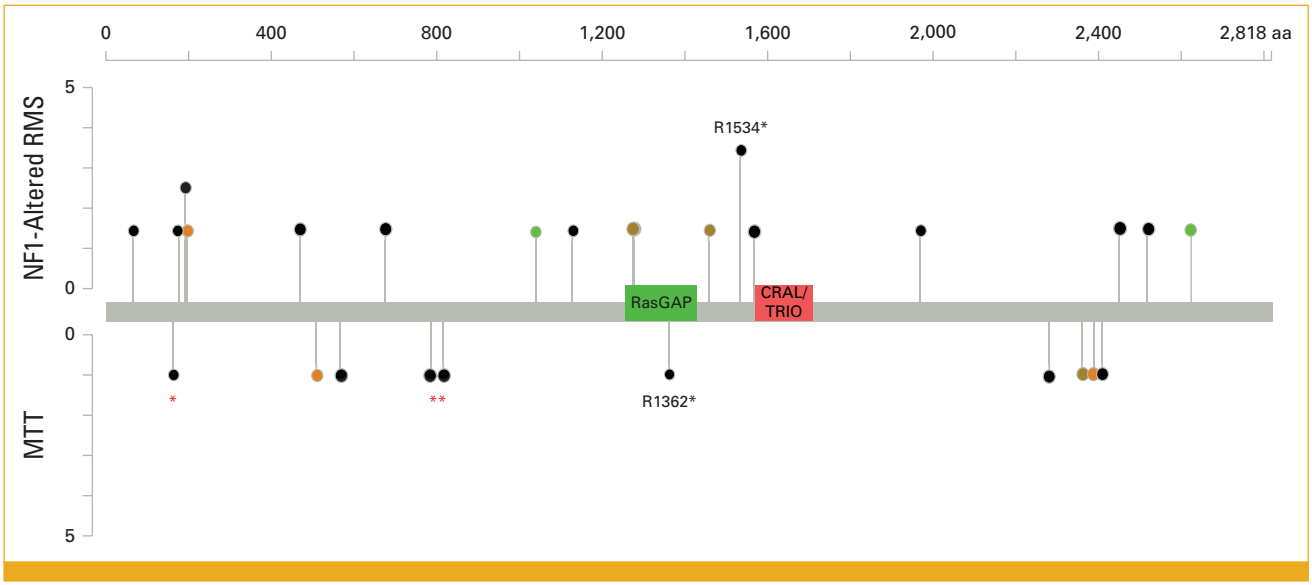

FIG 2. *NF1* somatic and germline alterations status and distribution in the two cohorts. Lollipop plot with the spectrum of *NF1* alterations, including *NF1*-mutant RMS tumors (above) and MTT tumors (below), representing the protein localization and type of *NF1* genomic alterations. In green, missense mutations; black, truncating mutations (nonsense, nonstop, frameshift deletion, frameshift insertion, splice site); brown, in-frame mutations (in-frame deletion, in-frame insertion); orange, splice mutations. Red star indicates germline alterations. MTT, malignant triton tumor; RMS, rhabdomyosarcoma.

Genomic Landscape and Co-Occurring Gene Alterations in *NF1*-RMS and *NF1*-MTT

The median tumor mutation burden (mt/mb) was three (range, 1-8) mt/mb among MTT with germline *NF1* mutations, two (range, 1-22) mt/mb among MTT without *NF1* germline mutations, and three (range, 1-22) mt/mb among *NF1*-mutant RMS.

Most NF1-Altered RMS Are Embryonal Subtype and Coexisting TP53 Mutations Are Associated With Poor Outcome

In the ERMS cohort, *TP53* missense or splicing alterations (8 of 22; 36%) and loss-of-function *BCOR* alterations (5 of 22; 23%) were the most common and mutually exclusive events (Fig 3). Three cases had hotspot *NRAS* alterations (Q61L, Q61K, G13V) and one *HRAS* amplification, which were mutually exclusive from *TP53* alterations. Four (18%) cases showed *MYC* amplifications which were mutually exclusive from *BCOR* alterations. *CDKN2A/B* deletions occurred in two (9%) cases. Arm-level copy number gain/amplifications were detected in 8q, 20p, and 20q in more than half of the cases. In total, 9 of 22 (41%) tumors had additional alterations in the RTK-RAS pathway. In three (14%) cases, no other pathogenic mutations were found apart from *NF1* alterations. Both high-risk ERMSs harbored alterations in *TP53* gene and *SOS1*, *NRAS* amplification in one case and *ATRX* splice mutation in the other. Among the intermediate-risk ERMS (n = 10), six had *TP53* alterations and two *CDKN2A/2B* deletions. Among the low-risk ERMS (n = 9) none had *TP53* or *CDKN2A/2B* alterations, instead harbored *BCOR* loss in three cases, *NRAS* or *HRAS* alterations in two cases, and the remaining exhibited multiple CNV rearrangements. No alterations of *SUZ12* and *EED* genes of the PRC2 complex were found in this cohort.

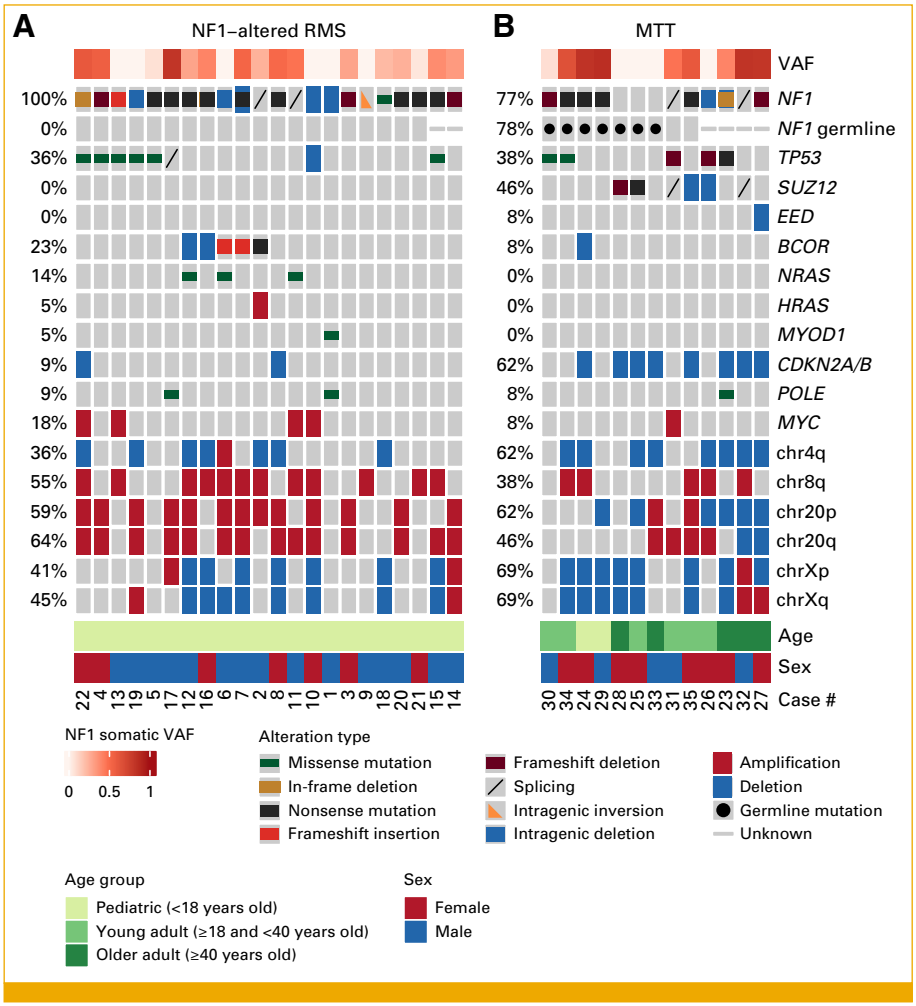

FIG 3. Oncoprint summary of molecular alterations in (A) 22 *NF1*-altered RMS compared with (B) 13 *NF1*-MTT. Each patient represents a column tagged by their case number below, and each gene query is listed in a row. Age groups and sex are shown color-coded. Mutation detection frequency (left column, %) is applied to each of the two cohorts tested by NGS. MTT, malignant triton tumor; NGS, next-generation sequencing; RMS, rhabdomyosarcoma; VAF, variant allelic frequency.

NF1-MTT Are Associated With a Unique Genomic Landscape Including CDKN2A/B and PRC2 Complex Loss-of-Function Alterations

The MTT group showed common *CDKN2A/2B* deep deletions (62%) and PRC2 complex loss-of-function alterations (54%), all except one being *SUZ12* alterations (nonsense mutation, frameshift deletion, or splicing) while one had an *EED* intragenic deletion. *SUZ12* or *EED* alterations were not detected in six cases, despite the loss of H3K27me3 expression in all, suggesting either undetected truncations or yet undefined mechanisms of PRC2 inactivation. Three patients with a proven history of NF1 and no detectable somatic *NF1* alterations harbored *CDKN2A/B* loss and PRC2 complex dysregulation through *SUZ12* truncating mutations (n = 2) or *NOTCH1* missense mutation (n = 1).

TP53 truncating or hotspot missense mutations occurred in 38% cases, which were mostly mutually exclusive from *CDKN2A/2B* alterations. All except one tumor had at least either a loss of *CDKN2A/2B* or *TP53* or a combined alteration of PRC2 complex genes and *TP53* (Fig 3; Appendix Table A4). One case (case 29) exhibited none of these alterations.

Survival Analysis

The *NF1*-altered RMS cohort had a median follow-up of 5.1 years (range, 0.5-11.6), with a 5-year event-free survival (EFS) of 44% and a 5-year overall survival (OS) of 70% (Appendix Fig A2A). No adverse factor was significant by univariate analysis. Trunk location (n = 2) was associated with worse EFS and OS (hazard ratio [HR], 1.704; $P < .001$) compared with other locations (Fig 4A). High-risk group was also associated with adverse OS (HR, 2.24; log-rank $P = .022$) while low-risk patients had a 5-year OS of 100% (Fig 4B). Patients with *NF1*-altered RMS with coexisting somatic *TP53* alterations had a 5-year OS of 24% compared with 90% in the *TP53* wild-type setting (HR, 2.38; log-rank $P = .0059$; Fig 4C). The six patients (four pediatric, two adult) who died of disease had *TP53* alterations (4 of 6), *CDKN2A* loss (1 of 6), or *MYOD1* L122R point mutation (1 of 6). The patients with active metastatic progressive disease after primary care harbor a *TP53* hotspot SNV.

The median follow-up time for the MTT group was 6.5 years (range, 3.6-16.7), and the median survival was 2.6 years. This group had an aggressive clinical outcome, with a 5-year EFS of 9.5% and a 5-year OS of 33% (Appendix Fig A2B). Nine patients developed relapse (six lung metastases), and 10 died of disease (Appendix Table A3). No clinical factors were significant by univariate analysis. No survival difference was observed between patients with germline *NF1* alterations or somatic *NF1* alterations only. No other molecular alterations were found to significantly affect survival in the MTT cohort.

Expression Patterns of Myogenic Markers by RT-qPCR

We further assessed the differential mRNA expression of key myogenic markers in *NF1*-mutant RMS, MTT, and normal skeletal muscle control by RT-qPCR (Appendix Fig A3). The results showed that the ERMS displayed significant upregulation of *DESM*, *MYOD1*, and *PAX7* myogenic genes akin to normal muscle while overexpression of *MYOG*, *PAX3*, and *PAX7* myogenic markers were detected in MTT.

DISCUSSION

RMS is the most frequent pediatric soft tissue sarcoma with a 70% survival rate.[15-18] In addition to RMS, other mostly unrelated tumor types may display rhabdomyosarcomatous phenotypes, such MPNST, malignant ectomesenchymoma, and so on. MTT is a rare, aggressive subtype of MPNST showing rhabdomyoblastic differentiation[19] and commonly associated with germline or sporadic *NF1* alterations.[6,20-22] As the distinction between MTT and *NF1*-RMS can be challenging and may have clinical impact, we sought to investigate the clinicopathologic findings and molecular landscape of a cohort of 22 *NF1*-mutant RMS compared with a group of 13 *NF1*-associated MTT, to better define their pathogenesis. We focused on *NF1*-altered RMS as it is the second most common genetic alteration in ERMS, with no previous study fully dedicated to this specific genotype to date, to our knowledge. Moreover, the shared *NF1* alterations with MTT further trigger diagnostic challenges.

First, patients with *NF1*-altered RMS showed a similar site predilection and risk group distribution compared with other large series of ERMS, including all genomic subsets.[1,19] Furthermore, the *NF1*-altered group shared mutational patterns with other cohorts of ERMS, with *BCOR, NRAS*, and *TP53* alterations as prevailing co-occurring events. However, previous reports described higher rates of these coexisting alterations, *BCOR* in 75%, *TP53* in 45%, and *NRAS* in 38% of cases[1] while our cohort exhibited lower rates of 23%, 36%, and 18%, respectively.

While this assumption deserves further larger-scale assessment, our focused analysis of *NF1*-mutant ERMS highlights that relapsed disease was confined to locoregional sites in all pediatric patients, contrasting with previous larger studies describing a third of ERMS relapses to be metastatic.[23] The 70% 5-year OS in our group was similar to 65%-70% for all-comers ERMS cohorts.[1,2] Previous published series of pediatric low-risk ERMS showed a 5-year survival of 75%-90%, with current efforts being directed toward dose-reduction chemotherapy.[1,17,24-26] Remarkably, our low-risk *NF1*-ERMS had 100% 5-year survival rate. The adverse prognostic impact of *TP53* alterations on ERMS survival is further confirmed within our cohort (HR, 2.38, $P = .0059$).[1,4,27] This finding provides additional support for using *TP53* alterations in current risk stratification,[28] especially in the context of intermediate-risk ERMS.

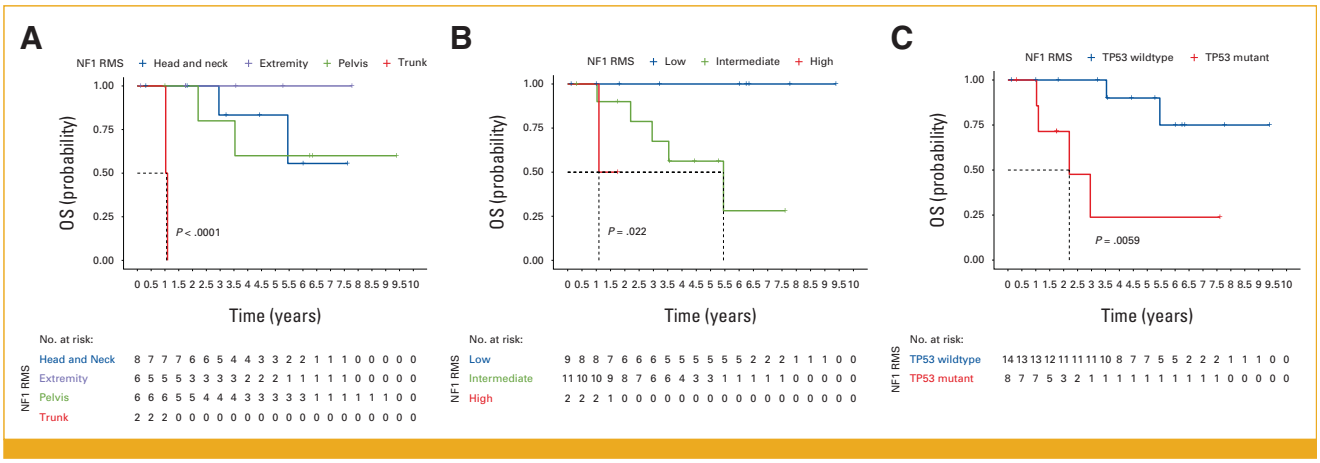

FIG 4. OS correlates with clinical and genomic factors in patients with *NF1*-altered RMS tumors. OS significant correlations included (A) trunk localization ($P < .0001$) compared with other sites (including extremity), (B) risk groups ($P = .022$), with low-risk tumors associated with 100% 5-year OS. (C) *TP53* alterations ($P = .0059$). OS, overall survival; RMS, rhabdomyosarcoma.

To our knowledge, our *NF1*-altered MTT group represents the largest and most comprehensive combined clinical and molecular investigation to date. The association with NF1 syndrome was documented in half of our cases, comparable with published series of conventional high-grade MPNST.[29-31] This finding suggests that the presence of *NF1* mutation in MTT does not imply a de facto NF1 syndrome setting. On the basis of the high *NF1* somatic variant allelic frequency ratio and no preexisting neurofibromas within the resected specimens, it is likely that in the remaining patients, the MTT occurred outside the NF1 syndrome. Our MTT cohort had a predilection for trunk anatomic sites (62%) compared with extremities (58%) in other high-grade MPNST.[32]

Only one MTT in this cohort occurred in the radiation field, a comparable incidence (7.5%) as described in all-comers high-grade MPNST.[33,34] Other than the systematic loss of function, no specific *NF1* alteration, whether germline or somatic, was associated with tumor phenotype, similar to MPNST.[30] Our NF1-MTT patient cohort represents a notably aggressive subset (5-year OS, 33%), in sharp contrast to large series of high-grade MPNST with a more favorable survival rates (5-year OS, 64%).[35] Various clinical factors such as tumor location do not appear to account for the stark difference in outcomes, as previously reported in MPNST cohorts.[6,31] Moreover, no genetic alterations, including the types of *NF1* alterations or any of the coexisting molecular events, had a survival impact on patients with NF1-MTT.

In keeping with the pathogenesis of conventional high-grade MPNSTs, the molecular landscape of *NF1*-MTT encompasses similar step-wise alterations, including *NF1* inactivation, *CDKN2A/B* loss, and subsequent loss-of-mutations in *EED* or *SUZ12* genes.[36,37] Akin to conventional MPNST, loss of H3K27me3 expression seems a reliable marker in confirming the diagnosis, being a surrogate of PRC2 complex alterations. This finding is particularly relevant in MTT as diagnostic pitfalls with ERMS are not infrequent, particularly in small biopsy material where the rhabdomyosarcomatous component is overrepresented. Thus, two of the three misclassified cases as ERMS with available material showed loss of H3K27me3 expression supporting an MTT diagnosis. Of note, 46% of MTT showing loss of H3K27me3 expression had no *EED* or *SUZ12* alterations by targeted sequencing. Importantly, no *SUZ12* and *EED* gene abnormalities were detected in RMS cases, lending further support using targeted sequencing in diagnostically challenging cases. Combining data with previous genomic studies, *NF1*-mutant ERMS harbor concurrent alterations in *CDKN2A/2B* in only 10%-25% of cases[1] while this alteration is present in 62% of MTT.

A number of striking differences emerged between the two groups, with ERMS occurring in younger patients (median, 18 years) primarily in the head and neck and pelvis,[1,4] whereas MTT are common in older individuals (median, 37 years), often affecting the trunk and extremities.[36] Our findings corroborated with earlier studies reveal that approximately 30%-45% of patients with MTT patients lack *NF1* germline alterations.[20-22] Furthermore, we underscore the rare occurrence of MTT in areas previously exposed to radiation.[6,38,39] H3K27me3 loss was systematic in our MTT cohort and frequently associated with PRC2 alterations. One previous study described H3K27me3 loss of expression in 25 ERMS tumors, suggesting that this marker is not suitable to discriminate ERMS from MTT, and instead, clinicopathologic characteristics should be used in this differential diagnosis.[40] By contrast, other studies have shown that H3K27me3 loss was exceptionally rare in RMS tumors.[41,42] The lack of PRC2 complex alterations in our cohort and in other large studies[1,4,43] strengthens the latter findings. Nevertheless, the complexity of distinguishing these two entities warrants a comprehensive approach using both

clinicopathologic and molecular findings. Additionally, the H3K27me3 loss of expression does not seem to consistently align with PRC2 complex alterations.

In conclusion, our study highlights that patients with *NF1*-mutant RMS demonstrate a 70% 5-year OS while cases with coexisting somatic *TP53* alterations had a dismal 25% 5-year OS compared with 90% in *TP53* wild-type setting. This result further supports the on-going ARST2032 trial excluding patients with high-risk genomic features (*TP53* and *MYOD1*) from dose reduction. Conversely, the MTT group followed a highly aggressive clinical course, with a dismal 33% 5-year OS and 46% risk of metastasis. The striking molecular differences between the two groups suggest that NGS can be used in diagnostically challenging cases, as well as to exclude the presence of *TP53* alterations shown to be associated with poor outcome in *NF1*-ERMS.

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DATA SHARING STATEMENT

Data will be available on reasonable request to the corresponding author.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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APPENDIX

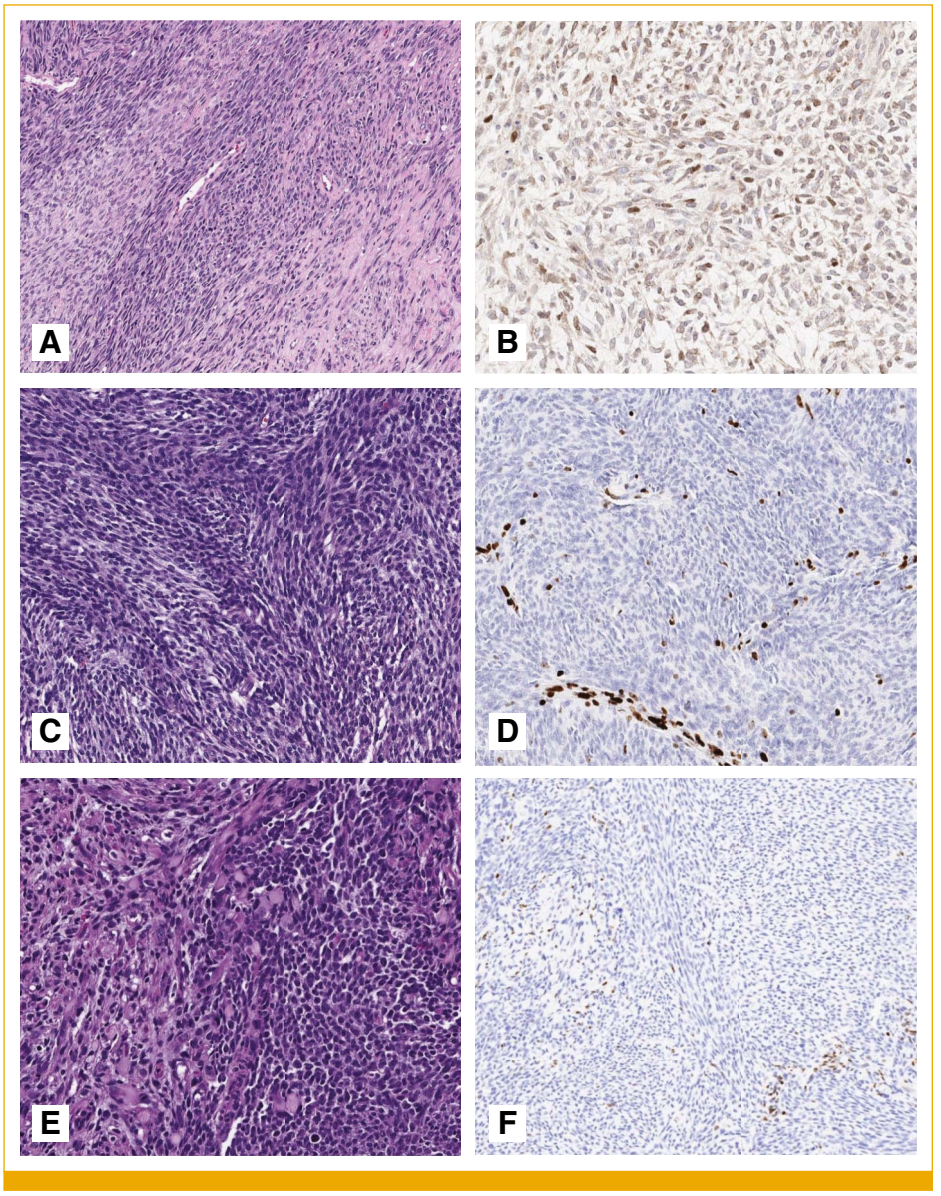

FIG A1. Pathologic features of the three cases of MTT that were initially diagnosed as RMS. (A and B, case 26) Microscopic features showing alternating cellular and more fibrotic areas, arranged in long fascicles and monomorphic cytology, which by immunohistochemistry showed scattered myogenin positive cells; (C and D, case 27) hypercellular undifferentiated spindle cell neoplasm arranged in intersecting fascicles showing complete loss of H3K27me3 expression; (E and F, case 29) biphasic neoplasm showing abrupt transition from an undifferentiated fascicular neoplasm to areas of more epithelioid growth with abundant eosinophilic cytoplasm, which by immunohistochemistry showed complete loss of H3K27me3 expression in both components. All three cases had up-front surgical resection of the tumor. In case 26, the misdiagnosis had clinical impact, being initially treated as RMS, including adjuvant chemotherapy (VAC) and radiotherapy (36.0 Gy). MTT, malignant triton tumor; RMS, rhabdomyosarcoma; VAC, vincristine, dactinomycin, and cyclophosphamide.

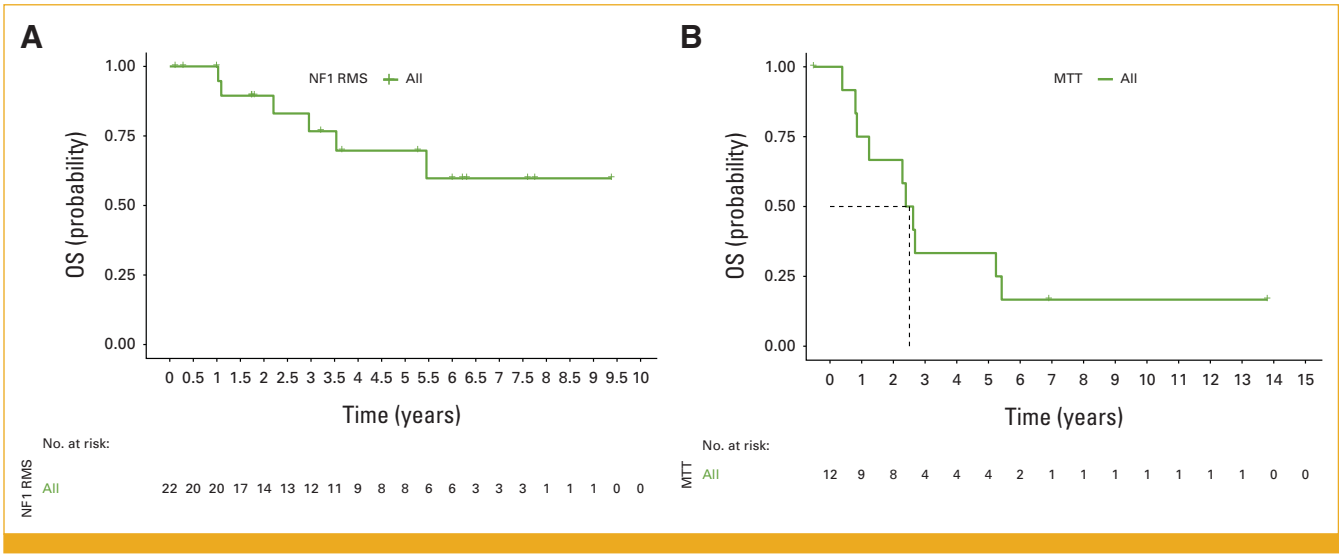

FIG A2. OS Kaplan-Meier curves. (A) *NF1*-altered RMS (n = 22); (B) *NF1*-MTT (n = 13). MTT, malignant triton tumor; OS, overall survival; RMS, rhabdomyosarcoma.

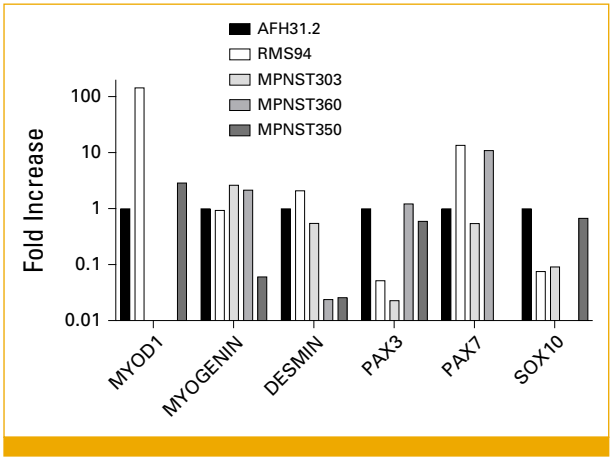

FIG A3. Expression levels of key myogenic and neural markers in a subset of NF1-ERMS and NF1-MTT, compared with normal muscle. Expression of *MYOD1*, *MYOG*, *DESM*, *PAX3*, *PAX7*, and *SOX10* is depicted in fold increase compared with normal muscle tissue (black). Missing bars are RNA levels undetectable. Frozen tumor tissues from one *NF1*-mutant ERMS (case 3) and three MTT samples (from cases 25, 32, 34) were available for RT-qPCR analysis. Case 25 exhibited high levels of *MYOD1*, *PAX3*, and *SOX10*, whereas cases 31 and 34 showed upregulation of *MYOG* and *PAX7* levels. Overall SOX10 expression was low in MTT, similar to normal muscle tissue. ERMS, embryonal rhabdomyosarcoma; MTT, malignant triton tumor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

TABLE A1. Primers Used for RT-qPCR Reactions

Gene	Forward Primer	Reverse Primer
MYOG	5′-AGATGTGTCTGTGGCCTTCC-3′	5′-AGCTGGCTTCCTAGCATCAG-3′
MYOD	5′-AGCACTACAGCGGCGACT-3′	5′-GCGACTCAGAAGGCACGTC-3′
PAX3	5′-AGCTCGGCGGTGTTTTTATCA-3′	5′-CTGCACAGGATCTTGGAGACG-3′
PAX7	5′-CGTGCTCAGAATCAAGTTCG-3′	5′-GTCAGGTTCCGACTCCACAT-3′
DESM	5′-GCTGCTGGACTTCTCACTGG-3′	5′-AGATGTGTCTGTGGCCTTCC-3′
SOX10	5′-ATGAACGCCTTCATGGTGTGGG-3′	5′-CGCTTGTCACTTTCGTTCAGC AG-3′

Abbreviation: RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

TABLE A2. Clinical Features of *NF1*-Mutant RMS Cohort

Case No.	Follow-Up Period, Years	Age at Diagnosis, Years	Sex	Location	Tumor Type	Risk	Status	Team (adult/peds)
1	11.96	20.9	Male	Pelvis	SRMS	Intermediate	DOD	P
2	10.37	17.3	Male	Pelvis	ERMS	Low	NED	P
3	8.93	37.0	Female	Extremity	ERMS	Low	NED	P
4	8.20	32.3	Female	Head and neck	ERMS	Intermediate	NED	A
5	7.85	28.2	Male	Thoracoabdominal wall	ERMS	Intermediate	DOD	A
6	7.11	2.3	Male	Pelvis	ERMS	Low	NED	P
7	7.01	16.5	Male	Pelvis	ERMS	Low	NED	P
8	10.26	4.6	Female	Head and neck	ERMS	Intermediate	DOD	P
9	7.09	6.3	Male	Head and neck	ERMS	Low	NED	P
10	6.08	28.5	Female	Extremity	ERMS	Intermediate	NED	A
11	5.93	70.5	Male	Extremity	ERMS	Intermediate	NED	A
12	4.93	4.3	Male	Head and neck	ERMS	Intermediate	NED	P
13	4.89	4.2	Male	Head and neck	ERMS	Intermediate	DOD	P
14	4.37	21.8	Male	Extremity	ERMS	Intermediate	NED	A
15	3.94	6.3	Male	Pelvis	ERMS	Intermediate	DOD	P
16	3.88	6.4	Female	Head and neck	ERMS	Low	NED	P
17	2.85	32.8	Male	Extremity	ERMS	High	NED	A
18	1.71	14.1	Male	Pelvis	ERMS	Low	NED	P
19	1.60	56.3	Male	Other	ERMS	High	DOD	A
20	1.38	18.2	Male	Extremity	ERMS	Low	NED	P
21	2.32	5.6	Female	Head and neck	ERMS	Low	NED	P
22	0.80	22.8	Female	Head and neck	ERMS	Intermediate	AWD	P

Abbreviations: AWD, alive with disease; DOD, died of disease; ERMS, embryonal RMS; NED, no evidence of disease; RMS, rhabdomyosarcoma; SRMS, spindle cell RMS.

TABLE A3. Clinical Features of *NF1*-Altered Malignant Triton Tumor Cohort

Case No.	Age at Diagnosis, Years	Follow-Up, Years	Sex	Tumor Location	Status	Team (adult/pediatrics)	Relapse Status	Surgery	Radiotherapy	Chemotherapy
23	53.4	3.6	Female	Chest wall	NED	A	NA	NA	NA	NA
24	3.0	6.7	Female	Lung	DOD	P	Regional	R0	Yes	Neoadjuvant
25	37.3	6.1	Female	Chest wall	DOD	A	Metastatic	R0	Yes	None received
26	39.6	9.4	Female	Thigh	NED	A	Local	R0	Yes	Adjuvant
27	65.0	5.5	Female	Thigh	DOD	A	Metastatic	R0	Yes	None received
28	77.7	8.2	Female	Chest wall	DOD	A	Metastatic	R0	Yes	None received
29	2.2	16.6	Male	Paratesticular	NED	P	No relapse	R0	No	Adjuvant
30	19.7	3.7	Male	Thigh	DOD	P	Metastatic	R1	Yes	Neoadjuvant
31	28.6	4.3	Male	Thigh	DOD	A	Progression	No	No	Neoadjuvant
32	51.8	7.5	Male	Chest wall	DOD	A	Progression	R0	Yes	Adjuvant
33	75.5	6.5	Male	Mediastinum	DOD	A	Metastatic	R0	No	Adjuvant
34	27.5	7.7	Female	Parapharyngeal	DOD	P	Metastatic	R0	Yes	Adjuvant
35	27.4	5.7	Female	Calf	DOD	A	Local	R0	Yes	Adjuvant

Abbreviations: DOD, died of disease; NA, not available; NED, no evidence of disease; R0, complete resection; R1, incomplete resection.

TABLE A4. Molecular Features of *NF1*-Altered Malignant Triton Tumor Cohort (n = 13)

Case No.	Altered Germline NF1	Clinical NF1 Syndrome	H3K27me3 Status	NF1 Somatic Alteration Type (VAF)	Somatic Variant-NF1 VAF/TC	*CDKN2A/2B* Alteration	PRC2 Complex Alteration (VAF)	*TP53* Alteration (VAF)
23	ND	NA	Loss	Inframe deletion (0.41)	1	Yes	No	Yes (0.90)
24	Yes	Yes	Loss	Truncating mutation (0.83)	1	Yes	No	No
25	ND	Yes	Loss	NEG	NEG	Yes	Yes (*SUZ12*, 0.47)	No
26	ND	NA	ND	Deep deletion	1	No	Yes (deletion)	Yes (0.76)
27	ND	NA	Loss	Truncating mutation (0.81)	0.74	Yes	Yes (deletion)	No
28	ND	Yes	ND	NEG	NEG	Yes	Yes (*SUZ12*, 0.22)	No
29	Yes	Yes	Loss	Truncating mutation (0.86)	1	No	No	No
30	Yes	Yes	Loss	Truncating mutation (0.08)	0.51	No	No	Yes (0.09)
31	WT	No	Loss	Splicing variant (0.47)	1	No	Yes (*SUZ12*, 0.42)	Yes (0.49)
32	ND	NA	Loss	Splicing variant (0.83)	1	Yes	Yes (*SUZ12*, 0.89)	No
33	ND	Yes	Loss	NEG	NEG	Yes	No	No
34	ND	Yes	Loss	Truncating mutation (0.66)	0.78	No	No	Yes (0.02)
35	ND	No	Loss	Truncating mutation (0.61)	1	Yes	Yes (deletion)	No

Abbreviations: NA, not available; ND, not done; NEG, negative; TC, tumor cellularity; VAF, variant allelic frequency; WT, wild-type.