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RESEARCH ARTICLE

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Changes on chromosome 11p15.5 as specific marker for embryonal rhabdomyosarcoma?

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Abstract

Rhabdomyosarcomas (RMS) constitute a heterogeneous spectrum of tumors with respect to clinical behavior and tumor morphology. The paternal uniparental disomy (pUPD) of 11p15.5 is a molecular change described mainly in embryonal RMS. In addition to LOH, UPD, the MLPA technique (ME030kit) also determines copy number variants and methylation of *H19* and *KCNQ1OT1* genes, which have not been systematically investigated in RMS. All 127 RMS tumors were divided by histology and PAX status into four groups, pleomorphic histology ($n = 2$); alveolar RMS PAX fusion-positive (PAX+; $n = 39$); embryonal RMS ($n = 70$) and fusion-negative RMS with alveolar pattern (PAX-RMS-AP; $n = 16$). The following changes were detected; negative ($n = 21$), pUPD ($n = 75$), gain of paternal allele ($n = 9$), loss of maternal allele ($n = 9$), hypermethylation of *H19* ($n = 6$), hypomethylation *of KCNQ1OT1* ($n = 6$), and deletion of *CDKN1C* ($n = 1$). We have shown no difference in the frequency of pUPD 11p15.5 in all groups. Thus, we have proven that changes in the 11p15.5 are not only specific to the embryonal RMS (ERMS), but are often also present in alveolar RMS (ARMS). We have found changes that have not yet been described in RMS. We also demonstrated new potential diagnostic markers for ERMS (paternal duplication and UPD of whole chromosome 11) and for ARMS PAX+ (hypomethylation KCNQ1OT1).

KEYWORDS
alveolar RMS, CDKN1C, embryonal RMS, H19, KCNQ1OT1, PAX, UPD

1 | INTRODUCTION

Rhabdomyosarcomas (RMS) represent a heterogeneous spectrum of tumors in terms of clinical behavior and tumor morphology.[1] RMS are the most common malignant soft tissue tumor seen in children and adolescence, with an annual incidence of 4.5 cases per million among children. Histologically, RMS resembles developing fetal striated skeletal muscle.[2–4] They are frequently composed of primitive cells, which only show a subtle evidence of lineage-specific differentiation; however, they may mature and, in some cases, reach a stage close to terminal differentiation resembling myotubes of a developing skeletal muscle. Approximately 50% of rhabdomyosarcoma cases are diagnosed in the first decade of life.[3] RMS is currently categorized by distinct histopathological subtypes, including embryonal, alveolar,

pleomorphic, and spindle cell/sclerosing; these have distinct molecular and clinical correlates.[4–8] Age also influences the incidence of these tumors. The embryonal RMS (ERMS) are the most common form in early childhood, but some data indicate a second peak in early adolescence.[3,4] Ras mutation (*NRAS*, *KRAS*, and/or *HRAS*) is noted in approximately 25% of ERMS.[9] Rarely, these mutations can be demonstrated in alveolar RMS (ARMS).[10] Compared to ARMS, ERMS generally show gain of whole chromosomes 2, 7, 8, 11, 12, 13, and 20.[11,12] Therefore, the copy number aberration is also different between ERMS and ARMS and can be helpful in supporting the diagnosis of ERMS in difficult cases. Bimodal peaks are not evident in children with ARMS. ARMS occur at all ages, but are more common in adolescents and young adults.[13,14] Cytogenetic studies identify a frequent t(2;13)(q35;q14) or variant t(1;13)(p36;q14) chromosomal translocation in most cases of ARMS. These translocations involve the *PAX3* gene on chromosome 2 or the *PAX7* gene on chromosome 1 and the *FOXO1* gene on chromosome 13 to generate *PAX3-FOXO1* or *PAX7-FOXO1* fusion genes, which encode fusion proteins with oncogenic activity. Molecular pathologic analysis of fusion status revealed that 80% of ARMS cases contain a *FOXO1* fusion (60% with *PAX3-FOXO1* fusion and 20% with *PAX7-FOXO1* fusion).[14,15] This finding is important because fusion-negative ARMS have molecular features reminiscent of ERMS. The clinical outcome of children with fusion-negative ARMS is similar to that of children with ERMS. In addition to the presence of metastases, FOXO1 status is the most important prognostic factor.[14–17] In contrast to ERMS and ARMS, WHO-classified pleomorphic RMS primarily occurs in adult males in their sixth decade of life.[18] Finally, spindle cell/sclerosing RMS are an uncommon subtype, accounting for 3%–10% of all cases of RMS. It affects both children and adults.

Acquired uniparental disomy (UPD), also known as copy neutral loss of heterozygosity (LOH), is a relatively common event in cancer.[19,20] Acquired UPD can occur in two different ways; loss of one chromosome followed by duplication of the remaining one leading to whole-chromosome UPD, whereas somatic recombination leads to segmental acquired UPD. In both cases, the copy number does not change. UPD has the potential to lead to homozygosity of existing aberrations such as mutation, deletion, methylation, histone-modification, or imprinted genes.[19] The paternal uniparental disomy (pUPD) of 11p15.5 is another example of molecular changes in RMS.[21–25] The classically described uniparental disomy of 11p15.5, as well as deletion of maternal allele, gain of paternal allele or *H19* hypermethylation alone and/or *KCNQ1OT1* hypomethylation has been described in Beckwith–Wiedemann syndrome (BWS).[26–28] BWS is associated with some tumors such as nephroblastoma and hepatoblastoma.[19] Similar genetic changes have been described in some nephroblastomas without BWS syndrome.[29]

In this article, we aimed to confirm data from literature that UPD or LOH of 11p15.5 is more frequent in ERMS than ARMS in a large cohort of patients.[4,24,28] However, our data showed that there was no difference in the presence of UPD and LOH between all three groups: the fusion-negative RMS with alveolar pattern (PAX-RMS-AP) group, the ERMS group, and the ARMS PAX-positive group. The MLPA technique also allowed us to investigate changes in the imprinted 11p15.5 region (methylation status of *H19*, *KCNQ1OT1*), which have not yet been investigated in RMS. Therefore, we were able to demonstrate possible new diagnostic markers.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

All the procedures of the study were approved by the Institute of Medical Ethics Committee of University Hospital Motol. All the patients signed an informed consent form at the time of admission; this form explained that the tissues and other samples might be used for scientific research but would not compromise patient data privacy.

A cohort of 127 (60 females and 67 males) patients with histologically confirmed rhabdomyosarcoma at University Hospital Motol seen between 1995 and 2018 were included in this study. The median age was 7 years. Among all, 79 patients were between 0 and 10 years, 33 were between 10 and 20, and 15 were more than 20. We used 92 frozen tissues and 35 formalin-fixed, paraffin-embedded tissues. All samples contained tumor cells (at least 50%). Data on clinical characteristics, including age and sex, were retrieved from medical records. None of the patients with somatic chromosomal abnormalities in the 11p15.5 region had any clinical manifestations of BWA. In patients, where, consent was obtained and germline DNA was available, we investigated chromosomal abnormalities in the 11p15.5 region. But, none of these abnormalities were demonstrated in any patient. Only one patient (PRG123) with ERMS had rasopathy (Costelo syndrome; *HRAS* Gly12Ser). The somatic mutations of *HRAS*, *NRAS*, and *KRAS* genes were examined by the target NGS in 122 out of 125 tumor samples. Overall, we found pathogenic mutations in these genes in 10 out of 122 samples (*HRAS*: 2× PAX-RMS-AP, 3× ERMS; *NRAS*: 1× ARMS PAX+, 1× ERMS; *KRAS*: 1× PAX+, 2× ERMS; Supporting Information, Table S2).

2.2 | DNA and RNA extraction

Total RNA was isolated from fresh frozen (92 cases) or paraffin-embedded sections (35 cases) using extraction with TRIzol Reagent (Life Technologies) or High Pure RNA Paraffin Kit from Roche (Roche), both according to the manufacturer's instructions. The genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue blocks using QIAamp DNA FFPE Tissue Kit (Qiagen) or fresh frozen sections using extraction with TRIzol Reagent (Life Technologies).

2.3 | *PAX3-FOXO1* and *PAX7-FOXO1* fusion detection

2.3.1 | PCR analysis

Complementary DNA (cDNA) was prepared from 10 µL of RNA in 20 µL of reaction volume. The reaction mixture contained Tris–HCl 50 mM, pH 8.3; KCl 75 mM; MgCl2 3 mM; dithiotreitol l0 mM;

dNTP 0.5 mM each; random hexamers 12.5 mM; and 50 units of MMLV reverse Transcriptase (Gibco BRL) according to the manufacturer's instructions. Reverse transcription included an incubation period of 60 min at 37°C. Qualitative RT-PCR for the fusion gene *PAX3-FOXO1*, *PAX7-FOXO1* was performed with primers which are listed in Table S1. Quantitative RT-PCR for fusion gene *PAX3/7--FOXO1* and was performed on LightCycler (Roche), using TaqMan technology. PAX3-FOXO1 and PAX7-FOXO1 primers and probes for RQ-RT-PCR were designed by TipMolBiol; Human beta 2 microglobulin was used as a housekeeping gene.[30] The expression of fusion genes was calculated using absolute quantification. A diluting line of plasmids was used for preparation of standard curves. Normalized expression (PAX3/7:FOXO1) was determined as a ratio between fusion transcript and B2-microglobulin levels. Each PCR run was performed in duplicate, and the mean value of the results was calculated.

2.3.2 | MS-MLPA

The SALSA MS-MLPA Probemix ME030 BWS/RSS assay was used for the detection of aberrant methylation of one or more sequences of the IC2 (KvDMR) and IC1 (H19DMR) domains in the 11p15 BWS/RSS region. This assay can also be used to detect deletions/duplications in the aforementioned chromosomal region (MRC-Holland). MS-MLPA was performed following the manufacturer's instructions.[31] Data were analyzed using Coffalyser Software (MRC-Holland).

2.3.3 | SNP array

SNP microarray study was carried out commercially either from Illumina (HumanCytoSNP-12 DNA Analysis Bead Chip Kit 300 K or HumanCytoSNP-850 K v1.2 Bead Chip Kit [Illumina®]). Images were captured with iScan. Data were analyzed (primary) using Illumina's GenomeStudio and BlueFuse Multi Software. The resolution was set as 0.1 Mb for deletion, 0.5 Mb for gain, and 3 Mb for UPD.

2.4 | LOH study

The presence of LOH was studied in 102 tumor samples. Each sample was compared with the constitutional DNA of the same patient obtained from bone marrow aspirate or from peripheral blood. All constitutional samples were without tumor cell contamination as confirmed by molecular techniques (negative results of RQ-RT-PCR for *MYOD1*, α/γ acetylcholine receptor, *PAX3-FOXO1*, and *PAX7-FOXO1*). A panel of six highly informative microsatellite markers was used (Table S1). Amplification was performed with primers labeled with fluorescent dye (6-FAM) in a final volume of 20 µL containing 50–100 ng of DNA template, 1–2 mM $MgCl_2$, 10 pM of each primer, 0.2 mM dNTPs, 1× reaction buffer, and 0.3 U Taq polymerase (Top-Bio). Amplification was performed in an automated thermal cycler with 5 min 95°C denaturation step followed by 30 cycles of 94°C denaturation for 30 s, annealing at the temperature of 60–66°C for 45 s, and extension at 72°C for 30 s. The last cycle was followed by a final extension at 72°C for 20 min. PCR products were detected by fragment analysis using an automated genetic analyzer ABI PRISM 3130 or 3500 (Applied Biosystems).

2.5 | Statistics

For statistical comparison, Fisher's exact test was used; this test assumed the null hypothesis (H0) that all variables are independent. If p value ≤0.05, we can reject the null hypothesis. All statistics were performed in R software version 4.0.3 (https://www.r-project.org/).

3 | RESULTS

3.1 | Histological features and *PAX3-7FOXO1* status of RMS

All the tumor specimens were histologically evaluated following the classification of soft tissue tumors. The ERMS were confirmed in 70 cases, PAX-RMS-AP in 16 cases, ARMS PAX+ in 39 cases (PAX3+ $n = 33$; PAX7+ $n = 6$), and 2 cases had pleomorphic histology (PML).

3.2 | Detection of genetic abnormalities

All 127 RMS tumors were investigated by MS-MLPA using Probemix ME030 BWS/RSS. We examined two PML samples, one showed pUPD and the other was negative. Due to the small number of samples and the impossibility of testing by SNP array and LOH11p technique, we included only ERMS, PAX-RMS-AP, and ARMS PAX+ samples for further analysis. We found that only 20 (16%) cases were negative out of 125 investigated samples. In the other 105 samples, the following mutations were detected: pUPD ($n = 74$), paternal duplication allele ($n = 9$), maternal deletion ($n = 9$), hypermethylation of *H19* ($n = 6$), hypomethylation of *KCNQ1OT1* ($n = 6$), and deletion of *CDKN1C* ($n = 1$; Figure 1D). CNV and UPD were compared by LOH study and SNP array. Results from MS-MLPA and second technique were done in 104 samples (83%). Thus, only 21 samples (17%) were without confirmation of the second method. Also, LOH study and SNP array are unable to detect the methylation status of *H19*, *KCNQ1OT1* genes. A comparison of all techniques is summarized in Figure 2. MS-MLPA + LOH study was investigated in 71 samples, MS-MLPA + SNP array in 4 samples, and all three technics in 22 samples. We found only three results discrepancies (two cases MS-MLPA and LOH study detected UPD, but SNP array did not; one case MS-MLPA and SNP array detected loss of allele, but LOH study did not). SNP array allows to detect the extent of UPD on chromosome 11. Thus, we can demonstrate whether it is a segmental change or a UPD of the whole chromosome 11. We examined the SNP array in 26 tumor samples and found UPD in 12 of them. Interestingly, in six

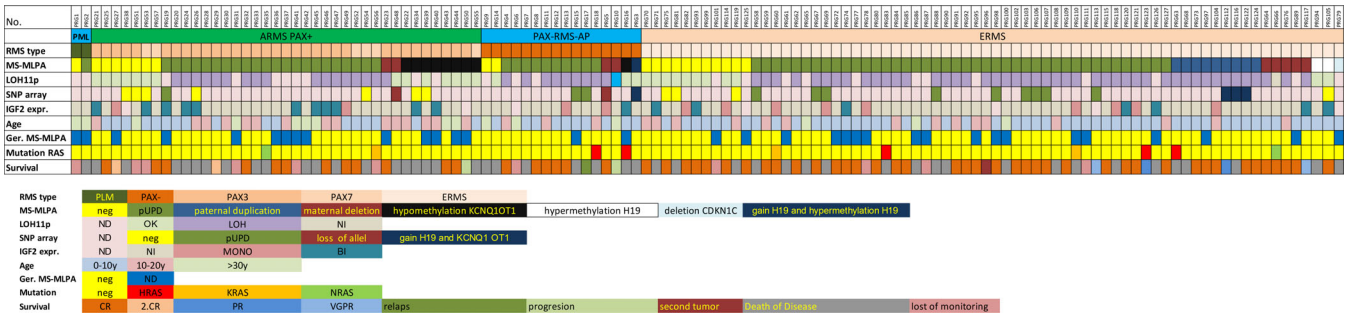


FIGURE 1 Genetic abnormalities. (A) Characterization of chromosomal abnormalities in ARMS PAX+. (B) Characterization of chromosomal abnormalities in PAX-RMS-AP. (C) Characterization of chromosomal abnormalities in ERMS. The percentage of chromosomal changes was calculated from the number of samples in each RMS subgroup (ARMS PAX+ (A), PAX-RMS-AP (B), and ERMS (C)). Thus, it shows the proportional representation of chromosomal changes in a given RMS subgroup. (D) Summary information on chromosomal abnormalities from all three groups (ARMS PAX+, PAX-RMS-AP, and ERMS). The percentage of chromosomal changes was calculated from all RMS samples. Thus, it shows the proportional representation of chromosomal changes in all RMS samples. (E) Statistical assessments of the changes with the largest difference (Fisher's exact test). ARMS, alveolar rhabdomyosarcomas; ERMS, embryonal rhabdomyosarcomas; KCNQ1OT1, Potassium Voltage-Gated Channel Subfamily Q Member 1 overlapping transcript 1; PAX, paired box 3 or 7 negative fusion; PAX−, paired box negative fusion; PAX+, paired box 3 or 7 positive fusion; pUPD, paternal uniparental disomy, H19, H19 gene; RMS, rhabdomyosarcomas.

samples, we found UPD of the whole chromosome 11 and it was always ERMS. Only three samples of ERMS had segmental UPD (ERMS vs. ARMS group was p value = 0.001932).

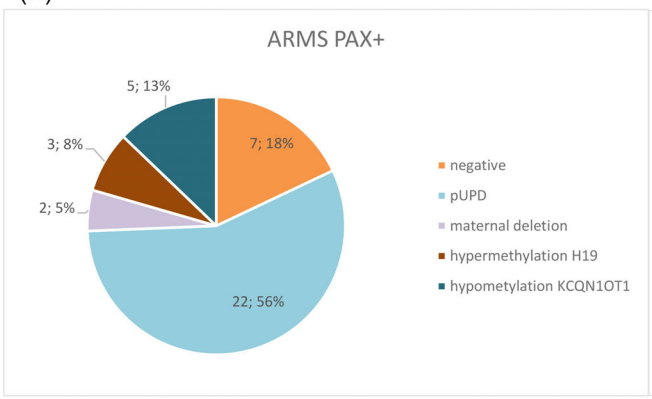
Using MS-MLPA technique, we demonstrated different changes and their numbers in ERMS, PAX-RMS-AP, and ARMS PAX+ (Figure 1A–C; percentages are expressed for a given group). The largest differences were demonstrated between ERMS and PAX-RMS-AP as well as ARMS PAX+ groups. The most common change demonstrated in all three groups was pUPD (n = 74; ERMS 42/70, PAX-RMS-AP 10/16, and ARMS PAX+ 22/39). Fisher's exact test showed no statistically significant difference between these groups (p value = 0.7452; Figure 1E). In contrast, we showed a statistically significant difference in paternal duplication, where this change was only found in ERMS (n = 9/70); when comparing ERMS/ARMS (PAX-RMS-AP, ARMS PAX+), p value = 0.004669. On the other hand, a change not found in ERMS is hypomethylation of *KCQN1OT1*. It was only found in PAX-RMS-AP (1/16) and ARMS PAX+ (5/39). When we evaluated all groups, p value = 0.006138, hence not statistically significant. However, when we compared ERMS versus ARMS PAX+ p value = 0.004928, showing significance. All statistical assessments of the changes with the largest differences are summarized in Figure 1E.

4 | DISCUSSION

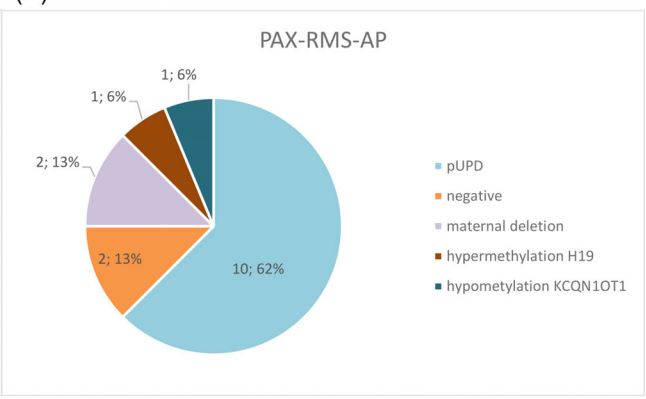
It is stated in the literature that the LOH or pUPD of 11p15.5 occurs predominantly or exclusively in the ERMS.[4,21,24,28] But Smith et al. had a hypothesis that there is a possibility of translocation independent pathway for development of ARMS. This is based on genetic alterations at the region 11p15 for ARMS cases lacking the common t(2;13) and t(1;13) translocations.[32] Our data show that both the ERMS and ARMS are associated with LOH at the 11p15 region with changes in methylation status. We first described that changes at the region 11p15 are a common event in the groups of ARMS patients (with or without translocations). We have statistically shown no difference in the frequency of pUPD 11p15.5 between ERMS, PAX-RMS-AP and ARMS PAX+ group (p value = 0.7452). Most of these LOH 11p15.5 results were verified by at least two techniques. Based on our data, LOH or pUPD 11p15.5 should not be used as an ERMS marker. Using the MLPA technique, we were able to demonstrate other changes in the imprinted 11p15.5 region (*H19*, *KCNQ1OT1*), which have not yet been investigated in RMS. Of the 106 changes detected in 11p15.5 region (*H19*, *KCNQ1OT1*), 31 were other than pUPD. We can divide them into three groups. First, where IGF2 was upregulated, the second group with downregulation of *CDKN1C* (p57Kip2), and the third, where both mechanisms were present at the same time. The first group contains a gain of paternal allele and hypermethylation of *H19*. The second group contains hypomethylation of *KCNQ1OT1* and the deletion of *CDKN1C*. The third group contains pUPD and loss of maternal allele.

The first group is dependent on the increased expression of the IGF2 protein, which is a protein hormone known to regulate cell proliferation, growth, migration, differentiation, and survival. The gene is parentally imprinted. Loss of imprinting of the *IGF2* gene is a recurrent observation in growth disorders that combine overgrowth with a variety of malignant tumors.[33] The mRNA *IGF2* overexpression can be directly regulated by methylation of *IGF2* gene. We did not prove methylation of P4 promotor of *IGF2* gene in our sample's cohort by MLPA (MLPA data did not show). Overexpression of mRNA *IGF2* may not always lead to overexpression of the IGF2 protein and activation[34] of the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway of the mitogen-activated protein kinase (MAPK) pathway or angiogenesis.[35] Downstream activation is dependent not only on IFG2 protein expression, but also on its receptors, and binding proteins.[33,34,36,37] Thus, changes in the IFG2 pathway must be well

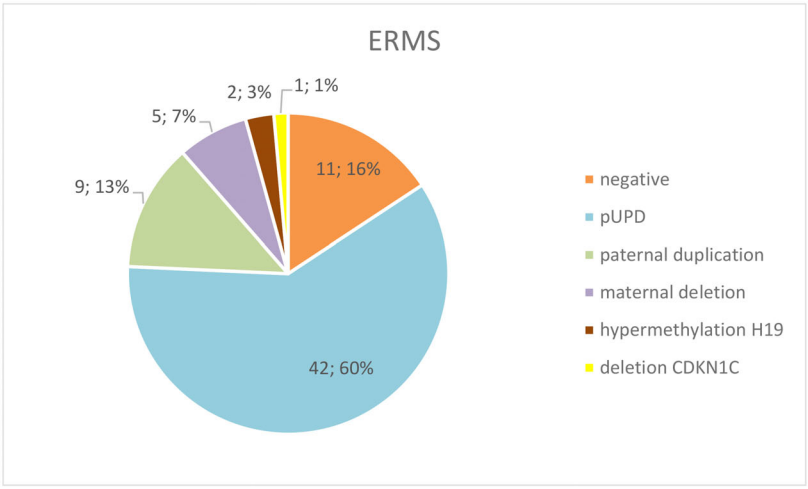
(A)



(B)



(C)



(D)

	PAX-RMS-AP	ERMS	ARMS PAX+	Total changes
negative	2 (1.6%)	11 (8.8%)	7 (5.6%)	20 (16%)
pUPD	10 (8%)	42 (33%)	22 (17.6%)	74 (59%)
paternal duplication	0	9 (7%)	0	9 (7%)
loss of maternal alelle	2 (1.6%)	5 (4%)	2 (1.6%)	9 (7%)
hypermethylation H19	1 (0.8%)	2 (1.6%)	3 (2.4%)	6 (4.8%)
hypometylation KCQN1OT1	1 (0.8%)	0	5 (4%)	6 (4.8%)
deletion CDKN1C	0	1 (0.8%)	0	1 (0.8%)

(E)

	ERMS/PAX-RMS-AP/ARMS PAX+ (p-value)	ERMS/ARMS (p-value)	ERMS/ARMS PAX+ (p-value)	ERMSE,PAX-RMS-AP/ARMS PAX+ (p-value)
pUPD	0.7452	0.5983	0.7007	0.6979
paternal duplication	0.0182	**0.004669**	0.02503	0.05595
hypometylation KCQN1OT1	0.006138	0.00618	**0.004928**	0.01125

FIGURE 2 Oncoplot—comparison of molecular genetics methods, histology, and clinical data. BI, bi-allelic expression; CR, complete remission; 2.CR, second complete remission; KCNQ1OT1, Potassium Voltage-Gated Channel Subfamily Q Member 1 overlapping transcript 1; LOH, Loss of heterozygosity; MONO, mono-allelic expression; ND, non done; Neg, negative; NI, non-informative; OK, no change; PAX3, paired box 3 positive fusion; PAX−, paired box negative fusion; PAX7+, paired box 7 positive fusion; PLM, pleomorphic RMS; PR, partial remission; pUPD, paternal uniparental disomy; VGPR, very good partial remission.

orchestrated, leading to tumor growth. Therefore, we need more information about this coordination in RMSs.

The second group depends on the decreased expression of the p57kip2, encoded by the *CDKN1C* gene, which is a member of the Cip/Kip family of cyclin-dependent-kinase inhibitors (CKIs), and plays a key role in mammalian development by regulating cell proliferation and differentiation in a number of different tissues.[38] Specifically, it has been reported that p57Kip2 inhibits the kinase activity of cyclin-CDK complexes in vitro, including cyclin E (A)/CDK2 and cyclin D1,2/CDK4.[39] *CDKN1C* is expressed after birth only in the heart, brain, lung, kidney, pancreas, skeletal muscle, testis, and placenta. *CDKN1C* is a paternally imprinted gene, expressed exclusively from the maternal allele.[37,40–42] *CDKN1C* defective expression is generally responsible for overgrowth disorders, such as the BWS syndrome, and also in certain cancer types.[15,37,43–48] Interestingly, Figliola et al. have proven that *CDKN1C* is a target of the muscle-specific factor MyoD1.[49] *MyoD1* induces *CDKN1C* expression by interacting with *CDKN1C* promoter or between direct interaction of *MyoD1* with *CDKN1C*.[49,50] Thus, the deletion of the *CDKN1C* gene may lead to impaired myoblast differentiation and rhabdomyosarcoma formation. Moreover, many authors have attributed p57Kip2 level as a valuable prognostic marker, since a decrease of its expression has been correlated to poor prognosis.[45,51] We included only one patient with ERMS in this group.

The third group is the most common in our cohort (pUPD 59%; loss of maternal allele 7%). Therefore, we assume that both overexpression of *IGF2* and downregulation of *CDKN1C* are involved in the tumorigenesis of RMS.

Moreover, we first demonstrated two changes that are more specific to the ERMS or ARMS group. The paternal duplication (7% cases) was demonstrated only in the ERMS group. This difference was shown to be statistically significant (p value = 0.004669), and thus could be a new diagnostic marker separating ERMS and ARMS; therefore, indicating possible differential activation of this pathway in RMS. In contrast, hypomethylation of *KCNQ1OT1* was demonstrated in one case only in the ARMS group (4.8%). In the case of PAX-RMS-AP, in which, we demonstrated the hypomethylation of *KCNQ1OT1*, we also investigated other possible fusions typical for ARMS (Archer sarcoma panel v3, data not shown here); but we did not detect any fusion. When we compared ERMSs and ARMSs, the result was not statistically significant (p value = 0.00618), but when we evaluated ERM versus ARMS PAX+, we showed statistical significance (p value = 0.004928). We have demonstrated not only a possible diagnostic marker, but that reduced *CDKN1C* expression may be more important for patients with ARMS than for ERMS.

The extent of UPD on chromosome 11 is another possible diagnostic feature that has not been described, as we have shown that UPD of the whole chromosome has only been demonstrated in ERMS (p value = 0.001932). We hypothesize that this finding is related to the fact that ERMS is generally characterized by whole chromosome aberrations.[12]

The IGF pathway still offers a wide variety of interesting developments that may contribute to the future development of growth factor-based therapies in RMS.[36,52–54] Accordingly, a number of small-molecule RTK inhibitors (RTKIs) and monoclonal antibodies (mAbs) targeting the IGF-binding domain on the Insulin-Like Growth Factor 1 Receptor (IGF1R) were developed for use in cancer treatment. To date, all of these strategies have failed in clinical trials, primarily owing to the onset of acquired resistance.[53] Instead, we support the option that by identifying and applying predictive biomarkers, a cohort of patients with IGF1R-driven tumors who will be more likely to respond positively to treatment, may be identified. ClinicalTrials.gov identifier (NCT number): NCT03041701, A Phase I/II Trial of the Insulin-Like Growth Factor 1 Receptor (IGF-1R) Antibody AMG479 (Ganitumab) in Combination with the Src Family Kinase (SFK) Inhibitor Dasatinib in Patients with ERMS and ARMS.

Our study showed that changes in the 11p15.5 are not specific to the ERMS but are also often present in the ARMS. Using the MLPA technique, we have described changes that have not yet been described in RMS. We demonstrated new potential diagnostic markers for ERMS (paternal duplication and UPD of whole chromosome 11) and for ARMS PAX+ (hypomethylation *KCNQ1OT1*). These prognostic features need to be validated in larger cohorts of patients.

These proven changes also lead us to the concept of using targeted therapy in patients with relapsing ERMS or ARMS, although it will be necessary to clarify a number of factors that may affect the success of such therapy.

AUTHOR CONTRIBUTIONS

Study design: Ales Vicha and Lenka Krskova. *MLPA*: Dagmar Voriskova and Lucie Stolova. *SNP array*: Lucie Stolova and Pavla Jencova. *LOH analysis*: Lenka Krskova. *Clinical data acquisition*: Eva Drahokoupilova and Anasuya Guha. *Histology examination*: Daniela Novakova-Kodetova. *NGS analysis*: Marie Kopecká and Ales Vicha. *Bioinformatics and biostatistics*: Petr Broz. *Writing—original draft*: Ales Vicha and Lenka Krskova. *Writing—review and editing*: All authors equally.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

This study includes clinical samples for which patients did not consent to extended data sharing and are therefore HIPAA protected.

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