

RESEARCH ARTICLE

Compound HRAS/PIK3CA Mutations in Chinese Patients with Alveolar Rhabdomyosarcomas

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Abstract

The rhabdomyosarcoma (RMS) is the most common type of soft tissue tumor in children and adolescents; yet only a few screens for oncogenic mutations have been conducted for RMS. To identify novel mutations and potential therapeutic targets, we conducted a high-throughput Sequenom mass spectrometry-based analysis of 238 known mutations in 19 oncogenes in 17 primary formalin-fixed paraffin-embedded RMS tissue samples and two RMS cell lines. Mutations were detected in 31.6% (6 of 19) of the RMS specimens. Specifically, mutations in the NRAS gene were found in 27.3% (3 of 11) of embryonal RMS cases, while mutations in NRAS, HRAS, and PIK3CA genes were identified in 37.5% (3 of 8) of alveolar RMS (ARMS) cases; moreover, PIK3CA mutations were found in 25% (2 of 8) of ARMS specimens. The results demonstrate that tumor profiling in archival tissue samples is a useful tool for identifying diagnostic markers and potential therapeutic targets and suggests that these HRAS/ PIK3CA mutations play a critical role in the genesis of RMS.

Keywords: Rhabdomyosarcoma - massARRAY system - mutation - oncogene - HRAS/PIK3CA

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Introduction

Rhabdomyosarcoma (RMS) is the most common type of soft tissue tumor in children and adolescents and can be classified as two main subtypes based on histologic and genetic criteria: the more prevalent embryonal rhabdomyosarcoma and the more aggressive alveolar rhabdomyosarcoma (EMRS and ARMS, respectively). Approximately 80% of ARMS have translocations involving PAX3 or PAX7 and FKHR (Sorensen et al., 2002). Meanwhile, ERMS is characterized by a loss of heterozygosity on the chromosome 11 short arm (11p15.5) (Anderson et al., 1999). Although much progress has been made in the past decades in terms of improving diagnosis and treatment of RMS, uncontrolled metastasis is a significant risk, and long-term survival for patients with metastatic RMS remains poor (< 30%) (De Giovanni et al., 2009). Recently, Xin et al. have reported that the 1-year survival rate of stage IV patients was estimated at 50%, with a 5-year survival rate of only 25% (Xin et al., 2013). Moreover, understanding of the genetic factors contributing to RMS development and progression remains limited.

Different tumor types harbor somatic gene mutations, which contribute to tumor development and can also serve as therapeutic targets. Mutations in the oncogenes RAS

and PTPN11 have been reported in RMS (Chen et al., 2006), while FGFR4 mutations are present in both ARMS and ERMS subtypes; moreover, FGFR4 knockdown or inhibition has been shown to decrease RMS cell proliferation in vitro and tumor burden in vivo (Taylor et al., 2009).

Sequenom MassARRAY technology uses a mass spectrometry-based genotyping approach that enables more sensitive mutational analysis than traditional Sanger sequencing. Furthermore, highly multiplexed PCR assays allow efficient high-throughput screening of large tumor sample sets and the identification of novel mutations in various tumor types (Thomas et al., 2007). However, a high-throughput oncogene mutation profiling study for RMS has been reported only one paper (Shukla et al., 2012).

The present study used the Sequenom MassARRAY platform to conduct a high-throughput sequencing analysis of 238 known mutations across 19 oncogenes in 17 RMS tissue samples and two RMS cell lines in order to identify novel mutations involved in RMS pathogenesis.

Materials and Methods

Patient samples and cell lines

Formalin-fixed paraffin-embedded (FFPE) RMS tissue

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Table 1. Summary of Mutations Identified in RMS

Sample ID	Subtype*	Gene_mutation	Amino acid substitution
R1	ERMS	NRAS_181C>A	Glutamine→Lysine (Q61K)
R5	ERMS	NRAS_181C>A	Glutamine→Lysine (Q61K)
R13	ARMS	NRAS_181C>A	Glutamine→Lysine (Q61K)
R16	ARMS	PIK3CA_1636C>A	Glutamine→Lysine (Q546K)
R18 (RD cell line)	ERMS	NRAS_183A>T	Glutamine→Histidine (Q61H)
R19 (PLA-802 cell line)	ARMS	HRAS_183G>T PIK3CA_1624G>A	Glutamine→Histidine (Q61H) Glutamic acid→Lysine (E542K)

*Based on histological assessment; RMS, rhabdomyosarcoma; ERMS, embryonal RMS; ARMS, alveolar RMS

Table 2. Association between Clinical and Pathologic Characteristics of RMS and RAS Mutations

	Variable	Cases	Mutation	No mutation	P*
Subtype	ERMS	10	2	8	1.000
	ARMS	7	1	6	
Grade	G2	5	1	4	1.000
	G3	12	2	10	
TNM stage	II	7	2	5	0.537
	III-IV	10	1	9	

*Fisher's exact test was used to compare variables; RMS, rhabdomyosarcoma; ERMS, embryonal RMS; ARMS, alveolar RMS; TNM, tumor-node-metastasis

samples were selected from the archives of the Department of Pathology of the First Affiliated Hospital, Shihezi University School of Medicine (Xinjiang, China). Written informed consent was obtained from all participating patients before enrollment in the study. This study was approved by the institutional ethics committee at the First Affiliated Hospital of Shihezi University School of Medicine and conducted in accordance with the ethical guidelines of the Declaration of Helsinki. The sample set included 10 ERMS and 7 ARMS. All original slides including haematoxylin-eosin and immunohistochemical staining from each case were reviewed by two senior pathologists. To identify samples as the ARMS subtype, the presence of PAX3-FKHR or PAX7-FKHR fusion genes was assessed by reverse transcription polymerase chain reaction. ERMS and ARMS cell lines (RD and PLA-802, respectively) were obtained from Shanghai Fuxiang Biological Technology Co. Ltd. (Shanghai, China). Data from patient records were used for a retrospective survival analysis with respect to the identified mutations.

Isolation of genomic DNA

Genomic DNA was extracted from tissue samples and cell lines using the QIAamp DNA FFPE Tissue and DNeasy Blood and Tissue kits, respectively (Qiagen, Hilden, Germany), following the manufacturer's protocol.

Mutational screen using MassARRAY and the OncoCarta panel

The MassARRAY system (Sequenom Inc., San Diego, CA, USA), which is based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and the OncoCarta Panel v1.0 (Sequenom), can detect 238 mutations in 19 genes (ABL1, JAK-2, CDK4, KRAS, HRAS, NRAS, AKT1, AKT2, KIT, EGFR, MET, ERBB2, PDGFA, BRAF, FGFR1, FGFR3,

PIK3CA, RET, and FLT3).

The procedure was based on the manufacturer's recommendations, with minor modifications. The initial PCR amplification was conducted in a reaction mixture (5 μ L) containing approximately 2 μ L sample DNA, 25 mmol/L MgCl₂, 250 mmol/L deoxynucleotide triphosphate (dNTP) mix, 0.5 μ M/500uM OncoCarta Primer Mix, and 5 units Sequenom PCR enzyme. The cycling conditions were as follows: 95°C for 2 min; 45 cycles each of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. Unincorporated dNTPs were dephosphorylated in a shrimp alkaline phosphatase cocktail. A single base extension reaction was performed in a 2 μ L iPLEX™ Pro Extension Reagent Set reaction with 0.755 μ L water, 0.2 μ L 10× buffer, 0.2 μ L Thermosequenase termination mix, 0.804 μ L OncoCarta primer mix, and 0.041 μ L Thermosequenase (Sequenom). The cycling conditions were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s; five cycles of 52°C for 5 s and 80°C for 5 s; and 72°C for 3 min. The reaction mixture was desalted and then analyzed using the MassARRAY Typer 4.0 software (Sequenom). SpectroCHIPs were analyzed using an Autoflex MALDI-TOF MS (Bruker AXS Inc./GmbH, Karlsruhe, Germany), and data were analyzed using the MassARRAY Typer Analyzer software 4.0.4.20 (Sequenom).

Statistical analysis

The SPSS v. 17 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Parameters of interest were compared using Fisher's exact test. Survival analyses were based on Kaplan-Meier life tables. Differences were considered statistically significant for two-sided *P* values < 0.05.

Results

Mutations were detected by Sequenom analysis in 31.6% (6 of 19) of RMS samples, in three different genes (NRAS, HRAS, and PIK3CA) at five nucleotide positions (Table 1). NRAS mutations were found in 27.3% (3 of 11) of ERMS specimens, while mutations in NRAS, HRAS, and PIK3CA were found in 37.5% (3 of 8) of ARMS specimens.

Mutations in genes belonging to the RAS family, i.e., NRAS and HRAS, were identified in 26.3% (5 of 19) of the samples, including both subtypes of RMS (27.3% or 3 of 11 for ERMS and 25% or 2 of 8 for ARMS). In the ERMS tissue or cell line, all of these were mutations in NRAS (two NRASQ61K and one NRASQ61H) (Figure 1). In ARMS specimens, one of the mutations was NRASQ61K,

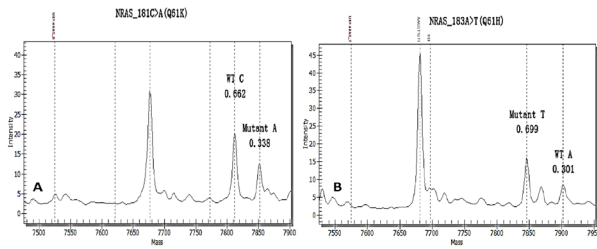


Figure 1. NRAS Mutations in ERMS. (A) Mass spectrogram of the NRASQ61K mutation in sample 1, indicating the substitution of CAA (Gln) by AAA (Lys) at codon 61. (B) Mass spectrogram of the NRASQ61H mutation in sample 18 (RD cell line), indicating a substitution of CAA (Gln) by CAT (His) at codon 61

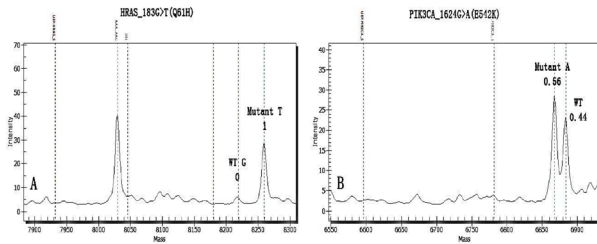


Figure 2. Compound HRAS and PIK3CA Mutations in ARMS (PLA-802 cell line). Mass spectrogram of (A) HRASQ61H and (B) PIK3CAE542K mutations in sample 19, indicating a Gln→His substitution at codon 61 and a GAA (Glu) to AAA (Lys) substitution at codon 542

the other was HRASQ61H.

PIK3CA mutations were identified in 10.5% (2 of 19) of RMS cases (i.e., 25% or 2 of 8 ARMS cases). Concurrent mutations in HRAS and PIK3CA were detected in the ARMS cell line PLA-802 (Figure 2).

There was no association between the presence of RAS mutations and RMA subtype ($P = 1.000$), and tumor stage ($P = 1.000$) or grade ($P = 0.537$) (Table 2), and the disease-free survival rate was similar for RMS patients with or without mutations ($P = 0.502$) (Figure 3).

Discussion

Many large-scale screens have been undertaken in order to characterize cancer genomes and identify somatic alterations that can hasten the development of targeted therapeutics. To date, two reports of mutational hotspot profiling in a large number of samples have emerged. One study found a mutation detection frequency of about 30% in 1000 tumor samples examined, which included breast, colorectal, and endometrial cancers, as well as sarcomas (Thomas et al., 2007). The other study consisted of a high-throughput Sequenom-based analysis in a large set of pediatric solid tumors, including neuroblastoma, Ewing's sarcoma, RMS, and desmoplastic small round cell tumors (Shukla et al., 2012). In this study, we investigated the mutational status of 19 cancer-associated genes in RMS in FFPE archival tissue samples and cell lines by using a mass spectrometry-based approach.

Mutations in NRAS, KRAS, HRAS, BRAF, PIK3CA, CTNNB1, PTPN11, and FGFR4 have been reported in 20.2% of RMS cases, including 28.3% of ERMS and 3.5% of ARMS cases (Shukla et al., 2012). These frequencies

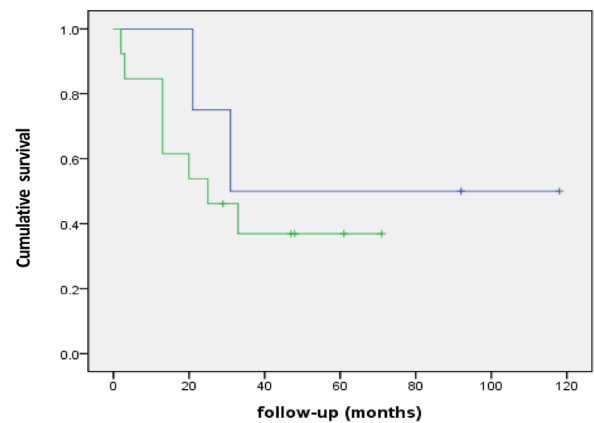


Figure 3. Cumulative Overall Survival Rate in RMS Patients Based on the Presence (blue) or Absence (green) of Mutations. Vertical lines in each curve represent censored data (i.e., cases living or lost to follow-up)

were lower than the values observed in the present study, in which mutations were identified in 31.6% of RMS cases. Mutations were detected in 27.3% of ERMS cases (in NRAS), while 37.5% of ARMS samples had mutations in NRAS, HRAS, and/or PIK3CA. Possible reasons for the difference in mutation rates between the two studies are the sample sizes (89 vs. 19 in this study) and patient ethnicity (European Caucasian vs. Chinese).

The earlier study also found that the mutation frequency for members of the RAS gene family was higher in ERMS than ARMS cases (11.7% vs. 3.5%, respectively) (Shukla et al., 2012). Moreover, these authors found HRASQ61K in the ERMS cell line SMS-CTR, as well as KRASG13D in an ARMS sample (Shukla et al., 2012). The results of other studies have variously shown that the frequency of RAS gene mutations in ERMS is approximately 35% (NRAS and KRAS) (Stratton et al., 1989), 22.6% (NRAS and HRAS missense mutations affecting codon 61, with no mutations detected in KRAS) (Martinelli et al., 2009), 42% (NRAS and HRAS) (Paulson et al., 2011), and 33.3% (HRAS) (Kratz et al., 2007). In the present study, RAS mutations were observed in both RMS subtypes at similar frequencies (27.3% and 25% in ERMS and ARMS, respectively). NRASQ61K or NRASQ61H mutations were observed in four samples, including the ERMS cell line RD, and HRASQ61H was found in the ARMS cell line PLA-802 (Table 1). Although it is not known why previous studies failed to identify RAS mutations in ARMS, members of this gene family clearly play a critical role in tumorigenesis in RMS. In support of this, a study involving a zebrafish model of RAS-induced RMS showed that tumors expressing markers typical of human RMS are morphologically similar to the embryonal subtype (Langenau et al., 2007).

Like RAS, PIK3CA is also one of the most commonly mutated oncogenes identified in human cancers. PIK3CA mutations were detected in 5% of ERMS samples in one study; two of the three mutations (E542K and E545K) were in the helical domain, while the third (H1047R) was located in the kinase domain (Shukla et al., 2012). In the present study, PIK3CA mutations were observed in ARMS at a frequency of 25%; both mutations (E542K, Q546K)

were in the same helical domain of the gene, suggesting that this genomic region is a mutational hotspot.

To our knowledge, this study is the first to report the identification of compound RAS/PIK3CA mutations in ARMS. The data suggest that the aggressive phenotype of ARMS may be associated with the presence of the double mutation and may thus play a major role in tumor progression, although further studies with a larger sample size are needed in order to determine whether these mutations have predictive value for the prognosis of RMS patients. In conclusion, mutational profiling in archival tissue samples can be a useful tool for identifying markers that can serve as therapeutic targets in RMS and other cancer types.

Acknowledgements

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