Phosphorylation profiles of protein kinases in alveolar and embryonal rhabdomyosarcoma

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Rhabdomyosarcoma is the most common pediatric soft-tissue sarcoma, which includes two major subtypes, alveolar and embryonal rhabdomyosarcoma. The mechanism of its oncogenesis is largely unknown. However, the oncogenic process of rhabdomyosarcoma involves multi-stages of signaling protein dysregulation characterized by prolonged activation of tyrosine and serine/threonine kinases. To better understand this protein dysregulation, we evaluated the phosphorylation profiles of multiple tyrosine and serine/threonine kinases to identify whether these protein kinases are activated in rhabdomyosarcoma. We applied immunohistochemistry with phospho-specific antibodies to examine phosphorylation levels of selected receptor and non-receptor tyrosine kinases, mammalian target of rapamycin (*mTOR*), *p70S6K*, and protein kinase C (*PKC*) isozymes on alveolar and embryonal rhabdomyosarcoma tissue microarray slides. Our results showed that the phosphorylation levels of these kinases are elevated in some rhabdomyosarcoma tissues compared to normal tissues. Phosphorylation levels of receptor and non-receptor tyrosine kinases are elevated between 26 and 68% in alveolar rhabdomyosarcoma and between 24 and 71% in embryonal rhabdomyosarcoma, respectively, compared to normal tissues. In addition, phosphorylation levels of *mTOR* and its downstream targets, *p70S6K*, *S6*, and *4EBP1*, are increased between 50 and 72% in both subtypes of rhabdomyosarcoma. Further, phosphorylation levels of *PKCα*, *PKCδ*, *PKCθ*, and *PKCζ/η* are upregulated between 57 and 69% in alveolar rhabdomyosarcoma and between 43 and 72% in embryonal rhabdomyosarcoma. This is the first report to create a phosphorylation profile of tyrosine and serine/threonine kinases involved in the *mTOR* and *PKC* pathways of alveolar and embryonal rhabdomyosarcoma. These protein kinases may play roles in the development or tumor progression of rhabdomyosarcomas and thus may serve as novel targets for therapeutic intervention.

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Rhabdomyosarcoma is the most common soft tissue sarcoma in childhood. Approximately 350 cases are newly diagnosed each year in the United States and they account for 4% of all childhood malignancies.¹ Most of these tumors arise in the head and neck region, genitourinary tract, and extremities. The two

main histological subtypes of rhabdomyosarcoma are embryonal rhabdomyosarcoma, which comprises more than half of all rhabdomyosarcoma cases, and alveolar rhabdomyosarcoma, which is less common but more aggressive. These two subtypes arise at different primary body sites and have different age patterns.² Clinically, the presentation of rhabdomyosarcoma is genetically heterogeneous. However, some genetic markers have been identified. Among them, approximately 75% of alveolar rhabdomyosarcoma are characterized by chromosomal translocation, most frequently, t(2;13)(q35;q14) or the variant t(1;13)(q36;q14).³ These translocations can disrupt either the *PAX3*

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or *PAX7* genes located on chromosomes 2 and 1 respectively, and the *FKHR* gene on chromosome 13, thereby generating the fusion protein *PAX3-FKHR* and *PAX7-FKHR* products. These fusion proteins function as chimeric transcription factors with increased transcription activity and have been shown to play a role in the dysregulation of the cell cycle in alveolar rhabdomyosarcoma cells.^{4,5}

Rhabdomyosarcoma is believed to arise from the regulatory disruption of proliferation and differentiation of skeletal muscle progenitor cells, but the molecular etiology of the disease remains unclear.⁶ To shed light on this question, recent studies have compared the gene expression pattern in cancer cells *versus* surrounding normal tissue cells.^{7,8} The current methods for studying gene expression in cancer cells focuses on the mRNA level using cDNA microarray or on the protein level using proteomics technologies.^{9–12} Although these useful and powerful techniques do lead to the identification of differential expression of certain proteins at the mRNA or protein levels, they may not be able to detect the difference of proteins at the level beyond the post-translational modifications between cancer and normal cells.

Protein phosphorylation is one of the key post-translational modifications to regulate protein function and appears to be most consistent with their biological activities. In normal cells, the phosphorylation state is under delicate control in that the protein kinases are usually phosphorylated transiently upon stimulation by growth factors or cytokines, whereas unstimulated cells have very low levels of phosphorylation of protein kinases. In cancer cells, however, some protein kinases show prolonged phosphorylation by upstream kinases or autophosphorylation and thus may be constitutively activated. This in turn leads to the constitutive activation of signal transduction pathways. Protein kinases, in particular many tyrosine kinases, are a major class of proto-oncogene products.^{13–16} Although tyrosine kinases account for less than 10% of all kinases, many of them are involved in growth signaling and cancer progression.^{17,18} These tyrosine kinases function in many diverse cellular processes such as growth, proliferation, mobility, survival, or tumor angiogenesis. The serine/threonine kinases represent another group of proteins important to cancer cell signaling.¹³ Serine/threonine kinases, such as mammalian target of rapamycin (*mTOR*) and some protein kinase C (*PKC*) isoforms are frequently phosphorylated and become activated in multiple cancers.^{19,20} Tyrosine kinases, *mTOR*, and some *PKC* isoforms are now regarded as excellent novel targets for cancer therapy.^{19,21–23} Given that protein kinases contribute as signaling mediators in tumorigenesis and tumor progression by activating downstream targets, and rhabdomyosarcomas are a substantial problem in pediatric oncology, there is a critical need to identify the phosphorylation profiles of signaling proteins in rhabdomyosarcomas.

We hypothesize that phosphorylation of some tyrosine and serine/threonine protein kinases is increased in rhabdomyosarcoma as compared to normal muscle cells. To address this question, we examined the phosphorylation of receptor and non-receptor tyrosine kinases and serine/threonine protein kinases involved in the *mTOR* and *PKC* pathways of alveolar and embryonal rhabdomyosarcoma using immunohistochemistry staining on multiple rhabdomyosarcoma specimens that were fixed on paraffin-embedded tissue microarray slides. Phospho-specific antibodies that recognize phosphorylated proteins in those pathways were used in this study. This is a novel approach and should allow for the identification of new protein kinases not previously known to be involved in rhabdomyosarcoma oncogenesis. Our results show that the phosphorylation levels of many receptor and non-receptor tyrosine kinases and serine/threonine kinases in the *mTOR* and *PKC* pathways are frequently elevated in multiple rhabdomyosarcomas as compared to matched normal muscle tissues. These activated protein kinases may serve as novel therapeutic targets for treatment of patients with rhabdomyosarcoma.

Materials and methods

Tissue Microarray Slides and Immunohistochemistry

Human rhabdomyosarcoma tissue microarray slides were obtained from the Children's Oncology Group (COG). The embryonal rhabdomyosarcoma tissue microarray block contains 32 unique embryonal rhabdomyosarcoma cases with two types of control tissues: one control tissue is normal skeletal muscle (five cases) and the other is alveolar rhabdomyosarcoma (five cases). Each case averaged three tissue cores (range: 1–9 cores), which were each 1.0 mm in diameter and randomly distributed throughout the block. The alveolar rhabdomyosarcoma tissue microarray also contains 32 unique cases with identical block design as defined above. In addition to five normal skeletal muscle control cases, five embryonal rhabdomyosarcoma cases were included as controls. Rhabdomyosarcoma diagnoses were made via review by COG and STSC (Soft Tissue Sarcoma Committee), designated reviewers, using the International Classification of Rhabdomyosarcomas.²⁴

These slides were baked at 60°C for 1 h, deparaffinized in xylene three times, transferred through two changes of 100% ethanol, and then dehydrated with graded ethanol. Antigen retrieval was carried out by boiling the slides in a beaker filled with 10 mM sodium citrate (pH 6.0) or 1 mM EDTA (pH 8.0). Endogenous peroxidase activity was quenched through 10 min incubation in 3% hydrogen peroxide. After antigen retrieval, the slides were briefly rinsed with 0.1% Tween/1 × TBS (0.1% TBST) two times and then washed three times for 10 min each

at room temperature to remove non-specific background binding. The protein blocking was performed by incubating the specimens in 5% normal goat serum or normal horse serum in 0.1% TBST for 1 h. Primary antibody was applied at 4°C overnight (1:50 dilution of rabbit monoclonal anti-human phospho-specific antibodies from Cell Signaling Technology, Beverly, MA, USA and Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in 0.1% TBST with normal serum. The phospho-specific antibodies included phospho-EGFR (Y1068), phospho-PDGFR-β (Y751), phospho-c-Met (Y1349), phospho-HER2/ErbB2 (Y1221/1222), phospho-VEGFR2 (Y1175), phospho-M-CSFR (Y723), phospho-FGFR (Y653/654), phospho-Bcr (Y177), phospho-c-Abl (Y245), phospho-c-Cbl (Y774), phospho-Src (Y416), phospho-JAK2 (Y1007/1008), phospho-mTOR (S2448), phospho-p70S6K (T389), phospho-S6 (S235/S236), phospho-4EBP1 (S65/T70), phospho-PKCδ (T505), phospho-PKCθ (T538), phospho-PKCα (S657), phospho-PKCζ/λ (T403/T410) (Cell Signaling Technology), phospho-FAK (Y576/Y577), phospho-PYK2 (Y402), and phospho-Shc (Y239/240) (Santa Cruz Biotechnology). After a series of rinses in TBS as described above, the bound antibody was subsequently detected using a VEC-TASTAIN ABC KIT from Vector Laboratories Inc. (Burlingame, CA, USA). The sections were then visualized with a 5–30 min incubation of 3-amino-9-ethylcarbazole high-sensitivity substrate chromogen from DakoCytomation (Carpinteria, CA, USA). The slides were then counterstained with hematoxylin and mounted with CRYSTAL/MOUNT (Biomedica Corp., Foster City, CA, USA) for long-term preservation.

Evaluation of Immunohistochemical Staining

Immunohistochemical staining was scored under microscope by eye. The staining intensity was graded relatively based on the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining.²⁵ Most or all of the tumor tissues showed staining in greater than 50% of the area. For specimens that were uninterpretable or in which most of the cancer tissues were missing, a score of not applicable (N/A) was given. Scoring of the tissue microarray was completed by at least two independent observers (LC and FH or KA and FH). Discrepant scores between the two observers were rescored to arrive at a single final score. For a tumor specimen to be considered positive, it had to have a score of 2 or greater from both observers.

Statistical Analysis

SAS software (version 9.1, SAS Institute Inc., Cary, NC, USA) was used for all statistical analysis. Fisher's exact probability test was used to investigate the correlation of phospho-protein kinase expression

with the following potential predictive variables: gender, age, stage at diagnosis, and tumor primary site. When Fisher's exact test is significant in comparison among multiple groups, we then categorized data into 2 × 2 tables and used Fisher's exact test to make significant conclusions. Spearman's model was used to investigate the association between phosphorylation levels of different protein kinases. In all analyses, a two-sided *P*-value of <0.05 was considered statistically significant.

Results

Increased Phosphorylation of Receptor Tyrosine Kinases in Rhabdomyosarcomas

More than 30 receptor tyrosine kinases have been reported to play a role in oncogenic transformation.¹³ Among them, *EGFR*, *Her2/ErbB2*, and *c-Met* are reported to be constitutively activated, promoting the activation of downstream signaling processes that induce cell growth, cell survival, and invasion of cancer cells.²⁶ These kinases become activated when they are phosphorylated by upstream kinases or through autophosphorylation. To further understand the phosphorylation profile of receptor and non-receptor tyrosine kinases in alveolar and embryonal rhabdomyosarcoma, we examined specimens that were fixed on paraffin-embedded tissue microarray slides (Table 1). The patient ages of these cases are between 0 and 19 with mean 9.1 in alveolar rhabdomyosarcoma and 5.8 in embryonal rhabdomyosarcoma, and most of the cases are in stage III (Table 1). Seven receptor tyrosine kinases, which include *c-Met*, *PDGFR-β*,

Table 1 Patient clinical characteristics

Characteristic	Number of cases	
	ARMS (n = 32)	ERMS (n = 32)
Gender		
Male	13	24
Female	19	8
Age (years)		
0–4	11	18
5–14	14	10
15–19	7	4
Mean	9.1	5.8
Stage		
I	5	7
II	5	4
III	15	14
IV	7	7
Primary site		
Head and neck	9	10
Extremity	13	3
Genitourinary	7	10
Others	3	9

EGFR, *M-CSFR*, *Her2/ErbB2*, *VEGFR-2/Flk-1*, and *FGFR*, were selected. These receptor tyrosine kinases fall into six subfamilies. The phosphorylation levels of these receptor tyrosine kinases are elevated between 26 and 68% in alveolar rhabdomyosarcoma and between 24 and 61% in embryonal rhabdomyosarcoma, respectively (Table 2). In all, 45% or greater of both alveolar and embryonal rhabdomyosarcoma cases demonstrated positive staining for tyrosine phosphorylation of *c-Met*, *PDGFR-β*, and *M-CSFR* suggesting that these pathways may play a role in the oncogenesis of both types of rhabdomyosarcomas. The increased level of tyrosine phosphorylation of *EGFR* is around 50% in alveolar rhabdomyosarcoma indicating the *EGFR* pathway may play a role in cancer progression in this type of rhabdomyosarcoma. Furthermore, the increased levels of tyrosine phosphorylation of *Her2/ErbB2*, *FGFR* and *VEGFR-2/Flk-1* are between 24 and 44%, suggesting that these pathways might also contribute to oncogenesis in rhabdomyosarcoma.

Table 2 Elevated phosphorylation of kinases and their downstream proteins in ARMS and ERMS patients^a

	Positive (%)	
	ARMS (n = 32)	ERMS (n = 32)
<i>Receptor tyrosine kinase</i>		
p-c-Met (Y1349)	68	57
p-PDGFR-β (Y751)	64	57
p-EGFR (Y1068)	50	31
p-M-CSFR (Y723)	45	61
p-HER2/ErbB2 (Y1221/1222)	44	24
p-VEGFR2 (Y1175)	32	52
p-FGFR (Y653/654)	26	38
<i>Non-receptor tyrosine kinase</i>		
p-JAK2 (Y1007/1008)	65	70
p-PYK2 (Y402)	30	48
p-Src (Y416)	26	43
p-c-Abl (Y245)	50	48
p-c-Cbl (Y774)	41	34
p-Bcr (Y177)	57	71
p-FAK (Y576/577)	63	64
p-Shc (Y239/240)	64	71
<i>mTOR pathway</i>		
p-mTOR (S2448)	60	68
p-p70S6K (T389)	65	72
p-S6 (S235/S236)	50	59
p-4EBP1 (S65/T70)	62	71
<i>PKC pathway</i>		
p-PKCα (T657)	68	43
p-PKCδ (T505)	69	64
p-PKCθ (T538)	65	69
p-PKCζ/λ (T403/T410)	57	72

^aThe immunostaining intensity was scored under microscope on the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Scoring of the tissue microarray was completed by at least two independent researchers. Scores of normal tissues are 0 or 1. Scores of 2 and 3 of IHC staining in ARMS and ERMS tissues were considered as positive. % positive: percentage of numbers of positive divided by the total number of specimens.

mas. Representative positive immunohistochemistry staining for *phospho-EGFR* and *phospho-c-Met* in rhabdomyosarcoma patient tissues are shown in Figure 1. The immunohistochemistry staining of corresponding phospho-protein in normal tissues were negative (Figure 1). The relationship between the expression of different phospho-receptor tyrosine kinases and patient gender, age, tumor stage, and primary sites was analyzed. Neither patient gender nor tumor stage was found to be statistically significantly correlated with the expression of phospho-receptor tyrosine kinases in both alveolar and embryonal rhabdomyosarcoma cases. Interestingly, the expression of *phospho-M-CSFR* was found to be significantly correlated with patient age in alveolar rhabdomyosarcoma patients ($P=0.0062$). The alveolar rhabdomyosarcoma cases that showed higher phosphorylation of *M-CSFR* (immunohistochemistry staining score of 2 or 3) are younger compared to those cases with lower *phospho-M-CSFR* levels (immunohistochemistry staining score of 0 or 1, Table 3). A total of 7 out of 10 alveolar rhabdomyosarcoma patients who have higher *p-M-CSFR* expression are in the age group 0–4 (data not shown). The expression of *phospho-FGFR* was found to be significantly correlated with patient age in embryonal rhabdomyosarcoma patients ($P=0.0266$). The embryonal rhabdomyosarcoma cases with higher phosphorylation of *FGFR* are older compared to those cases with lower *phospho-FGFR* levels (Table 3). A total of 8 out of 11 embryonal rhabdomyosarcoma patients who displayed higher *phospho-FGFR* expression are older than age 4 (data not shown). Furthermore, the expression of *phospho-PDGFR* was found to be significantly correlated with tumor primary sites in embryonal rhabdomyosarcoma ($P=0.0183$). The embryonal rhabdomyosarcoma cases that showed higher *phospho-PDGFR* levels arise more frequently from head and neck region as well as extremity, whereas those express lower *phospho-PDGFR* arise more frequently from other sites (Table 3). Statistical analysis also showed that the expression of some phospho-receptor tyrosine kinases is correlated with each other. For example, the expression of *phospho-c-Met* and *phospho-EGFR* is significantly correlated ($P<0.0001$) in both alveolar and embryonal rhabdomyosarcoma patients (Table 4). More correlations between the expressions of phosphorylated receptor tyrosine kinases are shown in Table 4.

Increased Phosphorylation Levels of Non-Receptor Tyrosine Kinases in Rhabdomyosarcomas

We also tested tyrosine phosphorylation levels of eight non-receptor kinases in alveolar and embryonal rhabdomyosarcoma respectively, which include *JAK2*, *PYK2*, *Src*, *c-Abl*, *c-Cbl*, *Bcr*, *FAK*, and *Shc*. Among them, *Src* activation has been shown to be correlated with cancer progression.¹⁵ Our results

Figure 1 Elevated phosphorylation levels of representative receptor tyrosine kinases, *EGFR* and *c-Met*, in rhabdomyosarcoma. Immunohistochemistry staining of alveolar and embryonal rhabdomyosarcoma tissue microarrays that contain both normal and cancer tissues were performed using phospho-EGFR (Tyr1068) antibody and phospho-c-Met (Tyr1349) antibody.

Table 3 Significant correlation between immunohistochemical expression of tyrosine kinases and clinical characteristics in ARMS and ERMS

	Age		P-value
	High ^a	Low ^a	
p-M-CSFR (ARMS)	0–4 years	5–19 years	0.0062
p-FGFR (ERMS)	5–19 years	0–4 years	0.0266
Primary sites			
p-PDGFR (ERMS)	Head and neck extremity	Genitourinary others	0.0183
p-Src (ERMS)	Head and neck extremity	Genitourinary others	0.0189

^aHigh, IHC-staining score of 2 and 3; low, IHC-staining score of 0 and 1.

showed that the phosphorylation levels of these eight non-receptor tyrosine kinases are elevated between 26 and 65% in alveolar rhabdomyosarcoma

and between 34 and 71% in embryonal rhabdomyosarcoma (Table 2). The increased levels of tyrosine phosphorylation of *JAK2*, *c-Abl*, *Bcr*, *FAK*, and *Shc*

Table 4 Correlation between immunohistochemical expression of receptor tyrosine kinases in ARMS and ERMS samples

	p-PDGFR			p-EGFR			p-M-CSFR			p-Her2/ErbB2			p-VEGFR			p-FGFR		
	A/B	%	P	A/B	%	P	A/B	%	P	A/B	(%)	P	A/B	(%)	P	A/B	(%)	P
p-c-Met	ARMS ERMS	13/24 11/28	54.2 39.3	0.0659 0.0001	12/23 8/28	52.2 28.6	8/20 10/23	40.0 43.5	0.1255 0.0116	9/23 5/28	39.1 17.6	0.0807 0.1683	9/20 9/23	45.0 39.1	0.0269 0.0349	6/24 9/28	25.0 32.1	0.0208 0.0008
p-PDGFR	ARMS ERMS				10/23 7/28	43.5 25.0	8/20 11/23	40.0 47.8	0.0046 0.0017	9/23 5/28	39.1 17.6	0.0010 0.1216	8/20 8/23	40.0 34.8	0.0112 0.1778	7/24 9/28	29.2 32.1	0.0021 0.0004
p-EGFR	ARMS ERMS						6/20 5/23	30.0 21.7	0.1276 0.1150	8/23 4/28	34.8 14.3	0.0280 0.0441	8/21 11/23	38.1 47.8	0.0016 0.0002	5/24 8/23	20.8 34.8	0.0195 0.0001
p-M-CSFR	ARMS ERMS												6/21 5/23	28.6 21.7	0.0411 0.0777	4/21 5/26	19.0 34.8	0.0029 0.0473
p-Her2/ErbB2	ARMS ERMS												6/21 6/23	28.6 26.1	0.1309 0.0128	5/26 3/22	19.2 13.6	0.1001 0.2168
p-VEGFR	ARMS ERMS												7/23	30.4		7/23	30.4	0.0895

A, the number of cases that have positive IHC staining in both proteins; B, total available case number; %, the percentage of A in B.

are over or around 50% in both alveolar and embryonal rhabdomyosarcoma suggesting that these five pathways may play important roles in the oncogenesis of both types of rhabdomyosarcomas. Furthermore, the increased levels of tyrosine phosphorylation of *PYK2*, *Src*, and *c-Cbl* are between 26 and 48% indicating these pathways might also be involved in the oncogenesis of both alveolar and embryonal rhabdomyosarcoma. Representative rhabdomyosarcoma patient specimens staining positive for *phospho-c-Cbl* and *phospho-Src* are shown in Figure 2. No significant correlation was found between the expression of phospho-non-receptor tyrosine kinases and patient gender, age or tumor stage in both alveolar and embryonal rhabdomyosarcoma. In embryonal rhabdomyosarcoma, the *phospho-Src* level is found to be significantly correlated with tumor primary sites ($P=0.0189$). The embryonal rhabdomyosarcoma cases that showed higher phosphorylation of *Src* arise more frequently from head and neck region and extremity (Table 3). Statistical analysis also showed that the expression of some phospho-non-receptor tyrosine kinase is correlated with each other. As an example, the expression of *phospho-Src* and *phospho-c-Abl* is statistically correlated in alveolar rhabdomyosarcoma ($P<0.0001$) and embryonal rhabdomyosarcoma ($P=0.0194$) patients (Table 5). Correlations between the expressions of phosphorylated non-receptor tyrosine kinases are detailed in Table 5.

The Phosphorylation Levels of Protein Kinases in mTOR Pathways are Elevated in Rhabdomyosarcomas

The serine/threonine protein kinases, *mTOR* and *p70S6K*, are among the most important downstream effectors in oncogenic receptor tyrosine kinase signaling.²⁷ We examined the phosphorylation levels of *mTOR* and *p70S6K* as well as their downstream-targeted proteins, *S6* and *4EBP1* that function in protein translation. Our results showed that phosphorylation levels of *mTOR* are elevated 60% in alveolar rhabdomyosarcoma and 68% in embryonal rhabdomyosarcoma. Phosphorylation of *p70S6K* and *S6* are also increased between 50 and 72% in both types of rhabdomyosarcoma (Table 2). Positive immunohistochemistry staining for *phospho-p70S6K* in rhabdomyosarcoma patient tissues compared to normal tissues are shown in Figure 3. No evidence of significant correlation was found between any clinicopathological features and the expression of phosphorylated *mTOR* or *p70S6K* in both alveolar and embryonal rhabdomyosarcoma patients. Statistical analysis showed that the expression of *phospho-mTOR* and *phospho-p70S6K* is significantly correlated in alveolar rhabdomyosarcoma ($P<0.0001$) and embryonal rhabdomyosarcoma ($P=0.0179$) patients (data not shown).

Figure 2 Elevated phosphorylation levels of representative non-receptor tyrosine kinases, *c-Cbl* and *Src*, in rhabdomyosarcoma. Immunohistochemistry staining of alveolar and embryonal rhabdomyosarcoma tissue microarrays that contain both normal and cancer tissues were performed using phospho-*c-Cbl* (Tyr774) and phospho-*Src* (Tyr416) antibodies.

Elevated Phosphorylation Levels of Protein Kinase C Isozymes in Rhabdomyosarcomas

The *PKC* enzyme family contains 12 different isozymes, many of which contribute to the regulation of diverse cellular response including proliferation, differentiation, and apoptosis.²⁸ The phosphorylation state of an isozyme is a crucial factor in determining activity.^{29,30} We examined four *PKC* isozymes which are serine/threonine kinases. Phosphorylation levels of *PKCα*, *PKCδ*, *PKCθ*, and *PKCζ/λ* are significantly upregulated between 57 and 69% in alveolar rhabdomyosarcoma, between 43 and 72% in embryonal rhabdomyosarcoma (Table 2). Positive immunohistochemistry staining for *phospho-PKCδ* of rhabdomyosarcoma patient tissues are shown in Figure 3. No significant correlation exists between any clinicopathological features

with the expression of *phospho-PKC* isozymes in both alveolar and embryonal rhabdomyosarcoma patients. The correlation between the expression of phosphorylated *PKCα* and alveolar rhabdomyosarcoma patient age is close to significance ($P=0.0757$). In alveolar rhabdomyosarcoma patients, the expression of *phospho-PKCθ* and *phospho-PKCδ* is found to be significantly correlated ($P=0.0078$, data not shown). In embryonal rhabdomyosarcoma patients, the expression of *phospho-PKCα* and *phospho-PKCδ* is significantly correlated ($P=0.0006$, data not shown).

Discussion

In this study, we identified for the first time, the phosphorylation levels of multiple tyrosine kinases

Table 5 Correlation between immunohistochemical expression of non-receptor tyrosine kinases in ARMS and ERMS samples

	p-PYK2			p-Src			p-c-Abl			p-c-Cbl			p-Bcr			p-FAK			p-Shc		
	A/B	%	P	A/B	%	P	A/B	%	P	A/B	%	P	A/B	%	P	A/B	%	P	A/B	%	P
p-JAK2	7/23	30.4	0.0020	4/21	19.0	0.3391	9/22	40.9	0.6406	6/22	27.3	0.1811	10/22	45.5	0.0322	10/20	50.0	0.1242	14/21	66.7	<0.0001
	ERMS	9/23	39.1	0.0245	7/23	30.4	0.6617	8/23	34.8	0.9956	7/23	30.4	0.6821	12/23	52.2	0.2071	14/23	60.9	0.0822	14/23	60.9
p-PYK2	ARMS	2/21	9.5	0.7871	5/22	22.7	0.2412	3/22	13.6	0.8710	6/22	27.3	0.0310	7/20	35.0	0.0143	7/21	33.3	0.0257	7/21	33.3
	ERMS	8/23	34.8	0.0078	8/23	34.8	0.0401	7/23	30.4	0.0116	11/23	47.8	0.0006	11/23	47.8	0.0030	9/23	39.1	0.0429	9/23	39.1
p-Src	ARMS				6/21	28.6	<0.0001	6/25	24.0	0.0023	2/21	9.5	0.8255	6/23	26.1	0.0523	5/24	20.8	0.5134	5/24	20.8
	ERMS				7/23	30.4	0.0194	7/28	25.0	0.0008	9/23	39.1	0.0108	10/28	35.7	0.1082	10/28	35.7	0.0940	10/28	35.7
p-c-Abl	ARMS																				
	ERMS																				
p-c-Cbl	ARMS																				
	ERMS																				
p-Bcr	ARMS																				
	ERMS																				
p-FAK	ARMS																				
	ERMS																				
	ERMS																				
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A, the number of cases that have positive IHC staining in both proteins; B, total available case number; %, the percentage of A in B.

as well as serine/threonine kinases in the mTOR and PKC pathways that are elevated in alveolar and embryonal rhabdomyosarcoma tissues. We showed that increased phosphorylation levels of seven selected receptor tyrosine kinases are between 24 and 68% in alveolar and embryonal rhabdomyosarcoma. Our results also showed that the phosphorylation levels of these eight non-receptor tyrosine kinases are elevated between 26 and 71% in alveolar and embryonal rhabdomyosarcoma. Further, we showed that increased phosphorylation levels of serine/threonine kinases in the mTOR and PKC pathways are between 43 and 72% in alveolar and embryonal rhabdomyosarcoma. We have also been able to obtain phosphorylation profiles of these selected tyrosine kinases and serine/threonine kinases in the mTOR and PKC pathways from rhabdomyosarcoma patient tissues. These protein kinases have been shown to contribute to cell proliferation and cell survival^{31–42} and most of them are likely to play crucial roles in tumor development and progression of rhabdomyosarcomas. Therefore, selective small molecular inhibitors that block these activated protein kinases may be useful therapeutic reagents.

Statistical analysis of these data showed that the elevated levels of phosphorylation of multiple protein kinases are correlated in rhabdomyosarcoma patient samples. The elevated phosphorylation of *EGFR* is significantly correlated with *c-Met*, *PDGFR*, *Her2/ErbB2*, *FGFR*, and *VEGFR*, respectively (*P*-values <0.05) but not with *M-CSFR*, whereas the elevated expression of *phospho-c-Met* is correlated with *phospho-EGFR*, *phospho-FGFR*, and *phospho-VEGFR* but not with *phospho-M-CSFR*, *phospho-PDGFR*, and *phospho-Her2/ErbB2* in alveolar rhabdomyosarcoma (Table 4). The elevated phosphorylation of *VEGFR* is correlated with *c-Met*, *EGFR*, *M-CSFR*, and *PDGFR*, respectively (*P*-values <0.05) and the elevated phosphorylation of *EGFR* is correlated with *c-Met*, *PDGFR*, and *M-CSFR* in alveolar rhabdomyosarcoma (Table 4). These results suggest that in these 32 alveolar rhabdomyosarcoma, there are at least two or more receptor tyrosine kinases whose phosphorylation is elevated in the same patient. In embryonal rhabdomyosarcoma, for example, the expression of *phospho-EGFR* is significantly correlated with *phospho-c-Met*, *phospho-PDGFR*, *phospho-Her2/ErbB2*, and *phospho-FGFR*, but not with *phospho-VEGFR* or *phospho-M-CSFR*. The elevated expression of *phospho-c-Met* is correlated with *phospho-EGFR*, *phospho-FGFR*, *phospho-M-CSFR*, *phospho-PDGFR*, and *phospho-VEGFR* but not with *phospho-Her2/ErbB2* (Table 4). These results also suggest that in these 32 embryonal rhabdomyosarcoma, there are at least two or more receptor tyrosine kinases whose phosphorylation is elevated in the same patient. Elevated expression of two or more receptor tyrosine kinases is presented in only alveolar or embryonal rhabdomyosarcoma but not in both subtypes. For example, in Table 3,

Figure 3 Elevated phosphorylation levels of representative serine/threonine kinases, *p70S6K* and *PKCδ*, in rhabdomyosarcoma. Immunohistochemistry staining of alveolar and embryonal rhabdomyosarcoma tissue microarrays that contain both normal and cancer tissues were performed using phospho-*p70S6K* (Thr389) and phospho-*PKCδ* (Thr505) antibodies.

the elevated expression of *phospho-PDGFR* is significantly correlated with *phospho-c-Met* only in embryonal rhabdomyosarcoma ($P=0.0001$) but not in alveolar rhabdomyosarcoma ($P=0.0659$) and the elevated expression of *phospho-VEGFR* is significantly correlated with *phospho-EGFR* only in alveolar rhabdomyosarcoma ($P=0.0016$) but not in embryonal rhabdomyosarcoma ($P=0.3441$). These results suggest that co-activation of these two receptor tyrosine kinases through their downstream signaling pathways may be important for the cooperation in tumor development or progression in either alveolar rhabdomyosarcoma or embryonal rhabdomyosarcoma but may not be important in another subtype. In addition, the elevated expression of several receptor tyrosine kinases is not associated with each other at all in both alveolar rhabdomyosarcoma and embryonal rhabdomyosar-

coma. The elevated expression of *phospho-M-CSFR* and *phospho-EGFR* ($P=0.1276$ in alveolar rhabdomyosarcoma and $P=0.1150$ in embryonal rhabdomyosarcoma) and *phospho-FGFR* and *phospho-VEGFR* ($P=0.2168$ in alveolar rhabdomyosarcoma and $P=0.0895$ in embryonal rhabdomyosarcoma) are not associated with each other in both alveolar and embryonal rhabdomyosarcoma (Table 4). These results suggest that co-activation of these two receptor tyrosine kinases and their downstream signaling pathways may not be relevant for the cooperation in tumor development or progression in either alveolar rhabdomyosarcoma or embryonal rhabdomyosarcoma.

Among the non-receptor tyrosine kinases, some of their increased phosphorylation is also correlated in both subtypes of rhabdomyosarcoma. For example, the expression of *PYK2* is significantly correlated

with *JAK2*, *Bcr*, *FAK*, and *Shc* in both alveolar and embryonal rhabdomyosarcoma (P -values <0.05 , Table 5). Furthermore, oncogene products that are activated in cancer such as *Src*, *c-Abl*, *Bcr*, and *c-Cbl* are associated with each other in some cases of alveolar and embryonal rhabdomyosarcoma. For example, significant correlations were observed between the expression of *phospho-Src* and *phospho-c-Abl*, *phospho-Src* and *phospho-c-Cbl*, *phospho-c-Abl* and *phospho-c-Cbl*, in both alveolar and embryonal rhabdomyosarcoma (Table 5). These findings suggest that the concurrent elevated phosphorylation and activation of at least two of these non-receptor tyrosine kinases that may function as oncogene products may contribute to the signaling pathway dysregulation and tumor formation or progression in rhabdomyosarcoma. The elevated phosphorylation of certain non-receptor tyrosine kinases are only associated with each other in one subtype of rhabdomyosarcoma. For instance, the significant association of increased levels of phosphorylation of *Bcr* and *c-Abl* only exists in embryonal rhabdomyosarcoma ($P=0.0014$) but not in alveolar rhabdomyosarcoma ($P=0.3582$, Table 5). In addition, the significant association of increased levels of phosphorylation of *Bcr* and *Src* is only observed in embryonal rhabdomyosarcoma ($P=0.0108$) but not in alveolar rhabdomyosarcoma ($P=0.8255$, Table 5), suggesting that the co-activation of *Bcr* and *c-Abl*, or *Bcr* and *Src* might play an important role in tumorigenesis or development of embryonal rhabdomyosarcoma. Further, the elevated phosphorylation of several receptor tyrosine kinases is not associated with each other at all in both alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma. For example in Table 5, the elevated phosphorylation of *JAK2* and *Src* or *c-Abl* and *FAK* is not significantly associated with each other in both alveolar and embryonal rhabdomyosarcoma (Table 5). This suggests that co-activation of these non-receptor tyrosine kinases and their downstream signaling pathways might not promote oncogenesis in alveolar and embryonal rhabdomyosarcoma. Instead, co-activation of *Src*, *c-Abl*, *FAK*, or *JAK2* with others, such as *Src* with *c-Abl*, *JAK2* with *Shc*, or *FAK* with *Bcr* (Table 5) might cooperate more efficiently in promoting tumor development or progression in both alveolar and embryonal rhabdomyosarcoma.

Findings from this pilot study may have potential clinical utility and may be used to optimize the molecular therapeutic targets for individualized cancer interventions. To the best of our knowledge, this is the first report to profile the phosphorylation levels of a variety of receptor tyrosine, non-receptor tyrosine, and serine/threonine kinases in the mTOR and PKC pathways in rhabdomyosarcomas. This report may provide a basis for future studies using small molecule compounds and other intervention methods that target these activated protein kinases in rhabdomyosarcomas.

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