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High ALK mRNA expression has a negative prognostic significance in rhabdomyosarcoma

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Background: Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase aberrantly expressed in cancer, but its clinical and functional importance remain controversial. Mutation or amplification of ALK, as well as its expression levels assessed by conventional immunohistochemistry methods, has been linked to prognosis in cancer, although with potential bias because of the semi-quantitative approaches. Herein, we measured ALK mRNA expression in rhabdomyosarcoma (RMS) and determined its clinical impact on patients' stratification and outcome.

Methods: Specimens were obtained from RMS patients and cell lines, and ALK expression was analysed by quantitative RT–PCR, western blotting, IHC, and copy number analysis.

Results: High ALK mRNA expression was detected in the vast majority of PAX3/7-FOXO1-positive tumours, whereas PAX3/7-FOXO1-negative RMS displayed considerably lower amounts of both mRNA and protein. Notably, ALK mRNA distinguished unfavourable PAX3/7-FOXO1-positive tumours from PAX3/7-FOXO1-negative RMS ($P<0.0001$), and also correlated with larger tumour size ($P<0.05$) and advanced clinical stage ($P<0.01$), independently of fusion gene status. High ALK mRNA levels were of prognostic relevance by Cox univariate regression analysis and correlated with increased risk of relapse ($P=0.001$) and survival ($P=0.01$), whereas by multivariate analysis elevated ALK mRNA expression resulted a negative prognostic marker when clinical stage was not included.

Conclusion: Quantitative assessment of *ALK* mRNA expression helps to improve risk stratification of RMS patients and identifies tumours with adverse biological characteristics and aggressive behaviour.

Rhabdomyosarcoma (RMS) is the commonest soft tissue malignancy among children and adolescents (Parham and Ellison, 2006), and can be classified into alveolar, embryonal, and the less frequent pleomorphic subtypes (Helman and Meltzer, 2003). More than two-thirds of all RMS are of embryonal histology (ERMS) and tend to occur at younger age, whereas alveolar rhabdomyosarcoma (ARMS) comprises the remaining 20–25% of cases and is a more aggressive malignancy (Barr *et al*, 2002). ARMS is characterised by specific chromosomal translocations that give rise to *PAX3/7-FOXO1* fusion genes, whereas ERMS frequently shows loss of heterozygosity at 11p15 and a significantly better prognosis (Barr *et al*, 2002). Detection of genetic abnormalities is important in the diagnosis of RMS, whereas the identification of potential biomarkers and factors that contribute to tumour growth and progression may lead to more effective risk-adapted therapies and improved outcome (Parham *et al*, 2007; Davicioni *et al*, 2009). Receptor tyrosine kinases (RTKs) are among the most interesting therapeutic targets in cancer, because of their involvement in signalling pathways that drive the malignant phenotype and influence proliferation and survival of tumour cells (Manning *et al*, 2002; Stommel *et al*, 2007). In RMS, this has been shown for EGFR (erbB1), Her2 (erbB2), IGF1R, c-Met, and PDGFR-α, whose

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expression varies according to histology, but the consequences of their aberrant activity are similar (Ganti *et al*, 2006; Rees *et al*, 2006; Taniguchi *et al*, 2008; Kim *et al*, 2009).

Anaplastic lymphoma kinase (ALK) is a RTK belonging to the insulin receptor superfamily that has been reported in RMS, but its role in the pathogenesis of this malignancy is still not known (Pillay *et al*, 2002). ALK can be detected as a fusion protein kinase or full-length receptor protein in several tumour types (Kelleher and McDermott, 2010), and in both cases it drives tumour biology through phosphorylation and activation of several downstream effector proteins. Inhibition or silencing of ALK leads to cell cycle arrest and apoptosis, supporting the concept that ALK is important for the development and progression of cancer (Barreca *et al*, 2011). Herein, we analysed ALK mRNA expression, copy number variation, and mutational status in RMS, with clinical and molecular correlation in a cohort of 87 patients, and assessed its activity *in vitro*. We demonstrated that *ALK* is upregulated in PAX3/7-FOXO1-positive ARMS, both at mRNA and protein levels, and is capable of signalling when properly stimulated. In these settings, ALK mRNA levels discriminated RMS patients with different outcomes, whereas ALK receptor activity regulated phosphorylation and activation of downstream target proteins. The results of this study may have a direct impact for the ongoing RMS risk stratification strategies, and major implications for future clinical studies and treatment protocols.

MATERIALS AND METHODS

Cell lines, primary tumours and normal controls. The cell lines examined in this study were: four PAX-FOXO1-positive (RH30, RH4, RH28, RC2) and one fusion-negative (RH18) ARMS, four ERMS (RD, SMS-CTR, CCA, RH36), three neuroblastoma (NB: LAN5, IMR32 and NB1), and one ALCL (KARPAS-299) cell lines. Cell culture conditions were in accord with the ATCC (Manassas, VA, USA) standard protocols. A total of 87 tissue samples from RMS patients enrolled in the pediatric sarcoma protocols RMS96, RMS4-99 and RMS2005 of the Italian Association of Pediatric Haematology and Oncology (AIEOP) was included after obtaining institutional review board approval. Diagnosis was reviewed by the AIEOP central panel of pathologists in all of the cases and confirmed by RT–PCR using primers for PAX3/7-FOXO1 (ARMS) and MyoD1 (ERMS). Fetal human skeletal muscle total RNA (Stratagene, La Jolla, CA, USA) was used as normal negative control.

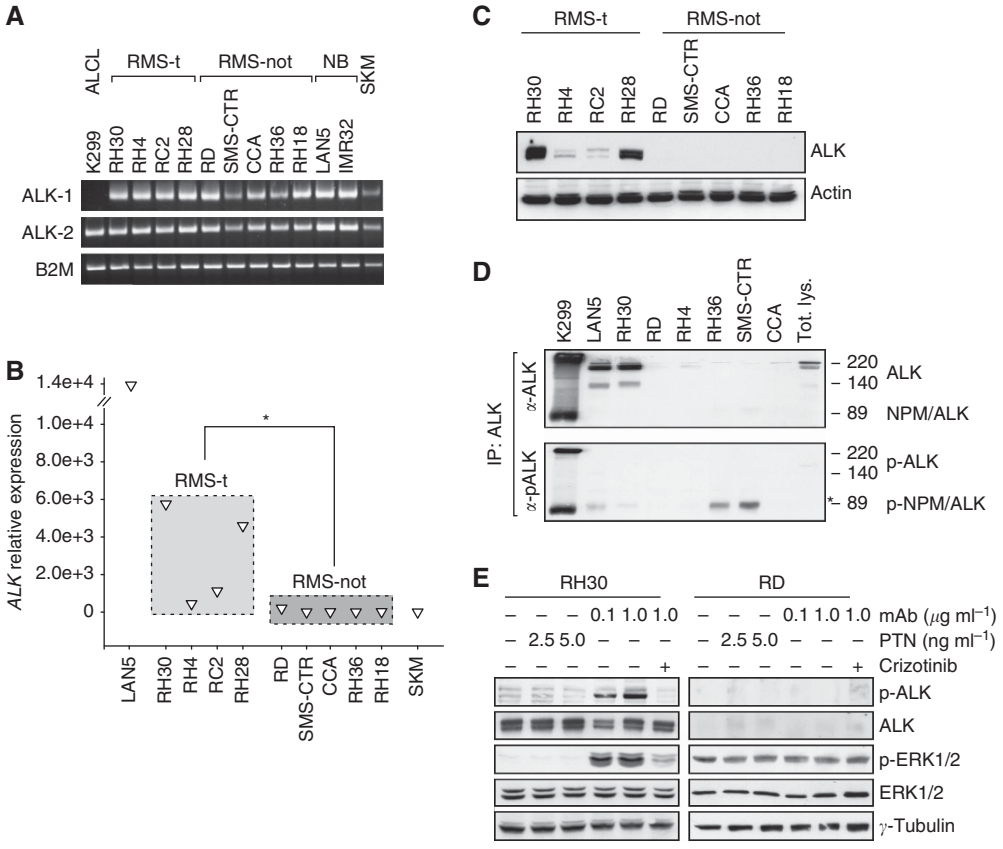


Figure 1. Expression analysis of ALK in RMS cell lines. (**A**) Expression of *ALK* mRNA in tumour cell lines, using primers spanning the extracellular region (ALK-1) and the intracytoplasmic portion (ALK-2) of *ALK* gene. *β2*-Microglobulin (B2M) was used as housekeeping gene. RMS-t, PAX3/7-FOXO1-positive RMS; RMS-not, PAX3/7-FOXO1-negative RMS; NB, neuroblastoma; SKM, fetal skeletal muscle; ALCL, anaplastic large cell lymphoma. (**B**) Relative *ALK* mRNA expression levels in RMS-t and RMS-not cell lines measured by qRT–PCR. LAN5 and SKM were included as positive and negative controls, respectively. Distribution of data is represented by box plot analysis (*dashed box*). *****$P<0.05$. (**C**) ALK protein expression in RMS-t and -not cell lines by western blotting, using *β*-actin as protein-loading control. (**D**) Total (*α-ALK*, upper panel) and phosphorylated (*α-pALK*, lower panel) ALK proteins detected in RMS-t and RMS-not cell lines after immunoprecipitation (*IP:ALK*). LAN5 and KARSPAS-299 (K299) cells were used as positive controls for ALK and NPM-ALK expression, respectively. LAN5 total lysates were used as input (*Tot.lys.*). Asterisk marks non-specific bands in RMS and NB cells. (**E**) ALK tyrosine phosphorylation in RH30 and RD cell lines exposed to pleiotrophin (*PTN*) or ALK agonist antibody (*mAb*) in the presence (+) or absence (−) of ALK inhibitor crizotinib. Total and phosphorylated ALK and ERK1/2 proteins were detected by western blotting. *γ*-Tubulin was included as loading control.

Cell lysis and immunoblotting. Cells in log-phase growth were collected and lysed as previously described (Bonvini *et al*, 2002), and western blot analysis was performed using standard methods. Antibodies used were: ALK (Invitrogen Corp., Carlsbad, CA, USA); phospho-ALK, ERK1/2 and phospho-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA); γ-tubulin (Sigma-Aldrich, St Louis, MO, USA); β-actin (Santa Cruz Biotechnology, Heidelberg, Germany). ALK agonist antibody mAb 16–39 was a generous gift from Professor Tadashi Yamamoto (University of Tokyo, Japan). ALK inhibitor crizotinib was from Selleckchem (Houston, TX, USA). ALK immunoprecipitation was performed using Invitrogen ALK primary antibodies and Protein-G sepharose beads (GE Healthcare Bio-Sciences AB, Stockholm, Sweden) (Bonvini *et al*, 2002).

DNA, RNA and protein preparation from cell lines and tumour specimens. High-molecular-weight DNA and total RNA were extracted from cell lines using the QIAamp DNA Mini Kit (Qiagen, Milan, Italy) and Trizol reagent (Invitrogen, Milan, Italy), respectively, whereas DNA, RNA and proteins were obtained from human samples by using Trizol reagent (Invitrogen) according to manufacturer's instructions.

RT–PCR and qRT–PCR analysis. PCR amplification of the extracellular (exons 1–3) and intracellular portion (exons 20–22) of *ALK* was performed as previously described (McDermott *et al*, 2008), whereas quantitative *ALK* mRNA expression was determined in cell lines and tumour samples by qRT–PCR using *GAPDH* (5′-TCCTCTGACTTCAACAGCGA-3′ and 5′-GGGTCT TACTCCTTGGAGGC-3′) as normalising reference gene. PCR reactions were performed in triplicate on ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA. USA) with SYBR green technology, and the relative expression of *ALK* was calculated by using the $2^{-\Delta\Delta CT}$ method.

ALK genomic copy number analysis. Primers and probe were designed to measure the number of genomic copies of *ALK* in cell lines and in human samples in accordance with Applied Biosystems' TaqMan requirements. *ALK* primers and probe were 5′-CCCGAGACATCTACAGGTGAGTAA-3′, 5′-GGGCTGAGG TGGAAGAGACA-3′ and 5′-ACTGCCTCACCCCTCCGGGC 3′-FAM labelled, respectively, whereas *RNA Pol IID* was the endogenous control gene selected to correct for aneuploidy (Williamson *et al*, 2005).

Immunohistochemistry. ALK expression analysis was performed on formalin-fixed paraffin-embedded RMS specimens, stained with ALK1 antibody (DAKO, Milan, Italy; 1 : 50), after deparaffinisation and antigen retrieval. Detection was performed using a biotinylated secondary antibody and a DAB chromogen in haematoxylin counterstained cells (Vectastain ABC Kit Elite, PK-6100, Vector Labs, Burlingame, CA, USA). Each case was considered positive when more than 50% of the cells had moderate-to-strong cytoplasmic staining.

Statistical analysis. Patients included in the study had a median follow-up of 2.3 years from diagnosis (range 0.02–11.4 years). A receiver-operator characteristic curve (ROC) was calculated to define the cutoff values of *ALK* mRNA predicting events or fusion gene status with the highest sensitivity and specificity. The areas under the ROC curves (AUCs) were analysed by the Hanley and McNeil method. Survival analysis was performed according to Kaplan–Meier method and differences were calculated by applying log-rank test. Overall survival (OS) was calculated from the date of diagnosis to the date of death for any cause or the last follow-up, whereas progression-free survival (PFS) was calculated from the date of diagnosis to the date of the first event (tumour progression or relapse) or the last follow-up. The association of *ALK* mRNA expression levels with specific clinical and molecular characteristics was analysed either by Student's *t*-test or Mann–Whitney *U*-test, using SAS statistical programme (SAS-PC, 9.2; SAS Institute Inc., Cary, NC, USA), whereas Cox proportional hazard regression model was applied to evaluate the prognostic potential of *ALK* mRNA expression regarding PFS and OS of RMS patients. The analysis was performed at both univariate and multivariate levels, with a level of significance defined at a probability value less than 0.05 ($P<0.05$) and 0.1 ($P<0.1$), respectively.

RESULTS

Expression and genomic status of ALK *in vitro*. ALK expression was measured in both ARMS and ERMS cell lines, and compared with that assessed in NB, ALCL, or fetal skeletal muscle cells. PCR detection of both extracellular and cytoplasmic portions of ALK

Table 1. Main clinical characteristics of RMS patients

Variable	ARMS-t ($n=35$)	ARMS-not ($n=13$)	ERMS ($n=39$)
Age, years			
⩽10	17	11	36
>10	18	2	3
Sex			
Male	11	7	22
Female	24	6	17
Site of disease			
Parameningeal	2	3	10
Orbit	0	1	4
Other head and neck	2	2	4
Extremity	18	3	3
Genitourinary	1	0	4
Bladder-prostate	1	0	8
Other	10	4	6
Unknown	1	0	0
Size, cm			
⩽5	8	3	9
>5	21	9	29
Not evaluable	1	0	0
Unknown	5	1	1
IRS group			
I	0	0	2
II	2	1	2
III	10	9	30
IV	23	3	4
Unknown	0	0	1
Evaluable ALK			
qRT–PCR	35	13	39
GCN	16	6	20
Survival analysis			
	31	10	30

Abbreviations: ARMS-t = PAX3/7-FOXO1-positive alveolar rhabdomyosarcoma; ARMS-not = PAX3/7-FOXO1-negative alveolar rhabdomyosarcoma; ERMS = embrional rhabdomyosarcoma; GCN = gene copy number; IRS = intergroup rhabdomyosarcoma study; qRT–PCR = quantitative real-time PCR; RMS = rhabdomyosarcoma. Patients and tumour characteristics of the 87 rhabdomyosarcomas used in this study are reported. Patients eligible for both *ALK* gene expression analysis (qRT-PCR and GCN) and survival analysis are also indicated.

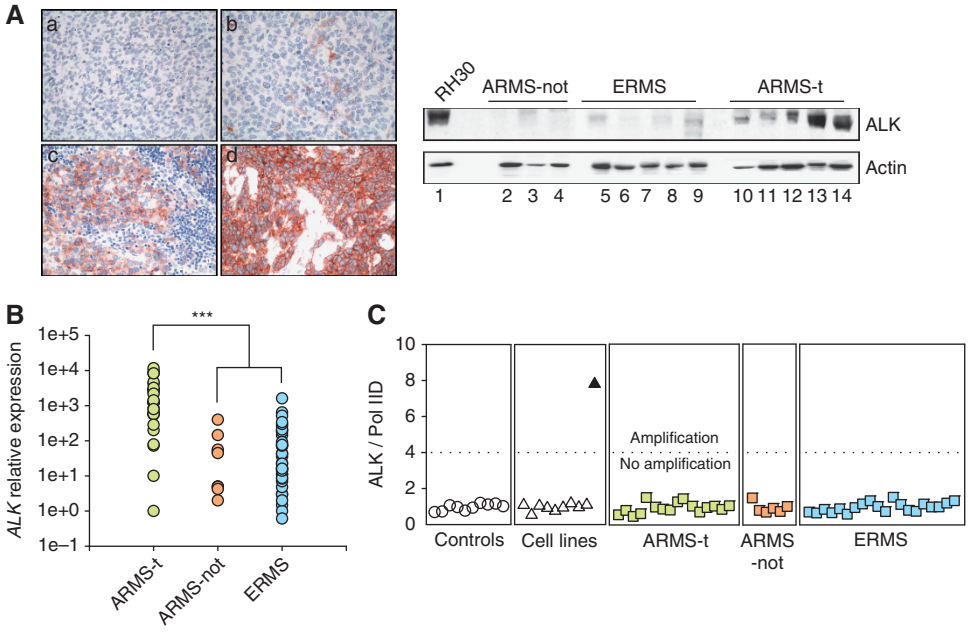


Figure 2. ALK mRNA and protein expression in RMS tumour specimens. (**A**) ALK protein expression analysis in PAX3/7-FOXO1-negative (ARMS-not, a; ERMS, b) and -positive (ARMS-t, c and d) RMS tumours, by immunohistochemistry (left) and western blotting (right). (**B**) Point plot analysis of ALK mRNA relative expression in PAX3/7-FOXO1-positive (*ARMS-t*) and -negative (*ARMS-not*) ARMS, and in ERMS tumours by qRT–PCR. *P*-values were calculated using the Student's *t*-test. ***$P<0.001$. (**C**) *ALK* gene amplification analysis in RMS tumours and cell lines, normalised for RNA Polymerase IID expression (*Pol IID*). Graph shows true *ALK* amplification in normal cells (open circles), tumour cell lines (RMS, open triangles; NB1, closed triangle), and RMS specimens (closed squares). Dotted line represents cutoff for definition of gene amplification (gene copy number $\geqslant 4$).

was carried out using different set of primers, in order to discriminate the expression of full-length ALK and truncated variants arising from chromosomal rearrangements, whereas western blot analysis was performed to determine endogenous protein expression and activity. We found that all RMS and NB cell lines expressed both the extracellular and cytoplasmic portions of ALK, whereas ALCL cells KARPAS-299 exhibited only the cytoplasmic portion of ALK as a result of variant NPM-ALK expression (Figure 1A). *ALK* mRNA was quantified by qRT–PCR, and its relative expression compared in PAX3/7-FOXO1-positive (RMS-t) and -negative (RMS-not) cell lines. *ALK* mRNA levels were significantly higher in ARMS cell lines expressing PAX3/7-FOXO1 (RMS-t) compared with ERMS (Figure 1B, $P=0.032$), and this correlated with higher ALK protein expression (Figure 1C, RMS-t *vs* RMS-not). However, when ALK was immunoprecipitated and probed with phosphospecific antibodies, no basal receptor phosphorylation, like that of NPM-ALK in ALCL cells, was observed (Figure 1D, lower panel), suggesting that ALK is not constitutively activated in these cells. To rule out any defect of RTK activity, we also conducted a mutagenesis screen in search of mutations in the *ALK* gene, and we analysed inducible ALK phosphorylation upon stimulation with putative ligand pleiotrophin (PTN) or ALK agonist monoclonal antibody mAb16-39. No point mutations were detected in the tyrosine kinase domain of *ALK* (exons 22–25, data not shown), and PTN failed to activate ALK when added to the cells (Figure 1E). In contrast, exposing PAX3/7-FOXO1-postive RH30 cells to mAb16-39 resulted in a dose-dependent increase of ALK phosphorylation, which was accompanied by the phosphorylation of downstream ERK1/2 kinase and completely prevented by the dual ALK-MET inhibitor crizotinib (Figure 1E). In contrast, neither mAb16-39 nor crizotinib changed basal ERK1/2 phosphorylation in ALK-negative RD ERMS cells.

ALK mRNA expression in RMS tumours. The expression of ALK was then measured in RMS human specimens (Table 1).

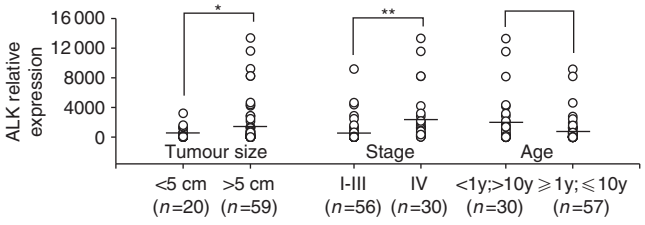
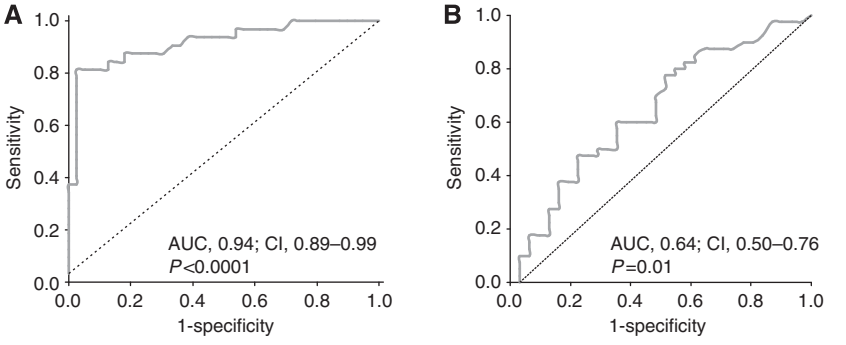


Figure 3. Correlation between *ALK* mRNA expression levels and clinicopathological parameters in RMS tumours. *ALK* mRNA levels, assessed by qRT–PCR and normalised to *GAPDH* housekeeping gene, were compared among groups classified by tumour size, staging, or age. *$P<0.05$; **$P<0.01$.

Consistent with our previous findings, ALK protein was barely detectable in PAX3/7-FOXO1-negative RMS, whereas it showed moderate-to-highly expression in PAX3/7-FOXO1-positive ARMS (Figure 2A). Quantitative ALK mRNA expression analysis confirmed that PAX3/7-FOXO1-positive ARMS tumours (ARMS-t) displayed higher ALK mRNA levels than fusion gene-negative RMS, with median expression values of 1405 (min. = 10/max. = 13 370 units) *vs* 23 (min. = 1/max. = 1614 units), respectively. This difference was statistically significant (Figure 2B, $P<0.001$), whereas it was not when *ALK* mRNA median expression levels of ARMS-not and ERMS were compared ($P=0.79$). Because DNA amplification can lead to increased mRNA expression, we also examined *ALK* DNA copy number for a set of RMS specimens (ARMS-t = 16; ARMS-not = 6; ERMS = 20) and cell lines ($n=9$), and compared with that measured in healthy individuals ($n=10$) or in cancer cells known for true *ALK* amplification (NB1). The results reported in Figure 2C demonstrated no obvious *ALK* gene amplification in RMS samples, when corrected for aneuploidy with the *RNA Pol IID* gene (Figure 2C, RMS median ALK/Pol IID = 1; NB1 ALK/Pol IID = 8), suggesting an altered regulation of *ALK* at the gene



PAX-FOXO1

	RMS-t	RMS-not	Total
ΔΔCT < 555	5	39	44
ΔΔCT ⩾ 555	26	1	27
Total	31	40	71

Event

	No	Yes	Total
ΔΔCT < 200	20	16	36
ΔΔCT ⩾ 200	7	28	35
Total	27	44	71

Figure 4. Receiver operating characteristic curve (ROC) analysis. ROCs showing the sensitivity and specificity of *ALK* mRNA as a parameter to classify RMS patients ($n=71$) on the basis of the fusion gene status (*PAX-FOXO1*, RMS-t *vs* RMS–not; $\Delta\Delta CT=555$) (**A**) or the risk of failure (*EVENT*, NO *vs* YES; $\Delta\Delta CT=200$) (**B**). *P*-values, AUC, and 95% CIs were computed by using SAS statistical programme. Abbreviations: AUC = area under the curve; CI, confidence interval.

promoter rather than at its genomic level. In addition, consistent with previous studies that demonstrated that ALK point mutations are uncommon in RMS cells (van Gaal *et al*, 2011; Yoshida *et al*, 2013), we found no significant variations in the ALK tyrosine kinase domain when bidirectional sequencing was performed (data not shown).

Prognostic implications of *ALK* mRNA expression. To investigate a possible correlation between high *ALK* mRNA expression and RMS patient outcome, we performed association analysis with known RMS prognostic factors, such as initial tumour size, clinical stage, and age at diagnosis. Data analysis of RMS patients showed a positive correlation between high *ALK* mRNA levels and advanced stage of disease (Stage IV, $P=0.01$) or tumour size (>5 cm, $P=0.04$), whereas ALK mRNA expression and age at diagnosis were not significantly associated (<1 to >10y, $P=0.06$; Figure 3). In addition, ROC curve analysis was carried out in RMS patients for which clinical follow-up was available, taking into account the fusion gene status (PAX3/7-FOXO1 expression) and patients' survival. We demonstrated that $\Delta\Delta CT=555$ was the ALK mRNA cutoff level capable of distinguishing with extremely high accuracy PAX3/7-FOXO1-positive RMS from fusion gene-negative tumours (Figure 4A, AUC = 0.94; 95% CI = 0.89–0.99, $P<0.0001$), whereas $\Delta\Delta CT=200$ was the discriminating value that best characterised patients according to risk of relapse (Figure 4B, AUC = 0.64; 95% CI = 0.50–0.76; $P=0.01$). Indeed, RMS patients with low *ALK* mRNA levels ($\Delta\Delta CT<200$) had a significant reduced risk of failure compared with those with high ALK mRNA expression ($\Delta\Delta CT>200$; 44% *vs* 80%, respectively), whereas tumours with very high *ALK* transcript levels ($\Delta\Delta CT>555$) were almost exclusively PAX3/7-FOXO1-positive RMS (RMS-t = 84%; RMS-not = 2.5%). The prognostic value of the quantitative *ALK* mRNA assessment was substantiated by Kaplan–Meier analysis for PFS and OS of RMS patients distinguished based on the specific quantitative *ALK* mRNA cutoff value $\Delta\Delta CT=200$, which demonstrated a significantly poorer prognosis of patients with high *ALK* mRNA levels (*ALK high* = $\Delta\Delta CT>200$), irrespective of tumour histology and fusion-gene status (Figure 5, log-rank test, PFS $P=0.001$, OS $P=0.01$). Cox regression analysis confirmed these data, in particular when *ALK* mRNA was included together with known RMS risk factors. In our cohort of 71 patients, clinical stage (Stage IV), histology (ARMS), fusion gene status (PAX3/7-FOXO1-positive), and ALK mRNA ($\Delta\Delta CT>200$) were all significantly associated with lower survival, whereas age and tumour size at diagnosis were not

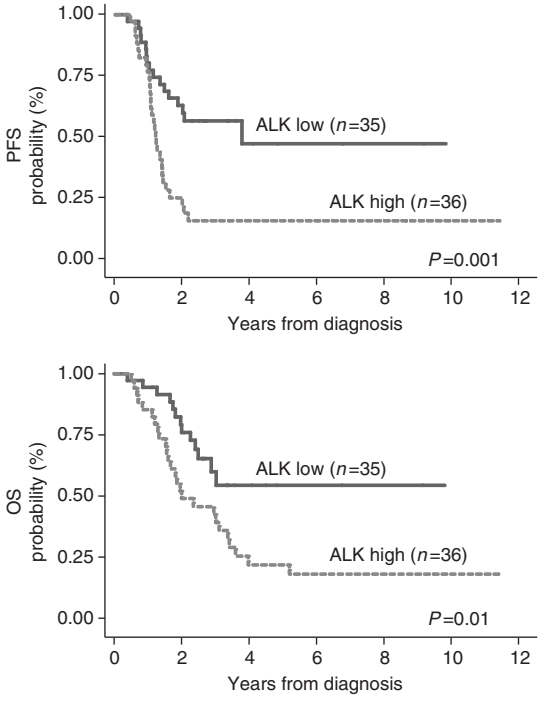


Figure 5. Prognostic significance of *ALK* gene expression. Kaplan–Meier and log-rank analysis for progression-free (PFS) and overall survival (OS) of RMS patients ($n=71$) based on specific quantitative *ALK* mRNA cutoff value (*ALK* low = $\Delta\Delta CT<200$; *ALK* high = $\Delta\Delta CT\geqslant200$) identify two subgroups of patients with significantly different outcome (PFS, $P=0.001$; OS, $P=0.01$).

(Table 2). ALK mRNA was a significant negative predictor of both PFS ($P=0.001$) and OS ($P=0.013$) in univariate analysis; whereas in multivariate analysis, RMS clinical stage was a stronger prognostic factor (*Stage*, OS: $P<0.0001$, HR = 3.8, 95% CI = 2.0–7.3; PFS: $P=0.0004$, HR = 3.0, 95% CI = 1.6–5.5). However, high *ALK* mRNA level was predictive on increased risk of relapse in multivariate analysis when tumour stage was not considered (*ALK* $\Delta\Delta CT\geqslant200$, PFS: $P=0.0017$, HR = 2.7, 95% CI = 1.5–5.1), as it was ARMS tumour histology in a model based on patient OS. Of note, ALK protein expression has been reported to correlate with ARMS histology, metastasis at presentation, and advanced clinical

Table 2. Summary of univariate and multivariate regression analysis performed in 71 RMS patients

		3-Years	Univ.	Multiv.		CI	3-Years	Univ.	Multiv.		CI
	Patients	OS (%)	*P*-value	*P*-value	HR	95%	PFS (%)	*P*-value	*P*-value	HR	95%
Sex											
Female	39	54 ± 9	0.62				36 ± 8	0.77			
Male	32	48 ± 9					34 ± 9				
Age, years											
1<;>10	25	38 ± 10	0.14				25 ± 9	0.09			
⩾1;⩽10	46	59 ± 8					43 ± 8				
Size, cm											
<5	20	68 ± 11	0.19				58 ± 11	0.27			
>5	44	51 ± 8					33 ± 7				
IRS group											
IV	28	26 ± 9	**<0,0001**[a]	**<0,0001**	3.8	2,0–7,3	12 ± 6	**0.0002**	**0.0004**	3.0	1,6–5,5
I–III	42	69 ± 8					55 ± 8				
ALK, ΔΔCT											
<200	36	60 ± 9	**0.013**				57 ± 8	**0.001**	(0,0017)[b]	(2,7)[b]	(1,5–5,1)[b]
⩾200	35	42 ± 9					16 ± 6				
Histology											
ERMS	31	67 ± 10	**0.006**	(0,0076)[b]	(2,7)[b]	(1,3–5,5)[b]	57 ± 10	**0.004**			
ARMS	40	41 ± 8					23 ± 7				
PAX3/7-FOXO1											
No	40	60 ± 9	**0.02**				54 ± 8	**0.0013**			
Yes	31	41 ± 9					16 ± 7				

Abbreviations: ALK = anaplastic lymphoma kinase; ARMS = alveolar rhabdomyosarcoma; CI = confidence interval; ERMS = embryonal rhabdomyosarcoma; HR = hazard ratio; IRS = intergroup rhabdomyosarcoma study; Multiv. = multivariate analysis; OS = overall survival; PFS = progression-free survival; Univ. = univariate analysis. Univariate and multivariate Cox regression analysis on OS and PFS, performed in 71 RMS patients with information on gender, age, tumour size, IRS stage, histology, fusion gene status (PAX3/7-FOXO1), and *ALK* mRNA expression level (ΔΔCT = 200).
[a]Significant values are indicated in bold.
[b]Significant values obtained without IRS group.

stage, but not with increased risk of relapse and death of RMS patients (van Gaal *et al*, 2011; Yoshida *et al*, 2013).

DISCUSSION

In this study, we examined the mRNA expression of *ALK*, a novel PAX3/FOXO1 target gene (Cao *et al*, 2010), in pediatric RMS tumours and cell lines, to discern the correlation with the clinical phenotype and establish the impact on patients' risk assessment. ALK is a RTK that can be expressed both as truncated fusion protein and full-length receptor kinase (Motegi *et al*, 2004; Soda *et al*, 2007), and it may induce malignant transformation both *in vivo* and *in vitro*. ALK overexpression correlates with advanced clinical stage and poor prognosis in several tumour types, and its inhibition results in a marked decrease of cell growth and survival (Mosse *et al*, 2008; Schulte *et al*, 2011). To date, several studies have reported on the protein expression of ALK in RMS and clinicopathological analysis using immunohistochemistry (Pillay *et al*, 2002; Corao *et al*, 2009; Yoshida *et al*, 2013). However, because of the different detection frequency and sensitivity observed, the clinical and functional importance of ALK in RMS remain uncertain yet.

Herein, we demonstrated that ALK expression varies broadly in RMS, with mRNA and protein levels significantly higher in PAX3/7-FOXO1-positive ARMS tumours and cell lines (Corao *et al*, 2009) compared with both ERMS and PAX3/7-FOXO1-negative ARMS (RMS-not). All the previous studies have used semi-quantitative methodologies to assess the expression of ALK in RMS, and according to these data, ALK protein expression was shown to be different in and between RMS subgroups (Corao *et al*, 2009; van Gaal *et al*, 2011; Yoshida *et al*, 2013). Herein, we provided the first evidence that ALK mRNA level, measured by quantitative RT–PCR, is a suitable marker for RMS patients stratification, as it is significantly higher in RMS tumours characterised by adverse clinicopathological parameters, such as the unfavourable PAX3/7-FOXO1-postive histology, advanced stage of disease, and larger tumour size at diagnosis, and also correlates with patients' increased risk of relapse and lower survival. In these settings, high *ALK* mRNA levels identified patients with higher risk of failure and poor outcome, and distinguished with high accuracy PAX3/7-FOXO1-positive ARMS and aggressive PAX3/7-FOXO1-negative tumours. Our findings are in accordance with metagene analysis performed by Williamson *et al*, which demonstrated that ALK gene expression highly discriminates PAX3/7-FOXO1-positive ARMS from fusion gene-negative RMS tumours (Williamson *et al*, 2010), but also with the observation that ALK protein may be predictive of poor patient survival in ARMS and metastatic ERMS (van Gaal *et al*, 2011; Lee *et al*, 2013). Indeed, we observed a significant trend towards a worse outcome of PAX3/7-FOXO1-negative RMS patients with high *ALK* mRNA levels, both when these patients

were defined according to the *ALK* median expression level and pre-specified cutoff values. Moreover, in Cox univariate regression analysis, high *ALK* mRNA levels ($\Delta\Delta CT > 200$) predicted a decreased PFS and OS independently of PAX3/7-FOXO1 expression, and Kaplan–Meier survival curves substantiated these conclusions. Although *ALK* mRNA did not turn out to be an independent prognostic marker in multivariate analysis considering RMS clinical stage, tumour size at diagnosis, age, and fusion gene status as risk factors, it had an unfavourable prognostic value when multivariate analysis was performed without considering tumour staging, supporting the concept that high *ALK* mRNA expression may be predictive of an unfavourable phenotype in primary RMS. Consistent with our results, ALK overexpression in NB predicts lower patient survival in a univariate Cox regression model using ALK mRNA as a continuous variable, whereas in a multivariate regression model, tumour stage, age, and 1p status result stronger independent negative variables (Schulte *et al*, 2011).

High ALK mRNA expression levels in RMS, however, were not due to specific ALK copy number gain or mutational events, because true ALK amplification or point mutations were not detected in both tumours and cell lines. In this study, we used a quantitative approach to detect true *ALK* amplification, and RNA Polymerase IID (*RNA Pol IID*) gene to correct for aneuploidy. No obvious amplification of the *ALK* gene was observed, whereas all the single substitutions detected after bidirectional sequencing of the *ALK* tyrosine kinase domain were synonymous or silent mutations. Consistently, true ALK amplification is rather uncommon in RMS and other tumour types (i.e., NB), either when it is measured by FISH or qRT–PCR (Schulte *et al*, 2011; Fleuren *et al*, 2013; Yoshida *et al*, 2013).

Finally, the strong correlation between *ALK* mRNA expression and patient outcome, together with the observation that ALK is capable of signalling in RMS cells, suggests that ALK is a RTK noteworthy for further investigation in this malignancy. To date, this is the first study that achieved inducible ALK phosphorylation in RMS cells *in vitro*, examined its activation and inhibited its activity. Although the role of ALK in RMS cells is still under investigation, we provided evidences that ALK may affect RMS cell signalling and be a potential target for small-molecule inhibitors in these cells.

In conclusion, this is the first study that implicates *ALK* mRNA as a negative factor in RMS and demonstrates that the quantitative assessment of *ALK* mRNA expression levels may be used to improve risk stratification of RMS patients independently of fusion gene status and tumour histology. These findings, although preliminary, warrant consideration for future treatment strategies of this malignancy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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