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 Melzter, 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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**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 (s-ALK, upper panel) and phosphorylated (p-ALK, lower panel) ALK proteins detected in RMS-t and RMS-not cell lines after immunoprecipitation (IP:ALK). LAN5 and KARPAS-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 Signalling 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'-GGGTCTTACTCTTGGAGGC-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’-GGGCCCTCATTGCTCACAAACCGA-3’ and 5’-GGGGCTGAGG-3’-FAM labelled, respectively, whereas RNA Pol IIID gene expression analysis (qRT-PCR and GCN) and survival analysis were also indicated.

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             |
| 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                      |                |                  |               |
| III                            | 0              | 0                | 2             |
| IV                             | 2              | 1                | 2             |
| Other                          | 10             | 9                | 30            |
| IV                             | 23             | 3                | 4             |
| Unknown                        | 0              | 0                | 1             |
| qRT–PCR                        | 35             | 13               | 39            |
| GCN                            | 16             | 6                | 20            |

| Abbreviations: ARMS-t = PAX3/7-FOXO1-positive alveolar rhabdomyosarcoma; ARMS-not = PAX3/7-FOXO1-negative alveolar rhabdomyosarcoma; ERMS = embryonal 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. |
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ALK mRNA expression in RMS tumours. The expression of ALK was then measured in RMS human specimens (Table 1). 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 level.

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 IIID 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 ≥4).

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.
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=0.09), 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  ΔΔCT=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  ΔΔCT=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 (ΔΔCT<200) had a significant reduced risk of failure compared with those with high ALK mRNA expression (ΔΔCT>200; 44% vs 80%, respectively), whereas tumours with very high ALK transcript levels (ΔΔCT>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 ΔΔCT=200, which demonstrated a significantly poorer prognosis of patients with high ALK mRNA levels (ALK high = ΔΔCT>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 (ΔΔCT>200) were all significantly associated with lower survival, whereas age and tumour size at diagnosis were not (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 ΔΔCT≥200, 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
In this study, we examined the mRNA expression of ALK in RMS, a novel PAX3/FOX01 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, 2001; 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-positive 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

| Table 2. Summary of univariate and multivariate regression analysis performed in 71 RMS patients |
|---------------------------------------------------------------|
| Patients | OS (%) | P-value | P-value | HR | 95% CI | OS (%) | P-value | P-value | HR | 95% CI |
| Sex       |        |         |         |     |        |        |         |         |     |        |
| Female    | 39     | 54 ± 7  | 48 ± 9  | 0.62| 36 ± 8| 34 ± 9  | 0.77   |
| Male      | 32     | 54 ± 4  | 48 ± 7  |     | 34 ± 4| 32 ± 7  |        |
| Age, years|        |         |         |     |        |        |         |         |     |        |
| 1<;>10    | 25     | 38 ± 10 | 99 ± 8  | 0.14| 25 ± 9| 43 ± 8  | 0.09   |
| 1<;>10    | 46     | 38 ± 10 | 99 ± 8  |     | 43 ± 10| 33 ± 7  |        |
| Size, cm  |        |         |         |     |        |        |         |         |     |        |
| <5        | 20     | 58 ± 11 | 99 ± 8  | 0.19| 58 ± 11| 33 ± 7  | 0.27   |
| >5        | 44     | 58 ± 11 | 99 ± 8  |     | 53 ± 11| 32 ± 7  |        |
| IRS group |        |         |         |     |        |        |         |         |     |        |
| IV        | 28     | 26 ± 9  | <0.0001*| 3.8| 0.0002| 0.004   | 3.0    | 1.6–5.5 |
| I–III     | 42     | 26 ± 9  | <0.0001*| 3.8| 0.0002| 0.004   | 3.0    | 1.6–5.5 |
| ALK, ΔΔCT |        |         |         |     |        |        |         |         |     |        |
| <200      | 36     | 60 ± 9  | 42 ± 9  | 0.013| 57 ± 8| 16 ± 6  | 0.001  | (0.0017)b| (2.7)b| (1.5–5.1)b |
| >200      | 35     | 60 ± 9  | 42 ± 9  |     | 53 ± 8| 16 ± 6  |        |
| Histology |        |         |         |     |        |        |         |         |     |        |
| ERMS      | 31     | 67 ± 10 | 41 ± 8  | 0.006| 57 ± 10| 23 ± 7  | 0.004  |
| ARMS      | 40     | 67 ± 10 | 41 ± 8  |     | 54 ± 10| 21 ± 7  |        |
| PAX3/7-FOXO1 |    |         |         |     |        |        |         |         |     |        |
| No        | 40     | 60 ± 9  | 41 ± 9  | 0.02 | 54 ± 8| 16 ± 7  | 0.0013 |
| Yes       | 31     | 60 ± 9  | 41 ± 9  |     | 54 ± 8| 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.

**DISCUSSION**

In this study, we examined the mRNA expression of ALK, a novel PAX3/FOX01 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 (Moses 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-positive 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 (AACT > 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 stage, 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 Ip 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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