Table 1 Clinically detected cases with *ALK* aberrations

No.	Age at diagnosis/sex	Origin	INSS	Base change predicted protein change	Chr. 2p/ploidy	Histology	Serum NSE (ng/mL)r)	Urinary VMA/HVA (μg/mgCr)	Therapy	Outcome
1. NB459	21 mo/male	Ad	4	3520T>C F1174L	MNA/doploid	Unfav	187.2	24.3/40.6	Cx →Res →Cx	Dead (11 mo)
2. NB484	26 mo/male	Ad	4	3522C>A F1174L	MNA/doploid	Unfav	370	9.32/71.4	Cx →Res →Cx	Alive (19 mo)
3. NB445	21 mo/male	Ad	4	3735C>A F1245L	MNA/doploid	Unfav	678.6	3.0/12.4	Cx→Res→Cx	Dead (22 mo)
4. NB351	0 mo/male	Med	4	3824G>A R1275Q	–/aneuploid	Fav	14	92.7/61.4	Pr. Res	Alive (65 mo)
5. NB448	10 mo/male	Cer	3	3824G>A R1275Q	Gain/aneuploid	Fav	23.1	43.4/34.6	Pr. Res→Cx	Alive with tumor (12 mo)
6. NB451	6 mo/female	Ret	1	3824G>A R1275Q	–/diploid	Fav	190	135/111	Pr. Res	Alive (24 mo)
7. NB452	25 mo/male	Ad	4	3824G>A R1275Q	MNA/diploid	Unfav	241.4	56.1/76.1	Cx→Res.→Cx	Dead (18 mo)
8. NB503	57 mo/male	Ret	3	3824G>A R1275Q	Gain/aneuploid	Unfav	1376	69.3/91.1	Res.→Cx (relapse→Cx)	Dead (4 mo)
9. NB055	14 mo/male	Ad	3	*ALK* amplification	MNA/diploid	Unfav	342.1	6.2/10.1	Res.→Cx	Dead (2 mo)
10. NB511	22 mo/female	Ad	4	*ALK* amplification	MNA/diploid	Unfav	268.1	47.2/60.6	Cx→Res.→Cx	Dead (28 mo)

Mo indicates months old; Ad, adrenal gland; Med, mediastinum; Cer, cervical; Ret, retroperitonium; Chr, chromosome; MNA, *MYCN* amplification; –, no remarkable changes of chr 2p; Gain, 3 to 5 copies of chr. 2p; Unfav, unfavorable; Fav, favorable; Pr. Res., Primary tumor resection; Cx, chemotherapy; Res, resection.

Table 2 Mass screening detected cases with *ALK* aberrations

No.	Age at diagnosis/sex	Origin	INSS	Base change predicted protein change	Chr. 2p/ploidy	Histology	Serum NSE (ng/mL)r)	Urinary VMA/HVA (μg/mgCr)	Therapy	Outcome
11. NB058	6 mo/female	Ad	1	3520T>G F1174V	Gain/aneuploid	Unfav	35.2	118.4/ 100.4	Pr. Res (relapse→Cx)	Dead (12 mo)
12. NB229	6 mo/male	Ad	4	3521T>C F1174S	–/aneuploid	Fav	41.9	210.9/41.9	Cx→Res→Cx	Alive (25 mo)
13. NB169	6 mo/male	Multifocal	1	3522C>A F1174L	– or Gain/diploid or aneuploid	Fav	30	127.4/40.3	Pr. Res→Cx	Alive (74 mo)
14. NB303	8 mo./female	Ad	3	3522C>A F1174L	Gain/aneuploid	Fav	17	43.4/75.9	Pr. Res. (relapse→Cx)	Alive (75 mo)
15. NB146	7 mo/male	Med	1	3733T>A F1245I	–/diploid	Fav	49.6	50/34	Pr. Res	Alive (89 mo)
16. NB524	8 mo/male	Ad	1	3735C>A F1245L	–/diploid	Fav	26.4	46.7/29.8	Pr. Res	Alive (51 mo)
17. NB204	8 mo/male	Ad	1	3746A>G D1249G	–/diploid	Fav	14.7	76.4/37	Pr. Res	Alive (90 mo)
18. NB367	6 mo/male	Ad	3	3824G>A R1275Q	Gain/aneuploid	Fav	8.9	59.2/38.4	Pr. Res	Alive (63 mo)
19. NB039	6 mo/male	Ad	4S	*ALK* amplification	MNA/diploid	Unfav	62.1	34.2/56.7	Cx→Res→Cx	Dead (35 mo)

Mo indicates months old; Ad, adrenal gland; Med, mediastinum; Cer, cervical; Ret, retroperitonium; Chr, chromosome; MNA, *MYCN* amplification; –, no remarkable changes of chr 2p; Gain, 3 to 5 copies of chr. 2p; Unfav, unfavorable; Fav, favorable; Pr. Res., Primary tumor resection; Cx, chemotherapy; Res, resection.

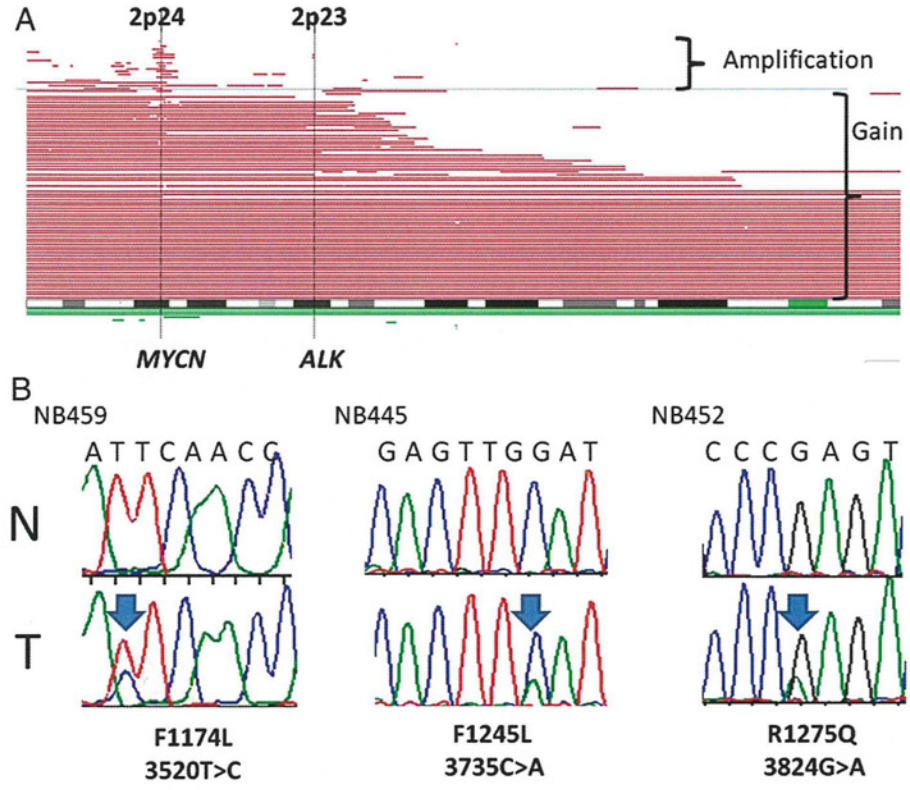

Fig. 1 Copy number gains and high-level amplifications in the short arm of chromosome 2 and representative *ALK* mutations in neuroblastoma. A, Each horizontal line indicates a region showing a simple copy number (CN) gain (CN <5, red bars, lower cases) and high-level amplification (CN >5, pink bars, upper cases) in each case. Most high-level amplifications involved the *MYCN* locus at 2p24, whereas the amplicons in 3 cases were found at 2p23, which contains the *ALK* locus. B, Representative cases with mutations in each exon of *ALK* TKD. N indicates constitutive DNA derived from peripheral blood cells of each patient; T, tumor DNA.

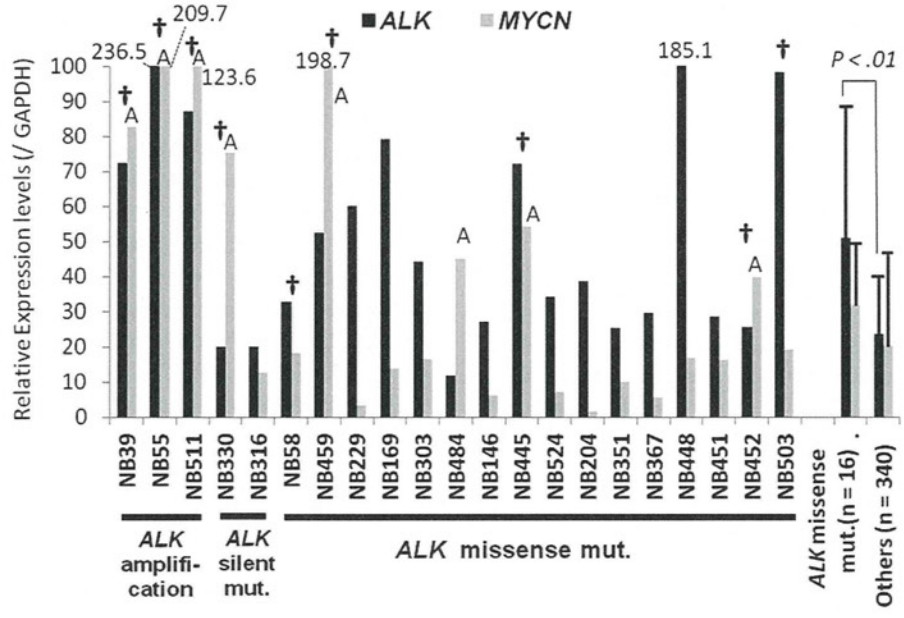

Fig. 2 Relative *ALK* and *MYCN* expression from neuroblastomas with *ALK* aberrations. The expression levels of *ALK* and *MYCN* in each sample were evaluated relative to the levels of *GAPDH*. The relative *ALK* expression levels in the tumors with *ALK* missense mutation were significantly higher than in those without mutations ($P < .01$). The 3 tumors with *ALK* amplification showed extremely high *ALK* expression. The relative *MYCN* expression levels were higher in the tumors with *ALK* aberrations but depended on *MYCN* amplification. A, *MYCN* amplification; †, died of disease; mut, mutation.

mutated *ALK* case (case 13) with multifocal tumors involving bilateral adrenal glands, mediastinum, and retroperitoneal tumors, bilateral adrenal tumors were aneuploid, whereas 2 extraadrenal tumors were diploid. Reviews of the treatments provided to the patients detected by mass screening found that primary resection was initially performed except for 1 INSS4 case. According to the Japanese infant neuroblastoma protocol, postoperative chemotherapy was not performed except for the INSS 4 case and the multifocal case. Among them, 2 cases (numbers 11 and 14) showed relapses of the tumor.

In the 3 cases with *ALK* amplifications, 1 INSS4 case (case 19) was detected by mass screening, whereas the other 2 (numbers 9 and 10) included an INSS3 tumor diagnosed at 14 months of age and an INSS4 tumor diagnosed at 22 months of age. All 3 patients had *MYCN* amplification and died. The survival curves of the patients with *ALK* aberrations (including missense mutations and amplifications) are shown in Fig. 3. Comparison of the survival rates of cases detected by mass screening showed progressive malignancy in the cases with *ALK* aberrations ($P = .002$). For the patients with *ALK* aberrations, survival rates of clinically detected cases were significantly worse than those detected by mass screening ($P = .025$). Three infants who were clinically detected were aneuploid with *MYCN* nonamplified tumors and showed favorable outcomes.

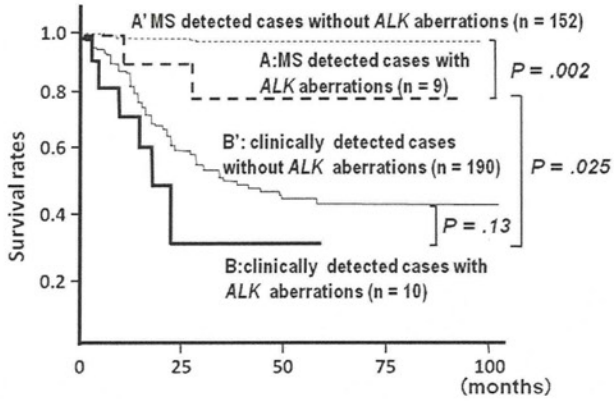

Fig. 3 Kaplan-Meier cumulative survival curves for neuroblastoma patients with mutant *ALK* or amplifications. A, For neuroblastoma patients detected by mass screening (MS), survival curve for those with tumors with *ALK* missense mutations or amplification (n = 9). A′, Survival curve of the remaining patients detected by mass screening (MS) (n = 152). Survival of patients with aberrant *ALK* tumors was significantly worse than others detected in the mass screening ($P = .002$). B, Survival curve of clinically detected patients with tumors with ALK missense mutations or amplifications (n = 10). B′, Survival curve of the remaining patients detected clinically (n = 190). Survival of the clinically detected patients with aberrant ALK tumors was worse but not significantly ($P = .13$), but significantly worse than that of the patients detected by mass screening (MS) with tumors with ALK missense mutations or amplification (n = 9) ($P = .025$). Lengths of follow-up phase in all cases were 62 ± 17 months (n = 361).

Therefore, the tumors with *ALK* aberrations detected in older patients (>18 months of age) showed high progressive malignancy.

In cases identified by mass screening, the histology of case 11 showed unfavorable histology because of a high mitosis-karyorrhexis index (MKI), and this patient died. Case 14 with INSS 3 also had a relapse, indicating that an activated *ALK* mutation might play a role in aggressively malignant transformation. To clarify the effects of an activated *ALK* mutation on patient outcome, a Cox regression analysis was carried out for all clinical cases and cases detected by mass screening. An activated *ALK* mutation was a significant independent prognostic factor in mass screening cases ($P = .029$) but not in clinically detected cases. Therefore, the activated *ALK* tumors that sometimes develop in infants might have high recurrent capacity even if detected by mass screening. Therefore, the early detection by screening was effective at improving the outcome of the patients with the activated *ALK* tumors.

3. Discussion

The *ALK* gene is involved in several chromosomal translocations or inversions that contribute to oncogenesis in several different tumor types, including anaplastic large cell lymphoma and lung cancer [19]. In anaplastic large cell lymphomas, the most common genetic aberration is a translocation that gives rise to oncogenic fusion proteins. On the other hand, recent analyses of neuroblastomas suggested that *ALK* is activated and may contribute to tumor development through gene amplification and specific mutations targeting the TKD [11,12]. Most mutations in ALK proteins are reportedly localized within the kinase domain and are assumed to produce constitutively activated proteins. ALK F1174L and ALK R1275Q mutants in Ba/F3 cells, a murine interleukin 3–dependent pro-B cell line, induced constitutive activation of ALK kinase with activation of downstream targets of ALK signaling [20]. Sequencing analysis of the ALK TKD revealed that mutations resulting in amino acid changes were found in 16 cases, and 12 of these 16 cases were detected in 2 hotspots of ALK F1174 and R1275. Because neuroblastoma tissues with other missense mutations (as well as these hot spot mutations) in TKD showed high ALK expression, all these missense mutations may lead to ALK activation. In patients with *ALK* amplification, *MYCN* amplification was also detected in the SNP arrays, suggesting that intragenic rearrangements may have occurred in a subset of advanced tumors from children with unfavorable outcomes [12]. Indeed, the expression levels of *ALK* in these 3 cases were very high, suggesting that *ALK* overexpression might also contribute to neuroblastoma progression. Passoni et al [21] also found that ALK overexpression was correlated with poor prognosis of neuroblastoma regardless of the TKD

mutation and *ALK* amplification. Thus, overexpression of ALK by activating mutation of the TKD or by amplification must be correlated with neuroblastoma progression. Meanwhile, silent mutations found in 2 cases did not show high ALK expression, suggesting that these silent mutations were unlikely to correlate with tumor progression.

For tumors analyzed at all clinical stages, the frequency of amino acid substitutions in the TKD or in *ALK* amplifications was 5.3%. These results were lower than those reported by Mosse et al [11] (12.4%) and by Caren et al (11.1%) [12]. This difference might be caused by the characteristics of the population analyzed in our report. That is, our study involved large numbers of early-stage neuroblastomas, especially those detected by mass screening projects. In the cases detected by mass screening, 1 amplified *ALK* case and 8 cases with activated *ALK* mutations were found, which constitute 5.6% of all cases detected by mass screening. This ratio is equivalent to that of the clinically detected cases, indicating that *ALK* mutation or amplification might be one of the early events of neuroblastoma progression. Meta-analysis of more than 700 neuroblastoma samples also showed that *ALK* mutations occurred in equal frequencies across all genomic subtypes, but F1174 mutations were observed in a high proportion of *MYCN*-amplified tumors [13]. In the present study, *ALK* F1174 mutations were detected in 2 amplified *MYCN* cases that were clinically diagnosed at more than 18 months of age and in 4 nonamplified *MYCN* cases that were detected by screening. Infant occult tumors with *ALK* F1174 mutations might readily develop into amplified *MYCN* tumors.

The clinical features of activated *ALK* neuroblastomas did not seem to be markedly different from other neuroblastomas. The outcomes of these patients seemed to depend mainly on clinical prognostic factors such as age at diagnosis, histology (INPC), stage (INSS), *MYCN* amplification, and DNA ploidy pattern. However, among ten INSS 1 to 3 infant cases, which included 8 cases detected by mass screening, underwent primary surgical resection, 3 suffered relapses. Because relapse and reoccurrence of tumors are very rare in infant neuroblastoma, *ALK* activation is one of the distinguishing risk factors in early-stage neuroblastomas. Moreover, the prognosis of patients with activated ALK tumors detected by screening was significantly better than that of the clinically detected activated ALK tumors. The diminishing or maturing neuroblastoma were frequently detected by screening in infancy, but early detection of activated ALK tumors by screening was considered to contribute to improvement in patient outcome. Therefore, in infants with early detected tumors, those with activated ALK tumors should undergo therapeutic strategies different from other cases.

In the 7 older patients consisting of one INSS3 and 6 INSS4 advanced cases, including 2 amplified *ALK* cases, 6 patients died and 1 recent case is alive under treatment, suggesting that ALK activation is also a risk factor in advanced stage neuroblastomas [11]. Survival curves of the clinically detected cases with aberrant *ALK* tumors seem to be worse in comparison with those without *ALK* aberrations, but not significantly.

In a recent report [13], F1174 and R1275 mutations were correlated with poor outcomes. In the 7 *ALK* mutated cases detected by mass screening, 3 relapsed and all had a F1174 or R1275 mutation, suggesting that F1174 and R1275 mutations might have a higher transforming capacity. These findings suggest that activated ALK tumors have high malignant potential regardless of whether they are discovered in infants or in screening procedures. The incidence of ALK activation in the tumors detected by mass screening was similar to that in clinically detected tumors. Therefore, patients with activated ALK neuroblastomas may require particularly aggressive treatments. In advanced neuroblastomas with ALK activation, ALK might be an attractive target for novel therapeutic strategies such as ALK kinase inhibitors, which are in development for specific targeted cancer therapy [22].

In the present series, one patient with multifocal neuroblastoma had an *ALK*-TKD activating mutation in his constitutional DNA as well as all neuroblastoma tumors derived from different sites. Since the *ALK* mutation is one of the major genes conferring familial neuroblastoma predisposition [11], *ALK* mutations may also cause multifocal neuroblastomas. To treat multifocal neuroblastoma cases, we should analyze *ALK* aberrations and perform genetic counseling to the family when an *ALK* germline mutation is apparent.

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Table 1 International Federation of Gynecology and Obstetrics classification of ovarian tumors[10]

Stage	Extent of disease
I	Tumor limited to the ovaries
Ia	Limited to one ovary; no ascites. No tumor on external surface; capsule intact
Ib	Limited to both ovaries; no ascites. No tumor on external surfaces, capsule intact
Ic	Limited to one or both ovaries but with tumor on surface of one or both ovaries; or with capsule ruptured; or with positive ascites or positive peritoneal washings
II	Tumor involving one or both ovaries with pelvic extension
IIa	Extension and/or metastases to uterus and/or tubes only
IIb	Extension to other pelvic tissues
IIc	As in IIa or IIb but with positive ascites or positive peritoneal washings; or with capsule ruptured
III	Tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal lymph nodes; extension to small bowel or omentum; superficial liver metastases
IIIa	Limited to true pelvis grossly with negative nodes but histologically confirmed microscopic seeding of abdominal peritoneal surfaces
IIIb	Limited to one or both ovaries with negative nodes but histologically confirmed implants of abdominal peritoneal surfaces, none > 2 cm diameter
IIIc	Abdominal implants > 2 cm diameter and/or positive retroperitoneal or inguinal nodes
IV	Tumor of one or both ovaries with distant metastases outside of peritoneal cavity; parenchymal liver metastases; pleural effusion, if present, must have positive cytology

oncologic control for many years, because in patients with adult-type GCT, relapses may occur even decades after diagnosis, although the survival rate is 95% if diagnosed in the early stages.

The presented case indicates the need to emphasize the possibility of occurrence of endocrinological disorders as a symptom of hormone-secreting tumors in children. GnRh-independent precocious puberty may be the first symptom of hormone-secreting ovarian tumors in children, even if tumors are not detectable in preliminary radiologic examinations. In such cases, profound diagnostics with systematic ultrasound examination are essential.

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Successful treatment for hepatoblastoma in a 1-year-old boy with trisomy 18

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Key words child, hepatoblastoma, trisomy 18.

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Hepatoblastoma (HB) is the most common primary hepatic tumor in children. Most HB cases are sporadic, but this tumor has been reported in association with Beckwith–Wiedmann syndrome, hemi-hypertrophy, Prader–Willi syndrome, familial adenomatous polyposis, and trisomy 18. Trisomy 18 is a devastating genetic disorder that is characterized by multiple

congenital anomalies. The mortality rate among infants with trisomy 18 is high as a result of cardiac and renal malformations, feeding difficulties, sepsis, and central apnea caused by nervous system defects. Although 90% of trisomy 18 patients die within the first year of life, a few cases of relatively long-term survival have been previously reported.[1]

Here we present a case of HB in a 1-year old boy with trisomy 18, cardiac malformation, and renal malformation.

Case report

A male neonate was born to a primigravida, 35-year-old mother and unrelated 40-year-old father, at 40 weeks 6 days of gestation. The pregnancy was uneventful and there was no remarkable family history. The infant's birthweight was 2538 g. He had several anomalies such as club foot, hyperdactyly, overlapping fingers and low-set fawn-like ears. On admission, ultrasonography (US) showed coarctation of the aorta and horseshoe kidney. On chromosome analysis the peripheral blood cells were found to be 47,XY, +18 chromosome (20/20).

At 14 months of age, he was referred to Nihon University Hospital with a mass in the right upper arm, which was pulsatile, with strong bruit sounds. On color Doppler US brachial artery aneurysm was diagnosed. It was improved by compression. His height was 62.4 cm (–5.6 SD), and bodyweight (BW) was 4820 g (–5.0 SD). His vital signs were stable. Abdominal examination indicated a firm mass in the upper abdomen. The mass was moderately tender with voluntary guarding but no peritoneal signs and no bruit sounds. Laboratory data were as follows: white blood cell count, 14 100/μL; red blood cell count, 214 × 10^4/μL; hemoglobin, 6.1 g/dL; hematocrit, 18.6%; platelet count, 39.7 × 10^4/μL; aspartate aminotransferase, 142 U/L; Total protein, total bilirubin, amylase, alkaline phosphatase, alanine aminotransferase, lactate dehydrogenase, and electrolytes were within normal range. Serum levels of carcinoembryonic antigen and carbohydrate antigen 19-9 and neuron-specific enolase were within normal range, but α-fetoprotein (AFP) was considerably elevated (141 900 ng/mL; normal range, <10 ng/mL). 24 h creatinine clearance (24 h Ccr) was low (47.6 mL/min). The ejection fraction of the heart was normal.

Abdominal US and computed tomography (CT) confirmed a solid mass (11.7 × 10.7 cm) in the right liver lobe. Doppler US showed lowered renal blood flow due to pressure of the tumor. Under suspicion of HB (pretreatment extent of disease; PRETEXT III), we started induction therapy according to the (Japanese Study Group for Pediatric Liver Tumor (JPLT) protocol. The doses of chemotherapy were modified by the BW conversion, while considering the renal function and cardiac function. At first, two courses of tetrahydropyranyl adriamycin (THP-ADR; 1 mg/kg BW × 0.3 on day 1) were given, and tumor size and AFP carefully evaluated for the effect of treatment. Cisplatin (CDDP; 1.33 mg/kg BW × 0.3 × 0.66 on days 1 and 2) was given as the third course. Because CDDP monotherapy did not seem as effective, a combination of THP-ADR and CDDP was given as the fourth chemotherapy course. At this point, we performed complete tumor resection by right hepatectomy. The resected specimen was a mass of 102 g, measuring 6.0 × 4.5 × 4.0 cm. A histological diagnosis of well-differentiated, fetal-type HB was made. Flow cytometry indicated a diploid pattern, and the karyotype of the tumor cells was 47,XY, +18. The patient's postoperative recovery was uneventful. Postoperatively the patient received two additional courses of THP-ADR/CDDP at the same dosage. AFP level was normalized within 7 weeks after surgery. He has remained well during 1.5 years of follow sup with no evidence of renal and cardiac failure. His growth and weight patterns are comparable to those of the pretreatment period (height –3.5 SD, weight –3.3 SD; Fig. 1). He is severely mentally affected.

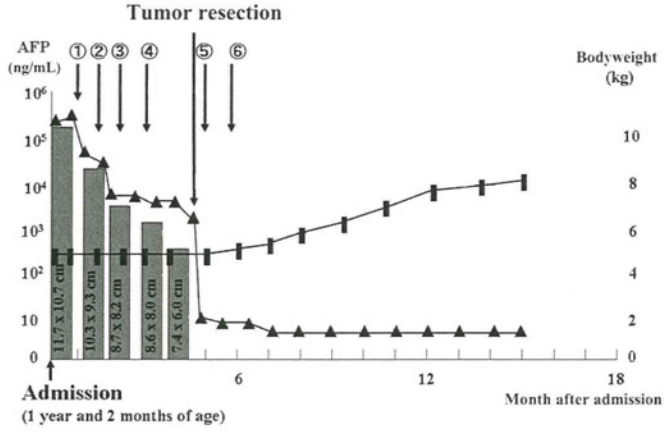

Fig. 1 Clinical course of the present patient. 1–6, chemotherapy courses. (▲) α-fetoprotein (AFP); (■) bodyweight. The gray boxes refer to the size of the tumor.

Discussion

We describe a case of HB in a 1-year-old boy with trisomy 18, cardiac and renal malformation, who was successfully treated with modified chemotherapy and surgery. The brachial artery aneurysm diagnosed in the present patient has not previously been reported, but some reports on arterial aneurysm of umbilical cord with trisomy 18 can be found in the literature.[2] Nine case reports including the present one, describe HB in trisomy 18 (Table 1).[3–10] Median age at diagnosis was 13.8 months (range, 3–33 months). Two patients were male and seven were female. The median birthweight was 2258 g (range, 1630–3300 g). Six patients had visceral anomalies and one had a renal anomaly. Only the present patient was treated with chemotherapy and surgery. Among published cases in four patients (3, 4, 5 and 8), complete tumor resection was performed. Three of them (patients 3, 5 and 8) and the present patient were free of the disease and were alive >1 year after surgery. Two patients (2 and 6) died of heart failure and three patients (1, 4 and 7) died of the disease. On histologic subtype, six patients had fetal type, one patient had embryonal type, and one patient had fetal–embryonal type. In recent years, several national and international cooperative studies have shown that the prognosis of HB can be improved dramatically by combining surgery and chemotherapy, such as CDDP and THP-ADR.[11] In Japan, the chemotherapy protocol using CDDP and THP-ADR (JPLT) has been the standard treatment since 1991. Due to the lack of

Table 1 Studies on hepatoblastoma in trisomy 18

Patient	Author (year), ref No.	Age (months)	Sex	Birthweight (g)	Karyotype	Visceral anomalies	Renal anomalies	Chemotherapy	Surgery	Histologic subtype	Prognosis	Cause of death
1	Dasouki and Barr (1987)[3]	33	F	1860	47,XX,+18	VSD	–	–	–	NA	Died at 33 months of age	Probably tumor
2	Mamlok *et al.* (1989)[4]	3	F	1800	47,XX,+18	ASD, VSD, PS, AS	–	–	–	Embryonal type	Died at 3 months of age	Heart failure
3	Tanaka *et al.* (1992)[5]	24	F	1750	47,XX,+18:46, XX=2:1	VSD	–	–	Right lobectomy	Fetal type	Well after surgery at 2 years 9 months	–
4	Bove *et al.* (1996)[6]	21	F	3300	48,XX,+18,+20	–	–	–	Right lobectomy	Fetal–embryonal type	Died at 26 months of age	Bone metastasis
5	Teraguchi *et al.* (1997)[7]	7	F	2722	47,XX,+18	VSD, PDA	–	–	Partial lobectomy	Fetal type	Well after surgery at 2 years 5 months	–
6	Maruyama *et al.* (2001)[8]	3	F	2464	47,XX,+18	VSD	–	–	–	Fetal type	Died at 5 months of age	Heart failure
7	Kitanovski *et al.* (2009)[9]	6	F	1630	47,XX,+18	–	–	–	–	Fetal type	Died at 7 months of age	Probably tumor
8	Fernandez *et al.* (2011)[10]	9	M	NA	47,XY,+18:46, XY=9:11	–	–	CDDP, 5-FU, VCR, ADR	LTX (after second resection)	Fetal type	Well after LTX at 2 years 4 months	–
9	Present series (2011)	18	M	2538	47,XY	CA	Horseshoe kidney	THP-ADR, CDDP	Right lobectomy	Fetal type	Well after surgery at 2 years 8 months	–

ADR, doxorubicin; AS, aortic stenosis; ASD, atrial septal defesct; CA, coarctation of the aorta; CDDP, cisplatin; 5-FU, 5-fluorouracil; LTX, liver transplant; NA, not available; PDA, patent ductus arteriosus; PS, pulmonary stenosis; THP-ADR, tetrahydropyranyl adriamycin; VCR, vincristine; VSD, ventricular septal defect.

experience of chemotherapy use in patients with trisomy 18, it was hard to predict the effects or side-effects of chemotherapy in the present patient. During chemotherapy we regularly followed heart and renal function and were able to use dosage-modified chemotherapy safely enough to achieve complete tumor resection. According to our limited experience, even in patients with trisomy 18 and organ anomalies, HB can sometimes be treated safely enough with modified chemotherapy and the patient can be cured. But the decision as to whether to treat HB in a patient with trisomy 18, is always a major ethical issue. We report the efficacy and safety of the treatment strategy involving chemotherapy and surgery for a patient with HB and trisomy 18.

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超低出生体重児にWilson-Mikity症候群とBeckwith-Wiedemann症候群を伴った肝芽腫の1例

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要 旨

超低出生体重児にWilson-Mikity症候群とBeckwith-Wiedemann症候群（BWS）を伴った肝芽腫の1例を報告する．症例は，8か月の女児．BWSに伴う腫瘍のスクリーニング検査で，肝腫瘤と血清alpha-fetoprotein（AFP）高値を指摘され紹介となった．Wilson-Mikity症候群による肺気腫と肺高血圧症により呼吸循環動態が不安定であり，開腹腫瘍生検は行わずに化学療法を開始した．CITA療法2コースの後に肝部分切除を施行した．術後にlow CITA療法4コース行い治療を終了した．本症例は，腫瘍完全切除後もAFP高値が遷延し，また，一過性の上昇を伴ったことより，血清lectin-reactive alpha-fetoprotein（AFP-L3）と合わせて腫瘍切除後の経過を観察した．

超低出生体重児にBeckwith-Wiedemann症候群（BWS）を伴った肝芽腫の腫瘍切除後におけるAFPとAFP-L3の推移について報告する．

索引用語：肝芽腫，血清alpha-fetoprotein（AFP），血清lectin-reactive alpha-fetoprotein（AFP-L3），超低出生体重児，Beckwith-Wiedemann症候群

I はじめに

肝芽腫は，Beckwith-Wiedemann症候群[1)2)]（以下，BWS），低出生体重児[3)-6)]においてその発症頻度が高い．一般に，血清alpha-fetoprotein値（以下，AFP）は，肝芽腫において信頼性の高い腫瘍マーカーで，半減期は5日前後とされる．一方，BWS児[7)8)]や低出生体重児[9)]では，肝芽腫を伴わなくとも，AFP高値が遷延するとされる．肝芽腫の完全切除後にAFPの推移を観察することは，治療効果判定や再発・転移巣の早期発見において重要である．

今回我々は，超低出生体重児にBWSを伴った肝芽腫に対して，AFPと血清lectin-reactive alpha-fetoprotein値（以下，AFP-L3）の推移を観察した1例を経験したので文献的考察を加えて報告する．

II 症 例

症例：8か月，女児．

主訴：スクリーニング検査における肝腫瘤指摘，AFP高値．

家族歴：特記すべきことなし．

出生歴：完全破水に伴う臍帯脱出を認め，在胎26週0日に緊急帝王切開にて719 gで出生した．Apgar scoreは2点／8点（1分／5分値）にて，neonatal intensive care unit（NICU）へ入室となった．

出生後経過：出生後から肺高血圧症を伴い，高頻度振動型人工呼吸器にて計44日間の人工呼吸器管理を要した．生後8週頃から胸部単純レントゲン写真で網状陰影，嚢胞状陰影を認め，Wilson-Mikity症候群と診断された．生後3か月頃から，巨舌，左片側肥大が顕著となり，BWSの診断となった．その後も呼吸循環管理のため，入院加療を継続していた．

現病歴：BWSに伴う腫瘍のスクリーニング検査を定期的に行っていたところ，生後8か月時に肝腫瘤とAFP高値を認め，肝芽腫の疑いで当院へ転院となった．

入院時現症・身体所見：身長51.5 cm，体重3,750 g，体温37.0度，心拍数90回／分，呼吸数40回／分，血中酸素飽和濃度92％（酸素0.5 l/min投与にて），血圧85/50 mmHg．臍突出，左半身肥大，巨舌を認めた．

胸部所見：陥没呼吸を認め，呼吸音が減弱し呼気の延長，II音の亢進を認めた．

腹部所見：右肋弓下1.5横指に肝臓を触知したが，腫

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瘤は触知しなかった．

血液・尿検査所見：AFP 133,000 ng/ml（AFP-L3: 6.5%）と高値を認めるも，その他の一般血液・尿検査ならびに腫瘍マーカーは正常であった．

血液ガス所見（酸素 0.5 *l*/min 投与下動脈血）：pH 7.344，CO_2 49.5 Torr，O_2 72.7 Torr，$HCO3^-$ 24.7 mEq/*l*，BE +0.2 mEq/*l*．

心臓超音波検査：左心駆出率 65%，右室圧 90.5 mmHg と著しい肺高血圧症，軽度の三尖弁閉鎖不全を認めた．

胸部単純レントゲン写真：肺野全体の網状陰影と正中を越え対側にまで拡大した気腫像を認めた（図 1）．

腹部造影 CT 検査：肝 S3，S4 にかけて不均一な造影効果を伴う 29.3×27.7 mm の腫瘤を認めた（図 2）．

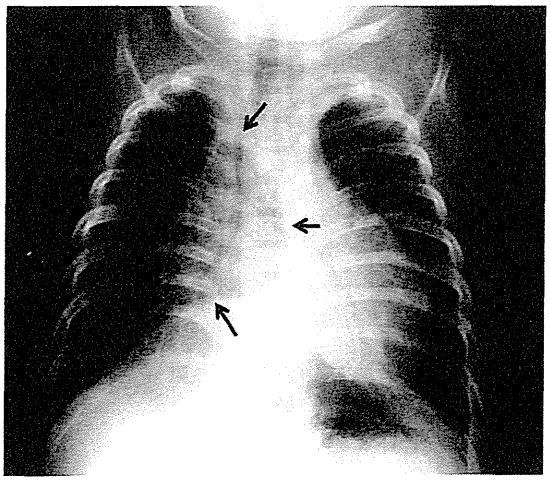

図 1　胸部単純レントゲン写真：肺野は全体的に網状で正中を越え対側にまで拡大した気腫像（矢印）を認める．

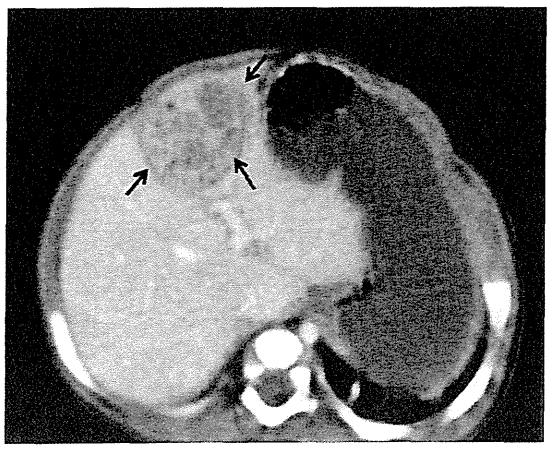

図 2　腹部造影 CT：肝 S3，S4 にかけて，造影効果をともなう内部不均一な 29.3 × 27.7 mm の腫瘤（矢印）を認める．

転院後経過：肺気腫，巨大ブラ，Wilson-Mikity 症候群などの呼吸器疾患及び，著しい肺高血圧症による心臓への侵襲を考慮し，開腹腫瘍生検は行わずに，肝芽腫（pre treatment extent of disease; PRETEXT II，日本小児外科学会悪性腫瘍委員会分類 stage II）の診断で，化学療法を開始した．日本小児肝癌スタディグループ (JPLT)-2 プロトコールに準じて治療を開始した．1 コース目は，CITA 療法（Tetrahydropyranyl-Adriamycin; THP-ADR 30 mg/m^2×2 days, day 1, 2，cisplatin; CDDP 80 mg/m^2×1 day, day 8）を 30% dose で行った．1 コース目で副作用はなく化学療法の安全性を確認し，2 コース目は，CITA 療法（THP-ADR 30 mg/m^2×2 days, day 2, 3，CDDP 80 mg/m^2×1 day, day 1）を 50% dose で行った．2 コース目終了後の腫瘍縮小率は 30%であった．腫瘍は，S3, 4 を占拠していたが，表在性であり，かつ主要血管と離れ，腫瘍辺縁から 5 mm 以上のマージンをもって部分切除可能と判断し，系統的肝切除は行わずに肝部分切除を施行した．化学療法後ではあるが，病理組織学的検査にて，胎児型 mitotically active subtype が主体で，一部に胎芽型の混在を認める hepatoblastoma の確定診断を得た．術後に low CITA 療法 4 コース（THP-ADR 30 mg/m^2×2 days, day 2, 3，CDDP 40 mg/m^2×1 day, day 1），1 コース目は 50% dose，2，3，4 コース目は full dose で行った．AFP と AFP-L3 の半減期は，肝切除後～化学療法終了までが 63.8 日と 315.7 日，化学療法終了後～正常化までが 57.6 日と 46.1 日であり，肝切除後正常化までの時間が一般的な他の肝芽腫患者に比して長かった（表 1）．術後補助化学療法中に AFP と AFP-L3 の低下が緩徐であったこと，一過性の上昇を認めたことより（図 3），頭・胸・腹部 CT 検査，腹部超音波検査，腫瘍シンチグラフィ，骨シンチグラフィを行うも，腫瘍残存や再発巣は認めなかった．肝切除後と化学療法の肝細胞障害後における肝再生に伴う AFP の変化を疑い，low CITA 療法 4 コース目の前後で hepatocyte growth factor（HGF）を検討した（図 4）．HGF は ALT，AST（データは表示せず）と同様に化学療法後 10 日前後で上昇し，30 日前後で化学療法前の値に戻った．化学療法後

表 1　肝切除後の AFP，AFP-L3 の半減期

半減期	肝切除後～化学療法終了	化学療法終了～AFP 正常化
AFP	63.8 日	57.6 日
AFP-L3	315.7 日	46.1 日

AFP; alpha-fetoprotein, AFP-L3; lectin-reactive alpha-fetoprotein

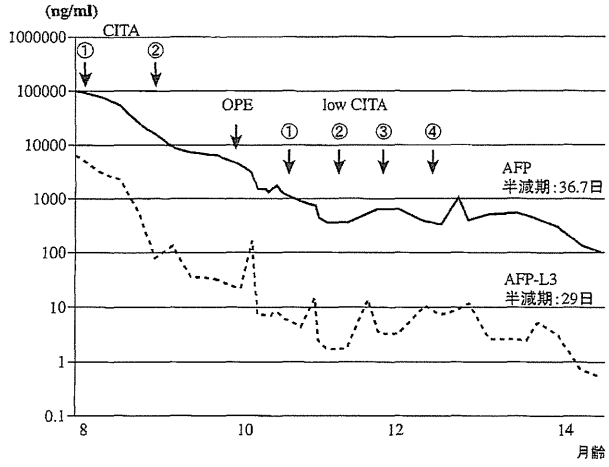

図3 入院後よりAFP正常化までの経過：AFP（正常値10 ng/ml以下）とAFP-L3（正常値10％以下）の低下は緩徐であり，一過性の上昇を認めた．AFP正常化までのAFP，AFP-L3の半減期は36.7日，29日であった．

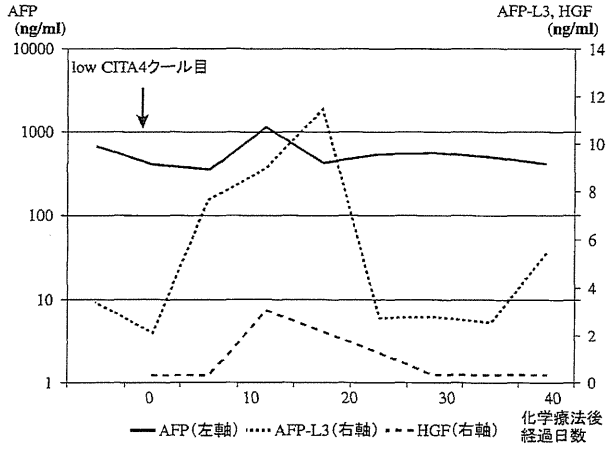

図4 Low CITA療法4コース目の前後におけるAFP，AFP-L3，およびHGFの推移：HGF（正常値0.40 ng/ml以下）はALT，ASTと同様に化学療法後10日前後で上昇し，30日前後で化学療法前の値に戻った．

のAFP，AFP-L3の倍加時間（doubling time）は，3.6日，27日であった．AFPの倍加時間はAFP-L3より短く，肝切除後と化学療法の肝細胞障害後における肝再生に伴うAFPの変化と判断した．最終化学療法が終了した時点でAFPとAFP-L3は正常値とならなかったが，慎重な経過観察の方針とし，治療開始から7か月後に退院となった．その後AFPとAFP-L3は緩徐に低下し，治療終了後1年（1歳8か月）で，AFP，AFP-L3は正常化した．治療終了後2年の現在，AFPとAFP-L3は正常域で再発を認めていない．

III 考 察

BWSは，臍帯脱出（exomphalos），巨舌（macroglossia），巨体（giantism）を主徴とし，症状の頭文字を合わせてEMG症候群とも言われる．BWSの1～3％に肝芽腫が合併するとされている[10)11)]．

肝芽腫の腫瘍マーカーとして用いられるAFPは，妊娠6～7週から胎児卵黄嚢，その後は胎児肝から産生される糖蛋白であり，血中のピーク値は，妊娠13週で3×10^7 ng/ml前後まで上昇し，生後約8か月前後で成人と同じレベルになる[12)13)]．また，AFPの半減期には個人差があり，約5日前後であるが，BWS患児においては健常児より長いとされ[7)8)]，Hamadaら[14)]によると33日の症例も確認されている．低出生体重児においてもAFPの半減期は，約7.7日と健常児より長いとされている[9)]．満期産の健常児に伴う肝芽腫であれば，Tsuchidaら[15)]の作成した血清AFP値推移表と比較すればよいが，BWS児や低出生体重児に肝芽腫を合併した場合，治療効果判定や再発の診断は難しい．Kinoshitaら[16)]は，AFP産生腫瘍に対するAFP-L3の測定の重要性を示した．肝芽腫においては，肝切除後のAFP低下よりもAFP-L3の低下が急速であり，肝切除後の評価としての有用性を報告している．したがって，BWS児や低出生体重児に伴う肝芽腫では，腫瘍産生成分であるAFP-L3の測定により腫瘍残存や治療効果判定を行う必要がある．自験例は在胎26週0日で出生し，修正月齢としても推移表の正常範囲内には入らなかった．また，表1に示したように，肝切除後から化学療法終了の期間では，AFP-L3の半減期がAFPよりも長く，肝切除後の評価に苦慮した．最終治療終了後は，Kinoshitaら[16)]の報告と同様にAFP-L3の半減期がAFPよりも短く，治療効果を比較的鋭敏に表していたと考えられた．

肝芽腫における肝切除後にAFPの変動を示す症例が報告されている[14)17)]．Hamadaら[14)]はBWSに伴う肝芽腫切除後のAFP高値ならびに一過性の上昇は，肝切除後に伴う肝細胞のrapid regenerationによるAFPの上昇と，腫瘍切除及び半減期の差異によるAFP低下とのアンバランスが原因と推察している．また，Kubotaら[17)]は肝芽腫切除後の化学療法中に再発巣を認めないにも関わらず，AFP上昇を呈した3症例を報告した．この3症例においてAFP-L3については調べられていないが，THP-ADRが共通の使用薬剤であり，AFP変動の原因と推察している．自験例では，治療経過中に一度の確認だが，図4に示したように，low CITA療法4クール後に肝細胞の増殖促進の指標とされるHGFは，ALT，AST

の上昇（データは表示せず）とともに一過性の上昇を示し，さらにAFPおよびAFP-L3も一過性上昇を認めた．この上昇時におけるAFP，AFP-L3の倍加時間は，3.6日，27日でAFPの方が短かった．腫瘍産生成分であるAFP-L3が変動していたことの原因は不明であるが，化学療法による肝細胞障害後のrapid regenerationのためAFPは上昇したと考えられた．肝芽腫の治療経過中に，AFP，AFP-L3の上昇を認める場合には，HGFを含めた検討が必要と思われた．

低出生体重児やBWSに肝芽腫を合併した症例は報告されているが，AFP-L3の推移に対する報告はない．今回我々は，超低出生体重児にBWSを伴う肝芽腫において，腫瘍残存のない完全切除後においてもAFP，AFP-L3が通常のAFP半減期通りに減少せず，治療効果判定や再発・転移の判断に苦慮した症例を経験した．BWS患児，低出生体重児における治療効果判定，再発・転移の判断には画像検査も併用しAFPとAFP-L3の推移や肝機能障害時のHGFを注意深く観察することが重要と思われた．

文　献

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A Case of Hepatoblastoma With Wilson-Mikity Syndrome and Beckwith-Wiedemann Syndrome in an Extremely Low Birth-Weight Infant

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We report a case of hepatoblastoma with Wilson-Mikity syndrome and Beckwith-Wiedemann syndrome (BWS) in an extremely low birth-weight infant. At 8 months of age, she was referred to our hospital due to liver tumor and elevated serum alpha-fetoprotein (AFP) levels upon screening for tumors with BWS. We started chemotherapy (CITA regimen) without an open biopsy, because of unstable conditions due to emphysema and pulmonary hypertension. After two courses of CITA regimen, she received tumor resection. Postoperatively, she received four courses of low CITA regimen. In this case, we carefully observed not only the level of AFP but lectin-reactive AFP (AFP-L3) because the levels of AFP and AFP-L3 showed gradual decreases with transient increases after complete tumor resection. Here we discussed changes of AFP and AFP-L3 levels after tumor resection in hepatoblastomas.

Key words: hepatoblastoma, serum alpha-fetoprotein (AFP), lectin-reactive serum alpha-fetoprotein (AFP-L3), extremely low birth weight infant, Beckwith-Wiedemann syndrome

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Anaplastic lymphoma kinase status in rhabdomyosarcomas

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Rhabdomyosarcoma is a rare soft tissue sarcoma that typically affects children, adolescents, and young adults. Despite treatment via a multidisciplinary approach, the prognosis of advance-stage rhabdomyosarcomas remains poor, and a new treatment strategy is needed. Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that is a potential target for specific inhibitors. In this study, we investigated 116 rhabdomyosarcomas using a polymer-based ALK immunostaining method and correlated the results with clinicopathological parameters. In addition, we examined *ALK* status using dual-color fluorescence *in situ* hybridization, PCR, and sequencing. In immunohistochemical analysis, ALK was detected in 2 (6%) of 33 embryonal rhabdomyosarcomas, 42 (69%) of 61 alveolar rhabdomyosarcomas, and 0 (0%) of 22 other subtypes, including pleomorphic, adult-spindle-cell/sclerosing, and epithelioid variants. Compared with ALK-negative alveolar rhabdomyosarcomas, ALK-positive ones are presented with metastatic spread more frequently and showed a greater extent of myogenin reactivity. Overall survival was not associated with ALK expression. *FOXO1* rearrangement was significantly associated with ALK immunoreactivity. The median *ALK* copy number was greater in ALK-positive tumors than in ALK-negative tumors. Most (93%) cases tested showed no selective increase in the *ALK* gene dosage. *ALK* selective amplification and low-level selective gain were noted in one and three cases, respectively. Further, a high-polysomy pattern ($\geq$4 *ALK* copies in $\geq$40% of cells) was observed in seven cases. A significant increase in the *ALK* copy number was exclusive to the ALK-immunopositive cohort, but it was uncommon, accounting for only 30% of the 37 ALK-positive rhabdomyosarcomas. *ALK* gene rearrangement was not observed in either cohort, while an *ALK* somatic mutation (I1277T) was found in one ALK-negative embryonal case. Although it remains controversial whether ALK expression without gene rearrangement is therapeutically relevant, this comprehensive analysis may help future studies on the utility of ALK-targeted therapy for patients with rhabdomyosarcoma.
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Keywords: anaplastic lymphoma kinase; fluorescence *in situ* hybridization; immunohistochemistry; rhabdomyosarcoma

Rhabdomyosarcoma is a rare sarcoma with skeletal muscle differentiation that typically affects children, adolescents, and young adults. It is histologically classified into the embryonal, alveolar, and pleomorphic subtypes,[1–3] although other rare variants like sclerosing, adult-spindle-cell, and epithelioid rhabdomyosarcomas have also been proposed.[4–7] Each subtype is characterized by a distinct epidemiology, clinical behavior, and genetic background.[1–3] For example, alveolar rhabdomyosarcoma affects older patients compared with the embryonal subtype, is associated with poor survival, and in most cases, is characterized by a specific t(2;13) or t(1;13) translocation, which results in the *PAX3-FOXO1* or *PAX7-FOXO1* fusion gene, respectively.[3] Although multidisciplinary treatment has dramatically improved prognosis, the survival rate of those affected by metastatic rhabdomyosarcoma remains low. Therefore, a new treatment strategy is needed.

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase whose gene *ALK* is located on chromosome 2. The *ALK* gene is rearranged in

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anaplastic large cell lymphoma,[8] ALK-positive B-cell lymphoma,[9] inflammatory myofibroblastic tumor,[10] and a small subset of lung carcinoma[11] and renal cell carcinoma,[12,13] while it is mutated in neuroblastoma[14] and in rare thyroid cancers.[15] The ALK protein is constitutively expressed in these *ALK*-associated cancers, whereby it has a critical oncogenic role. ALK has recently received significant clinical attention because it is targetable by small molecule kinase inhibitors. The ALK inhibitor crizotinib showed a marked overall response and good disease control rates in patients with *ALK*-rearranged lung cancers,[16] and similar encouraging results were reported for this drug in the case of an inflammatory myofibroblastic tumor[17] and anaplastic large cell lymphomas.[18]

Considering several reports that have documented ALK expression in rhabdomyosarcomas,[19–23] we speculated that ALK may also have an oncogenic role in these sarcomas, and that they may be treatable with ALK inhibitors. We first decided to clarify the incidence of ALK expression and genomic changes in rhabdomyosarcomas in relation to clinicopathological parameters. We investigated ALK expression in a large number of cases using a previously validated ALK immunostaining method[24] and correlated the results with the tumor subtype, *FOXO1* rearrangement, and clinicopathological findings. In addition, we determined *ALK* gene status (rearrangement, changes in copy number, and somatic mutations) in these sarcomas.

Materials and methods

Case Selection

This study was approved by the institutional review board. We retrieved 116 rhabdomyosarcoma specimens, each from a unique patient, from the pathology files of the National Cancer Center Hospital and the University of Tokyo Hospital (both in Tokyo, Japan). All hematoxylin and eosin-stained sections were reviewed along with the relevant immunostaining data, and the diagnoses were confirmed. All the tumors were positive for desmin and one or both of the myoregulatory proteins (myogenin and myoD1).[25] Of note, our study population predominantly included adults: 60% of the patients were ≥18 years of age (median age, 20 years). All the tumors were reclassified on the basis of the latest World Health Organization scheme[1–3] and the recent literature,[4–7] into embryonal ($n=33$), alveolar ($n=61$), pleomorphic ($n=8$), adult-spindle-cell/sclerosing ($n=12$), and epithelioid rhabdomyosarcomas ($n=2$). The adult-spindle-cell type was separately categorized because it is considered distinct from pediatric homonymous tumor (ie, a subset of the embryonal type) because of its more aggressive behavior and frequent association with sclerosing features.[7]

ALK Immunohistochemistry

Four-micrometer-thick sections of the tumors were deparaffinized. Heat-induced epitope retrieval was performed using the targeted retrieval solution pH9 (Dako, Carpinteria, CA, USA). The slides were treated with 3% hydrogen peroxide for 20 min to block endogenous peroxidase activity. They were then incubated with primary antibody against ALK protein (1:40, 5A4; Abcam, Cambridge, UK) for 30 min at room temperature. Immunoreactions were detected using EnVision-FLEX+ (Dako). The reactions were visualized using 3,3′ diaminobenzidine followed by counterstaining with hematoxylin. We had previously shown that this staining condition yielded perfect concordance between immunoreactivity and the *ALK* rearrangement status in lung cancers.[24] The staining results were categorized as negative (<5%), focally positive (5—50%), and diffusely positive (>50%), whereas the staining intensity was graded as weak, moderate, or strong.

Correlation with Clinical Parameters

The clinical data were correlated with the ALK immunoexpression status in the case of alveolar rhabdomyosarcoma. The clinical parameters studied were age, sex, primary tumor site (head and neck, urogenital/perianal, extremity, or trunk), whether the tumor initially presented with metastatic spread to the lymph nodes or distant sites, whether the tested specimen had been obtained before or after chemotherapy, whether the tested specimen was sampled from the primary site or metastatic site, and overall survival.

Correlation with Histological Parameters

Select histological parameters were correlated with the ALK immunoexpression status in the case of alveolar rhabdomyosarcoma. The histological parameters studied were the presence of a pure solid growth pattern, the presence of a mixture with additional embryonal rhabdomyosarcomatous morphology, the presence of nuclear pleomorphism (at least at a focal level), and the percentage of myogenin-immunopositive cells.

FOXO1 and *ALK* Gene Rearrangement Status

Fluorescence in situ hybridization (FISH) analysis to detect gene rearrangement was performed on formalin-fixed, paraffin-embedded, 4-μm-thick tumor sections. Break-apart probes were used for the *FOXO1* (Vysis LSI FOXO1 Dual Color, Abbott Molecular, Abbott Park, IL, USA) and *ALK* genes (Vysis LSI ALK Dual Color, Abbott Molecular) in accordance with the manufacturer's instructions. At least 50 nonoverlapping tumor cells were examined. Cases in which >20% of the cells showed split signals or isolated 3′ signals were considered positive for gene rearrangement.

ALK Copy Number Evaluation

Changes in the *ALK* copy number were determined using FISH on formalin-fixed, paraffin-embedded 4-μm-thick tumor sections. A dual-color probe set was used, in which the *ALK* gene was labeled with Texas Red (red) and the paracentromeric sequence of the chromosome 2 (*CEN2p*) was labeled with FITC (green) (GSP laboratory, Kawasaki, Kanagawa, Japan). FISH images were captured using the Metafer Slide Scanning Platform (MetaSystems, Altlussheim, Germany) to facilitate analysis. In order to minimize the influence of truncation artifacts, we evaluated $50-100$ nonoverlapping tumor cells with an adequate nuclear area that exhibited at least one each of green and red signals. The specific *ALK* gene copy number was analyzed by determining the ratio of the copy number of *ALK* to that of *CEN2p*, and copy number gain was classified as no selective gain (median *ALK/CEN2p* $=1$), low-level selective gain ($1<$median *ALK/CEN2p* <2), or selective amplification (median *ALK/CEN2p* ≥ 2). When there was no selective copy number gain (ie, median *ALK/CEN2p* $=1$), we focused on the absolute *ALK* copy number per cell. An increase in this parameter was categorized as no polysomy (≥ 4 *ALK* gene copy in $<10\%$ of cells), low polysomy (≥ 4 *ALK* gene copy in $10-<40\%$ of cells), and high polysomy (≥ 4 *ALK* gene copy in $\geq 40\%$ cells), similar to the criteria widely used in thoracic oncology.[26]

ALK Mutation Analysis

We extracted DNA from formalin-fixed paraffin-embedded tumor sections. Exons 23 and 25 of the *ALK* gene, which harbor hotspots of activating mutations, were amplified by PCR using EX *Taq* HS polymerase (TAKARA, Otsu, Shiga, Japan). The primers used were ALK-EX23F, 5′-GCCTTTAT ACATTGTAGCTGC-3′; ALK-EX23R, 5′-TCGGAGGA AGGACTTGAGGTC-3′; ALK-EX25F, 5′-TCTTCCCA GAGACATTGCTGC-3′; and ALK-EX25R, 5′-GGTAG AAAGTTGACAGGGTAC-3′ The PCR products were purified (QIAquick PCR purification kit; Qiagen, Valencia, CA, USA) and analyzed by sequencing with the same primers (Big Dye sequencing kit; Applied Biosystems, Carlsbad, CA, USA). Somatic mutations were evaluated by sequencing matched normal DNA extracted from muscle tissues. Somatic amino acid substitution was functionally evaluated using the Polyphen2 and MutationAssessor algorithms (http://genetics.bwh.harvard.edu/pph2/ and http://mutationassessor.org/, respectively).

Statistical Analysis

All data analyses were performed using SPSS version 20.0 (IBM Corporation, Somers, NY, USA). Fisher's exact test was used for categorical data, and Mann–Whitney's U test was used for continuous data. Overall survival, measured from the date of clinical presentation, was calculated using the Kaplan–Meier method, and survival difference was compared using the log-rank test. All P values were two tailed, and $P<0.05$ was considered significant.

Results

Correlation between ALK Expression and Tumor Subtype

As shown in Table 1, ALK expression was detected in 2 (6%) of 33 embryonal, 42 (69%) of 61 alveolar, 0 (0%) of 8 pleomorphic, 0 (0%) of 12 adult-spindle-cell/sclerosing, and 0 (0%) of 2 epithelioid rhabdomyosarcomas. ALK expression was significantly associated with tumor subtype ($P<0.001$). Among the 44 ALK-positive cases, 11 showed strong staining intensity, 14 showed moderate intensity, and 19 showed weak intensity (Figure 1). The staining extent was diffuse in 40 cases and focal in 4 cases. The median rate of positive cells was 80%. In immunopositive cases, the reaction was at the plasma membrane and/or cytoplasm with frequent paranuclear Golgi accentuation; no nuclear reactivity was seen.

Correlation between ALK Expression and Clinicopathologic Parameters in Alveolar Rhabdomyosarcoma

As shown in Table 2, the age, sex, primary tumor site, chemotherapy status, and sample type were not significantly associated with ALK immunoreactivity. ALK-positive and ALK-negative alveolar rhabdomyosarcomas initially presented with metastasis in 67% and 32% of the cases, respectively, and the difference was statistically significant ($P=0.014$). Further, overall survival did not differ between the groups ($P=0.96$, Figure 2). Among the histological parameters, pure solid growth pattern and mixture with embryonal morphology were not significantly associated with ALK status. All three alveolar rhabdomyosarcomas with at least focal nuclear pleomorphism were ALK immunonegative ($P=0.027$).

Table 1 ALK immunoreactivity in rhabdomyosarcoma subtypes

Subtype	*ALK Positive*	*ALK Negative*	*Total*
Embryonal	2 (6%)	31	33
Alveolar	42 (69%)	19	61
Pleomorphic	0 (0%)	8	8
Adult-spindle-cell/sclerosing	0 (0%)	12	12
Epithelioid	0 (0%)	2	2

Abbreviation: ALK, anaplastic lymphoma kinase.
$P<0.001$ (alveolar *vs* non-alveolar).

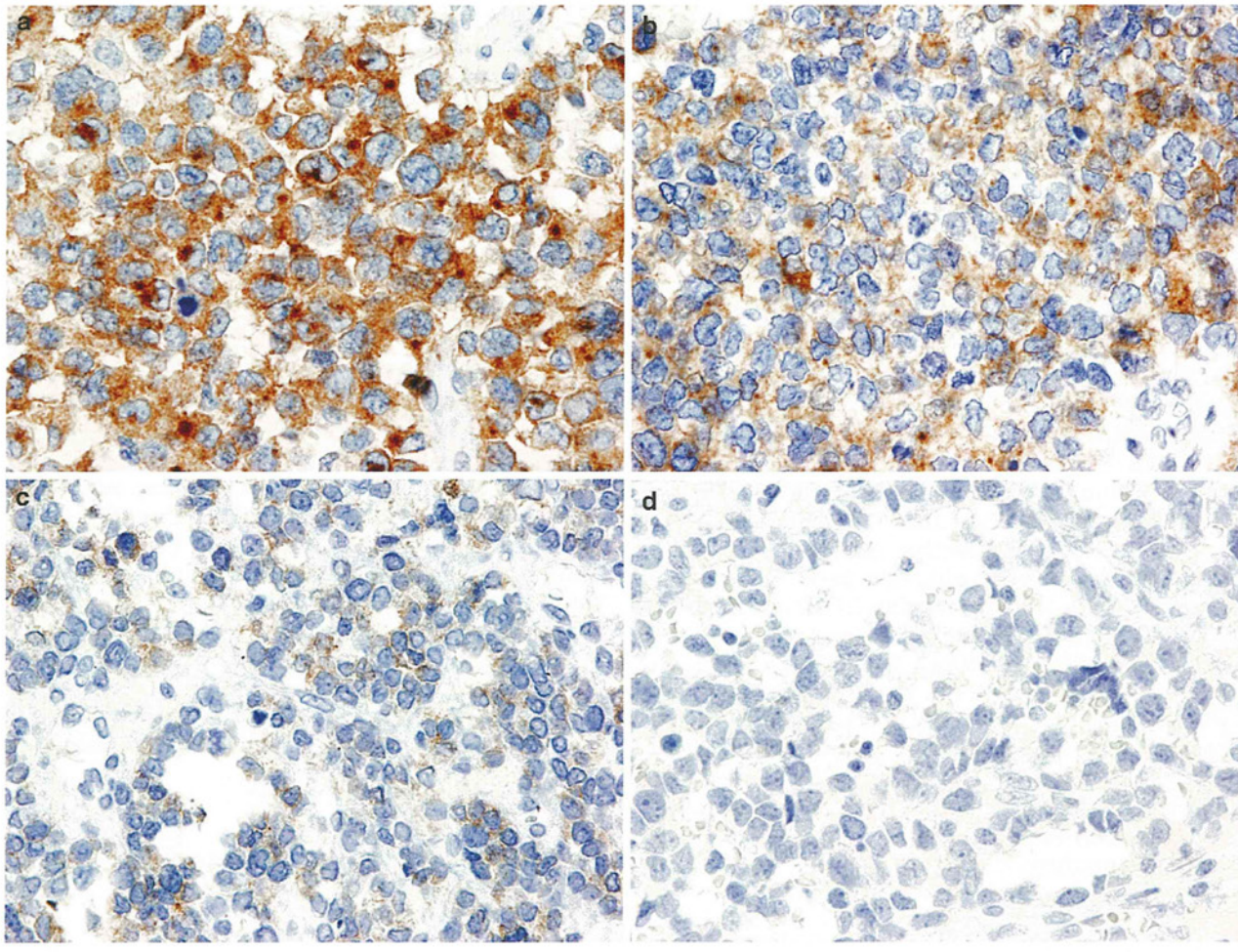

Figure 1 ALK immunoreactivity in rhabdomyosarcomas. The staining was (**a**) strongly positive, (**b**) moderately positive, (**c**) weakly positive, or (**d**) negative.

Table 2 Correlation between ALK immunoreactivity and clinicopathological parameters in alveolar rhabdomyosarcoma

Clinicopathological Characteristics		*ALK positive (N=42)*	*ALK negative (N=19)*	P
Age (years)	Median	19	18	0.97
	Range	2–49	4–53	
Sex	Male:female	20: 22	9: 10	1.00
Site	Head and neck	20	6	0.19
	Urogenital/perianal	9	3	
	Extremity	12	7	
	Trunk	1	3	
Metastasis at presentation		28	6	0.014
Status post chemotherapy		16	9	0.58
Primary:metastasis		22:20	12:7	0.58
Solid growth pattern only		8	2	0.49
Nuclear pleomorphism		0	3	0.027
Mixture with embryonal morphology		1	2	0.23
Median myogenin-positive cells (%)		95	60	0.001

Abbreviation: ALK, anaplastic lymphoma kinase.

The median rate of myogenin-immunopositive cells in the ALK-positive alveolar rhabdomyosarcomas was 95%, which was significantly higher than that (60%) in ALK-negative ones ($P=0.001$).

Correlation between ALK Expression and *FOXO1* Status in Alveolar rhabdomyosarcoma

FOXO1 gene rearrangement status was examined in all the alveolar rhabdomyosarcomas and select

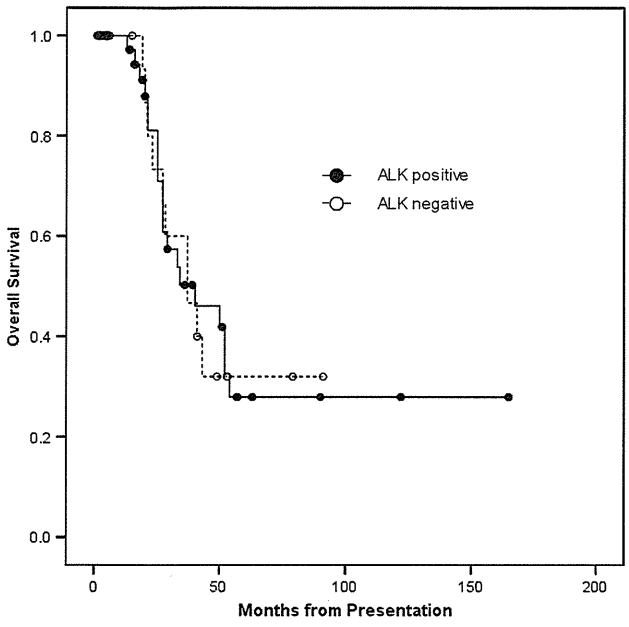

Figure 2 Kaplan–Meier analysis of overall survival of 42 patients with ALK-positive alveolar rhabdomyosarcoma and 19 patients with ALK-negative alveolar rhabdomyosarcoma. No significant difference in survival was observed between the groups as determined by the log-rank test ($P=0.96$).

Table 3 Correlation between ALK immunoreactivity and *FOXO1* rearrangement status in alveolar rhabdomyosarcoma

	ALK positive	*ALK negative*
FOXO1 rearranged	33	8
FOXO1 non-rearranged	0	6

Abbreviation: ALK, anaplastic lymphoma kinase.
$P<0.001$.

non-alveolar cases. Among 47 alveolar tumors successfully studied, 41 cases (87%) showed *FOXO1* rearrangement, while 6 cases were negative for *FOXO1* rearrangement. In contrast, none of the 18 non-alveolar tumors studied, including both ALK-positive embryonal cases, showed *FOXO1* rearrangement. All the 33 ALK-positive alveolar tumors and 8 of the 14 ALK-negative alveolar tumors showed *FOXO1* rearrangement (Table 3). Thus, ALK immunoexpression and *FOXO1* status were significantly correlated ($P<0.001$).

ALK Rearrangement Status

The *ALK* gene rearrangement status was determined in select ALK-positive and ALK-negative cases. None of the 24 cases (12 ALK positive, 12 ALK negative) tested showed *ALK* rearrangement.

Changes in *ALK* Copy Number

Changes in the *ALK* copy number were examined in all the alveolar rhabdomyosarcomas and select

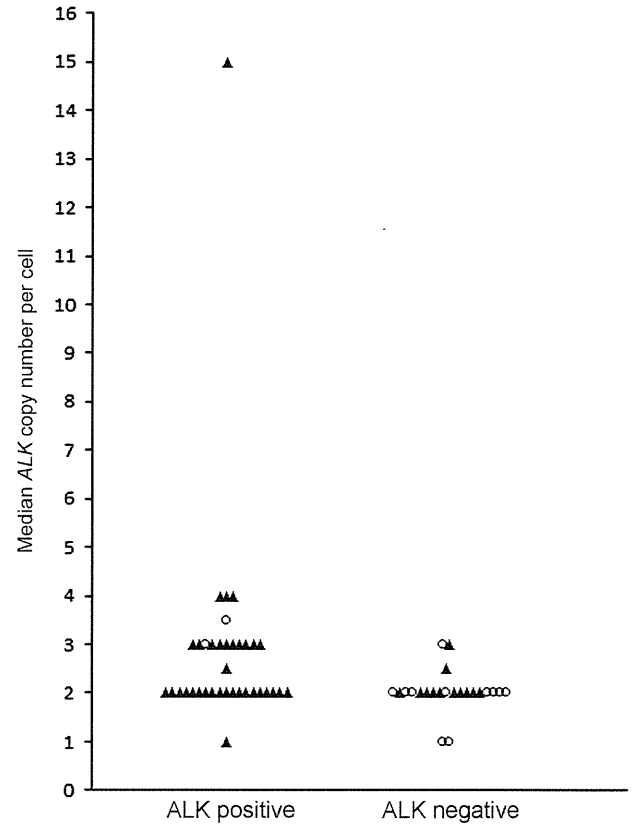

Figure 3 The median *ALK* copy number per cell determined by FISH in 37 ALK-positive and 23 ALK-negative rhabdomyosarcomas. ▲ indicates the alveolar subtype, and ○ indicates non-alveolar subtypes. ALK-positive tumors carried a significantly greater number of *ALK* gene copies than ALK-negative tumors did ($P=0.005$).

non-alveolar cases. Both ALK-positive embryonal rhabdomyosarcomas were included in the analysis. FISH was successful for 47 alveolar (35 ALK positive and 12 ALK negative) and 13 non-alveolar (2 ALK positive and 11 ALK negative) tumors. The median *ALK* copy number per cell was significantly greater in the ALK-positive tumors than the ALK-negative ones ($P=0.005$, Figure 3). However, the median *ALK/CEN2p* ratio did not differ significantly between these tumors ($P=0.106$, Figure 4), and most cases (56 cases, 93%) showed no selective increase in the *ALK* gene dosage (median *ALK/CEN2p* = 1, Figure 5a). Selective amplification was seen in one alveolar rhabdomyosarcoma (median *ALK/CEN2p* = 7, Figure 5b), which was that of a post-chemotherapy metastatic tumor that primarily occurred in the finger of an adult man, and the tumor showed strong diffuse ALK expression. Low selective gain was identified in one alveolar and two embryonal tumors (Figure 5c), and all three cases were positive for ALK expression. Tumor cells with ≥ 4 *ALK* copies were significantly more common in ALK-positive tumors than in ALK-negative tumors ($P=0.013$, Figure 6). Among the 56 cases that showed no selective increase in the *ALK* copy number, 29 showed no polysomy, 20 showed low

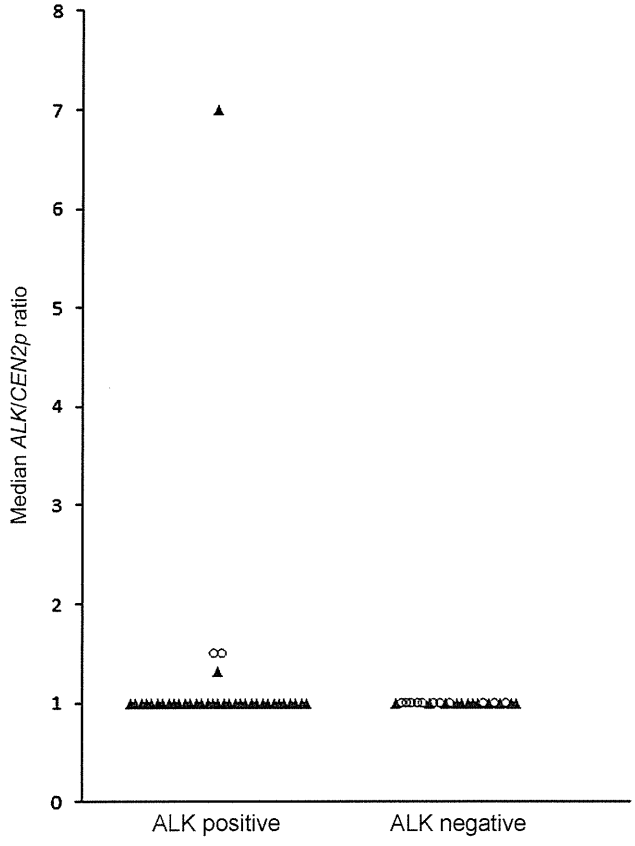

Figure 4 The median *ALK/CEN2p* ratio determined by FISH in 37 ALK-positive and 23 ALK-negative rhabdomyosarcomas. ▲ indicates the alveolar subtype, and ○ indicates non-alveolar subtypes. The vast majority (93%) of tumors showed a median *ALK/CEN2p* ratio of 1, and collectively, the ratio was not significantly different between the ALK-positive and ALK-negative groups ($P=0.106$). However, all four cases with a median *ALK/CEN2p* >1 were ALK immunopositive.

polysomy, and 7 showed high polysomy (Figure 5d). The latter pattern did not seem to merely represent the near-tetraploidy typical of alveolar rhabdomyosarcoma,[27,28] because six of the seven tumors with this pattern consisted of ≥10% cells with ≥5 *ALK* copies. All seven cases with the high-polysomy pattern were ALK immunopositive, while 26 ALK-positive and 23 ALK-negative tumors showed the no- or low-polysomy pattern. In summary, all 11 cases with selective *ALK* amplification, low selective gain, or high polysomy were ALK immunopositive. Overall, these genomic abnormalities were uncommon, accounting for 18% of the 60 rhabdomyosarcomas tested and 30% of the 37 ALK-positive rhabdomyosarcomas.

ALK Mutation Status

ALK mutation was examined in 34 cases, and the analysis was successful in 19 cases (7 ALK positive, 12 ALK negative). Only one case, a metastatic embryonal rhabdomyosarcoma in an adult male, showed a heterozygous somatic mutation (c.3830G>A, p.I1277T) of the receptor tyrosine kinase domain. This tumor was immunonegative for ALK and was located primarily in the heart. Functional evaluation of amino acid substitution using Polyphen2 and MutationAssessor predicted that this mutation may cause harmful effects (probably damaging or medium functional impact). This substitution is not found in the Catalog of Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/).

Discussion

Previous studies showed that alveolar rhabdomyosarcomas were more commonly immunopositive for ALK than other subtypes were (Table 4). Our series showed an even sharper difference in ALK reactivity between alveolar (69%) and other (4%) subtypes, which may be because of the differences in the staining protocol. Specifically, we used the 5A4 antibody and a polymer-based system, whereas most prior studies applied a more conventional method using the ALK1 antibody and the avidin-biotin system. Recent reports have suggested that ALK1/avidin-biotin method may result in suboptimal concordance between immunoreactivity and genomic status.[29–31] The differential ALK staining in rhabdomyosarcoma may carry diagnostic value. Subtyping rhabdomyosarcoma can be challenging, particularly when alveolar tumor shows a solid pattern and embryonal tumor shows dense cellularity without an apparent myxoid matrix.[32] In addition, sclerosing rhabdomyosarcoma may mimic the alveolar subtype.[4,6] Although *FOXO1* rearrangement assays may be useful for classification, immunohistochemical analysis is more accessible and cost effective. ALK staining has already gained wide application in diagnostic pathology, and optimized staining protocols like the one used here may become prevalent given the increased interest in ALK-targeted therapy. Our data suggest that ALK may be a useful marker for distinguishing the alveolar subtype from other subtypes of rhabdomyosarcomas, and this finding needs to be confirmed in a larger cohort.

ALK-positive and ALK-negative alveolar rhabdomyosarcomas were not clinicopathologically different in many respects, including overall survival. However, we found that ALK-positive cases presented with metastasis more commonly than ALK-negative cases. Although additional studies are needed for confirmation, it appears that ALK immunoreactivity may identify a subset of alveolar rhabdomyosarcoma that has a higher proclivity to metastatic spread. On histological examination, nuclear pleomorphism was exclusively observed in ALK-negative alveolar rhabdomyosarcomas, and ALK-negative alveolar cases harbored a smaller proportion of myogenin-positive cells than

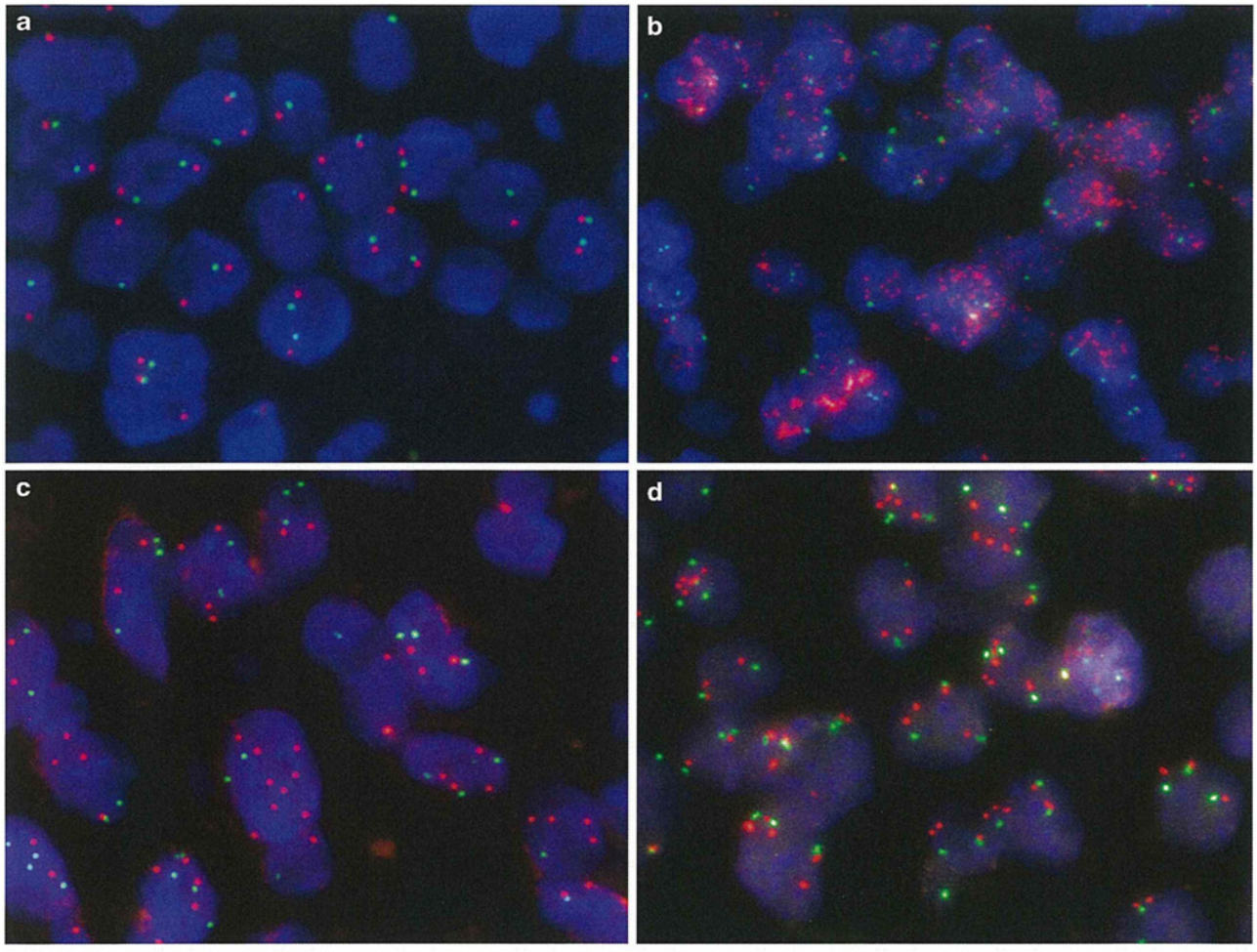

Figure 5 Changes in the *ALK* copy number in rhabdomyosarcomas determined by dual-color FISH. Red signals represent the *ALK* gene and green signals represent the reference sequence (*CEN2p*). (**a**) Most tumors tested showed no selective increase in *ALK* gene dosage (*ALK/CEN2p* = 1). (**b**) One tumor showed selective *ALK* amplification. (**c**) Three tumors showed low selective gain defined as 1 < median *ALK/CEN2p* < 2. (**d**) Seven tumors showed a high-polysomy pattern defined as ≥4 *ALK* copies in ≥40% cells with *ALK/CEN2p* = 1.

ALK-positive ones. Classic alveolar rhabdomyosarcoma is characterized by uniform cytology and a greater extent of myogenin staining compared with non-alveolar subtype.[25,33] Therefore, the presence of pleomorphic tumors and less extensive myogenin positivity in the ALK-negative alveolar cases may suggest that this subgroup includes non-classical examples. Some of these might be close to (or might actually represent) the non-alveolar subtype that is indistinguishable from alveolar rhabdomyosarcoma on the basis of morphological criteria alone.

ALK immunoreactivity and *FOXO1* status were strongly correlated in our cohort. This result is in agreement with several array-based analyses[34–37] in which *ALK* was found to be one of the most differentially expressed genes in *FOXO1* fusion-positive alveolar rhabdomyosarcoma as compared with fusion-negative alveolar and embryonal rhabdomyosarcoma. This association indicates that ALK may be a target of the PAX-FOXO1 chimeric protein. In support of this hypothesis, Davicioni *et al*[36] showed that *ALK* was upregulated when *PAX-FOXO1* was transfected into a fusion-negative cell line. Cao and associates[38] also showed that introducing small hairpin RNA against *PAX3-FOXO1* into a fusion-positive cell line down-regulated ALK expression. Further, the regulatory sequences of the *ALK* gene were shown to contain a high-affinity binding site to the PAX3-FOXO1 protein.[38] Although a previous study[20] found no association between *FOXO1* rearrangement and ALK immunoreactivity, this discordance may be owing to the difference in the number of cases studied, the ALK staining protocol used, and the molecular method employed to determine the gene rearrangement status.

The ALK immunoreactivity and *ALK* gene copy number were also found to be significantly correlated in our series. Notably, *ALK* selective amplification, low selective gain, and high polysomy were observed exclusively in ALK-immunopositive cases. Nevertheless, these significant copy number changes were relatively uncommon and were observed only in 30% of the ALK-positive tumors. In particular, true selective gene amplification was rare: it was observed in only 3% of the