의학박사 학위논문

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Prognosis prediction with immunohistochemical features and *MYCN* amplification in neuroblastoma

by

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Abstract

Introduction: Neuroblastoma is a childhood malignancy which showed highly heterogeneous clinical course. Many genetic and genomic features as well as clinical characteristics have been studied and incorporated in the modern prognostic classification of neuroblastoma to divide the risk groups. In this study, *MYCN* amplification, *anaplastic lymphoma kinase* (*ALK*) mutation and amplification, ALK protein expression, loss of the nuclear ATRX protein, and TERT protein expression were studied to investigate if there are any correlations between these molecular characteristics and clinical features or outcomes.

Materials and methods: Among the 104 patients who were histologically diagnosed with neuroblastoma and treated at Seoul National University Children's Hospital between January 2002 to July 2012, 72 patients whose initial tumor samples were evaluable were enrolled in this study. Formalin-fixed, paraffin-embedded tumor tissues obtained during surgery or biopsy were used. Mutation analysis for exons 23, 24, and 25 of the *ALK* gene was performed with PCR amplification and direct sequencing. Immunohistochemical staining was performed on 4-μm thick tissue microarray sections using an ALK monoclonal antibody, a polyclonal antibody against ATRX and a monoclonal anti-TERT antibody. Fluorescence *in situ* hybridization (FISH) was performed to investigate *MYCN* and *ALK*

amplification.

Results: Seven (10.0%) patients had *MYCN* amplification at initial diagnosis. Patients who had *MYCN* amplification were statistically younger than the other patients (1.6±0.8 years vs 3.1±3.7 years, P=0.010), and relapse rate was significantly higher in patients with *MYCN* amplification compared to the other patients (77.8% vs 19.4%, P<0.001). Forty (55.6%) patients showed ALK expression, and incidence ALK expression increased according to the increasing tumor stage (P=0.001). Relapse rate was significantly higher in ALK+ patients compared to the ALK- patients (47.5% vs 11.3%, P=0.007). *ALK* mutation was found only in 2 (4.1%) patients among 49 patients whose tumor samples were sufficient for DNA extraction, and *ALK* amplification was not found in any of the 65 patients whose tumor samples were evaluable for *ALK* FISH study. Nine (13.0%) patients showed loss of nuclear ATRX protein, and loss of nuclear ATRX protein was found in 2 different populations. One is the older, stage IV patients showing indolent disease course with ALK expression, and the other is young children with lower stage ALK- neuroblastomas having better prognosis. Patients without TERT expression seemed to be associated with high relapse rate, but there was no statistical significance.

Conclusion: Both *MYCN* amplification and ALK expression had strong prognostic significance in this study. Although *ALK* mutation was rare and no amplification was observed, ALK protein expression was found in significant

number of patients and was correlated with advanced stage neuroblastoma and poor outcome. With these results, *ALK* targeted therapy could be considered as a valid therapeutic strategy for relapsed/refractory neuroblastoma patients having ALK expression. Regarding the meaning of ATRX and TERT expression in neuroblastoma, further study is required.

Key words: Neuroblastoma, ALK, ATRX, TERT

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List of abbreviations and symbols

ALK anaplastic lymphoma kinase

ALT alternative lengthening of telomere

ATRX alpha thalassemia/mental retardation syndrome X-linked

FFPE formalin-fixed, paraffin-embedded

FISH fluorescence *in situ* hybridization

HDCT/aPBSCT high dose chemotherapy and autologous peripheral blood stem cell transplantation

IHC immunohistochemistry

INRG International Neuroblastoma Risk Group

INSS International Neuroblastoma Staging System

NPM nucleophosmin

PCR polymerase chain reaction

TERC telomerase RNA component

TERT telomerase reverse transcriptase

TMA tissue microarray

TRM treatment-related mortality

Introduction

Neuroblastoma is a childhood malignancy that arises from precursor cells of the sympathetic nervous system or the adrenal medulla. It is the most common extracranial solid tumor of childhood and accounts for 12% of all childhood cancer-related deaths [1, 2]. The clinical course is highly heterogeneous, ranging from spontaneous regression without any therapeutic intervention to very fatal cases showing rapid progression and death despite modern intensive multimodal treatment. Because of these clinical heterogeneities, different therapeutic approaches are needed in the treatment of neuroblastoma. Clinical features such as age at diagnosis and stage has been used to predict outcome and to divide the risk groups traditionally. In addition to these clinical characteristics, many genetic and genomic features have been studied and incorporated in the modern prognostic classification of neuroblastoma [3].

Amplification of *MYCN* oncogene was one of the first reported genetic markers for highly aggressive and advanced stage neuroblastoma [4]. *MYCN* amplification is known to be observed in approximately 20% of cases and remains a powerful prognostic factor indicating poor outcome [5]. Tumor cell DNA ploidy is also known to be independently correlated with the outcome, with near-triploidy associated with an excellent outcome and diploidy/tetraploidy correlating with a poorer outcome [6, 7]. With the

development of high-throughput techniques such as chromosomal or array CGH, numerous recurrent large-scale genomic imbalances, including loss of heterozygosity of chromosome regions 1p, 3p, and 11q, along with gain of chromosome 1q and 17q has been reported to be associated with poor outcome of neuroblastoma [8]. Recent International Neuroblastoma Risk Group (INRG) project reported that segmental chromosomal alterations, rather than the single genetic markers have prognostic impact in neuroblastoma [9].

Activating mutation of *anaplastic lymphoma kinase* (*ALK*) gene in neuroblastoma has been reported by several groups since 2008 [10-13]. ALK is a tyrosine receptor kinase which is encoded by *ALK* gene located on the short arm of chromosome 2 (2p23.2) [14]. The oncogenic potential of the ALK was first identified in anaplastic large cell lymphoma through the formation of a nucleophosmin (NPM)–ALK fusion protein with constitutive kinase activity by chromosomal translocation t(2;5)(p23;q35). One of the first reports of *ALK* in neuroblastoma was the germline *ALK* mutation in neuroblastoma pedigree. Mossé *et al* demonstrated that germline *ALK* mutations are responsible for the susceptibility to the development of hereditary neuroblastoma [13]. Subsequently, somatic *ALK* mutations have been reported in 6-12% of sporadic neuroblastoma and these mutations were located mostly within the tyrosine kinase domain [11]. In addition to point mutations, *ALK* amplification was reported in about 2–6 % of neuroblastoma

cases by several studies [15, 16]. The oncogenic potential of ALK was demonstrated by several *in vitro* and *in vivo* studies. The F1174L and R1275Q variants possessed the capacity to transform IL-3-dependent murine hematopoietic Ba/F3 cells to cytokine-independent growth, and two human neuroblastoma cell lines harboring the F1174L mutation were sensitive to the inhibitor [11]. In another study, the mutated kinases were autophosphorylated and displayed increased kinase activity showing the capacity to form tumors in nude mice [10].

Maintenance of telomere is essential for cancer cell survival. There are 2 different mechanisms of telomere maintenance, one is through increased expression of telomerases and the other is through the alternative lengthening of telomere (ALT) pathway [17, 18]. The former is related to the telomerase reverse transcriptase (TERT) expression, and the latter is known to be associated with *alpha thalassemia/mental retardation syndrome X-linked (ATRX)* gene mutation [17, 19, 20]. Recently, *ATRX* mutation was found especially in neuroblastoma of older children and adolescents. *ATRX* mutations have been associated with loss of the nuclear ATRX protein, longer telomeres and neuroblastomas without *MYCN* amplification. This mutation was related to indolent, but progressive course of neuroblastoma [21].

In this study, *MYCN* amplification, *anaplastic lymphoma kinase* (*ALK*) mutation and amplification, ALK protein expression, loss of the nuclear ATRX protein, and TERT protein expression were studied to investigate if

there are any correlations between these molecular characteristics and clinical features or outcomes.

Materials and methods

Patients and specimens

Patients who were histologically diagnosed with neuroblastoma and treated at Seoul National University Children's Hospital from January 2002 to July 2012 were enrolled in this study. A list of patients was obtained from the hospital's computerized database. A total of 104 patients were included initially, but 14 patients whose initial tumor samples were not available were excluded. After review of hematoxylin–eosin slides, additional 5 patients were excluded due to inadequate tumor specimen. Tissue microarray (TMA) was prepared with tumor samples of remaining 86 patients, and 13 patients were excluded again due to insufficient tumor specimen in TMA slides. Finally, 72 patients were evaluated in this study.

Clinical data were retrospectively collected including gender, age at diagnosis, tumor location, International Neuroblastoma Staging System (INSS) stage [1], treatment history, clinical follow-up, and final outcome.

Formalin-fixed, paraffin-embedded (FFPE) tumor tissue obtained during surgery or biopsy was used. For the patients with somatic mutation in their tumor tissue, peripheral blood was used to evaluate whether the mutations were somatic or germline mutation.

This study was approved by the Institutional Review Board of Seoul National University Hospital (H-1103-003-352).

Immunohistochemical staining

The hematoxylin–eosin sections were reviewed by pathologist, TMA was prepared from FFPE tissue of neuroblastoma sample. Immunohistochemical (IHC) staining was performed on 4-μm thick TMA sections using an ALK monoclonal antibody (Novocastra, Leica Biosystems; Newcastle, UK), a polyclonal antibody against ATRX (Sigma-Aldrich Corporation; St. Louis, Missouri, USA) and a monoclonal mouse anti-TERT antibody (Novus Biologicals; Littleton, Colorado, USA). The TMA sections were deparaffinized and rehydrated, and heat-induced epitope retrieval was performed by heating in Tris–EDTA buffer, pH 9.0, at 100℃ for 15 minutes. The intensity (0-3+) of positive cells were counted and scored by pathologist. For ALK and TERT protein expression, a staining intensity score of 2 or more was defined as positive. Loss of the nuclear ATRX protein was defined as a staining intensity score of 0 or 1.

Fluorescence *in situ* hybridization (FISH)

FISH was performed on unstained TMA section to investigate *MYCN* and *ALK* amplification. Probes used in this study included *MYCN* (2p24.1; orange; Vysis, Downers Grove, IL, USA), CEP2 (2p11.1-q11.1; green; Vysis, Downers Grove, IL, USA) and an *ALK* Dual Color Break Apart rearrangement Probe (2p23; Vysis, Abbott Laboratories, Abbott Park, IL, USA). Values for each signal and the ratios of red/green signals were reported in at least 50 non-overlapping nuclei per specimen. *MYCN/ALK* amplification was defined as over 4-fold increase of the *MYCN/ALK* signal number in relation to the number of control probe signals according to the previous studies [3, 22, 23].

Mutation analysis

The genomic DNA was extracted from FFPE tumor tissue. Three to five 10 μm thick sections were used for DNA extraction. For samples in which tumor contents were below 70%, tumor area was selectively scraped from the slides. Paraffin was removed from FFPE sections using xylene followed by ethanol washes, and then tissue was lysed with lysis buffer and proteinase K overnight at 56℃. Lysate was incubated at 90℃ for 15 minutes to remove DNA crosslinks, and then genomic DNA was extracted using QIAamp DNA FFPE Tissue Kit (QIAGEN; Hilden, Germany). For the DNA extraction from

peripheral blood lymphocytes, the QIAamp DNA Mini Kit (QIAGEN; Hilden, Germany) was used.

Polymerase chain reaction (PCR) amplification for exons 23, 24, and 25 of the *ALK* gene was performed. *ALK* PCR was performed in a 20 uL reaction mixture containing 5 uL of extracted template DNA, 2 uL of 10 pmol primers, and 10 uL of HotStarTaq Master Mix Kit (QIAGEN; Hilden, Germany). PCR products were subjected to electrophoresis on 2% agarose gels and were purified using a QIAquick PCR purification kit (QIAGEN; Hilden, Germany). Direct sequencing was performed using the BigDye(R) Terminator v3.1 Cycle Sequencing kits (Applied Biosystems, Foster City, USA) on the ABI PRISM 3730XL Analyzer (Applied Biosystems, Foster City, USA). The primer sets used in this study are listed in Table 1.

Table 1. Primer sequences and PCR conditions

Exon		Primer sequences	Annealing temperature (℃)
ALK exon 23	F	AGATTTGCCCAGACTCAGCTC	58
	R	GGTCTCTCGGAGGAAGGACT	
ALK exon 24	F	CTTCTGTCTCCCCACAGAGC	58
	R	AAGCACACAGATCAGCGACA	
ALK exon 25	F	TGATGGCCGTTGTACACTCA	58
	R	CTGAGGTGGAAGAGACAGGC	

Statistical analysis

Differences between categorical variables were measured by chi-square test or Fisher's exact test, and differences between means in continuous variables were calculated with student *T*-test. Kaplan-Meier method and log-rank univariate comparisons were used to estimate survival. Survival analysis was done except 3 patients who did not have sufficient follow-up duration after initial diagnosis because of loss to follow-up. SPSS version 21.0 was used for all statistical analyses, and statistical significance was accepted when $P<0.05$.

Results

Clinical characteristics

A total of 72 patients were enrolled in this study. The clinical characteristics of the patients were summarized in Table 2. The median age was 2.0 years (0.0-16.7 years). Primary sites were retroperitoneum in 55 (76.4%) patients, posterior mediastinum in 15 (20.8%), and neck in 2 (2.8%) patients.

Treatment was decided according to the patient's age, stage, and *MYCN* status. General treatment guideline was as follows: stage I, local surgery; stage II, local surgery ± chemotherapy; stage III, chemotherapy + local surgery ± radiotherapy ± Interleukin-2/isotretinoin; stage III with residual disease and stage IV (≥ 1 year or with *MYCN* amplification), chemotherapy + local surgery + high dose chemotherapy and autologous peripheral blood stem cell transplantation (HDCT/aPBSCT) + radiotherapy + Interleukin-2/isotretinoin; stage III with residual disease and stage IV (< 1 year without *MYCN* amplification), chemotherapy + local surgery ± radiotherapy ± isotretinoin. One patient with stage IVs underwent HDCT/aPBSCT because of *MYCN* amplification.

Sixteen patients relapsed showing 29.3% of relapse rate, and treatment-related mortality (TRM) occurred in 3 patients. Clinical and molecular characteristics of relapsed patients were summarized in Table 3. Median time to relapse from diagnosis was 23.6 months (3.4-83.7 months), and median

time to death from relapse was 5.6 months (1.2-55.3 months) in the 13 patients who died of disease.

Table 2. Characteristics of patients

Age, Median (range), months	2.0 (0.0-16.7)
Sex, No. (%)	
Male	36 (50.0)
Female	36 (50.0)
Primary site, No. (%)	
Abdomen	55 (76.4)
Posterior mediastinum	15 (20.8)
Neck	2 (2.8)
INSS stage, No. (%)	
Stage I	16 (22.2)
Stage II	8 (11.1)
Stage III	16 (22.2)
Stage IV	31 (43.1)
Stage IVs	1 (1.4)
***MYCN* amplification, No. (%)**	
Positive	7/70 (10.0)
Negative	63/70 (90.0)
ALK IHC, No. (%)	
Positive	40 (55.6)
Negative	32 (44.4)
ATRX IHC, No. (%)	
Loss	9/69 (13.0)
No loss	60/69 (87.0)
TERT IHC, No. (%)	
Positive	42/71 (59.2)
Negative	29/71 (40.8)

MYCN amplification

MYCN amplification was evaluable in 70 tumor samples. Seven (10.0%) patients had *MYCN* amplification at initial diagnosis. Patients who had *MYCN* amplification were statistically younger than the other patients (1.6±0.8 years vs 3.1±3.7 years, *P*=0.010). Among the 7 patients, 6 patients were stage IV, and 1 patient had stage IVs disease. All the patients with *MYCN* amplification showed ALK+ by IHC and loss of ATRX protein was not observed in these patients. TERT expression was not associated with *MYCN* amplification. Relapse rate was significantly higher in patients with *MYCN* amplification compared to the other patients (77.8% vs 19.4%, *P*<0.001, Figure 1). Among the relapsed patients, time to relapse from diagnosis was significantly shorter in patients with *MYCN* amplification (11.7±6.9 months) compared to that of the other patients without *MYCN* amplification (33.7±20.8 months) (*P*=0.009).

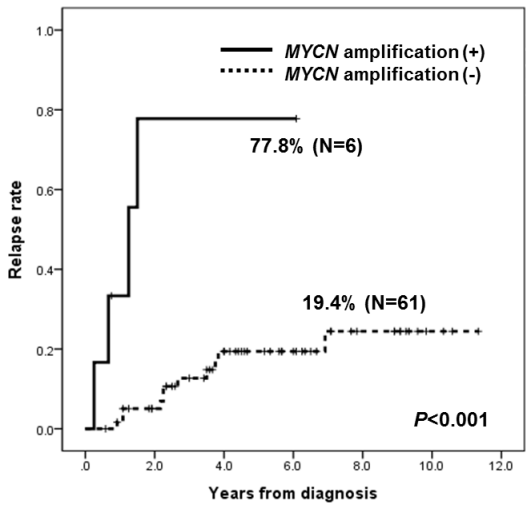

Figure 1. Association of *MYCN* amplification with relapse rate. Relapse rate was significantly higher in patients with *MYCN* amplification compared to the other patients.

Table 3. Summary of clinical and molecular characteristics of relapsed patients

Patient No.	Sex	Age at diagnosis (years)	INSS Stage	*MYCN* amplification	ALK IHC	ATRX IHC	TERT IHC	Time to relapse (months)	Outcome, survival time from relapse (months)
4	M	3.2	IV	-	-	No loss	-	45.1	AWD, 90.4
6	M	9.8	III	-	-	No loss	+	42.5	DOD, 42.0
23	M	12.2	IV	-	+	Loss	+	46.5	DOD, 55.2
27	M	1.5	IV	-	+	No loss	-	83.7	AWD, 1.0
28	M	7.0	III	-	-	No loss	+	11.8	DOD, 3.7
31	M	7.6	IV	-	+	No loss	-	32.8	DOD, 20.4
33	F	5.3	IV	NA	+	No loss	-	21.4	DOD, 1.9
37	F	0.5	IV	+	+	No loss	+	3.4	DOD, 1.2
38	F	3.6	IV	-	+	Loss	-	27.9	DOD, 55.3
47	M	4.6	IV	-	+	Loss	+	25.9	DOD, 5.6
50	M	2.0	IV	+	+	No loss	+	18.8	DOD, 25.5
67	M	3.3	IV	-	+	No loss	+	27.5	AWD, 17.4
72	M	2.3	IV	+	+	No loss	-	9.0	DOD, 5.8
75	F	1.9	IV	+	+	No loss	-	15.6	DOD, 4.3
76	F	2.7	IV	-	+	No loss	-	13.5	DOD, 1.8
81	M	4.4	IV	-	+	No loss	+	13.2	DOD, 2.2

AWD, alive with disease; DOD, died of disease; F, Female; INSS, International Neuroblastoma Staging System; NA, not assessable

ALK protein expression

ALK protein expression was evaluable in 72 tumor samples, and 40 (55.6%) patients showed ALK+. Incidence of ALK+ increased according to the increasing tumor stage (*P*=0.001) (Table 4). There was no difference of patient's age between the ALK- and ALK+ patients (3.0±3.9 years vs 3.1±3.2 years, *P*=0.88). ALK+ was not associated with loss of nuclear ATRX protein or TERT protein expression.

Relapse rate was significantly higher in ALK+ patients compared to the other patients (47.5% vs 11.3%, *P*=0.007) (Figure 2-A). Three patients with ALK- relapsed, and 2 of them had stage III diseases. When the survival analysis was limited to the stage III-IV disease, the relapse rate of ALK+ patients had tendency to be higher compared to that of ALK- patients (54.5% vs 24.3%, *P*=0.124) (Figure 2-B).

Table 4. ALK expression according to stage

ALK expression by IHC	INSS stage			
	I	II	III	IV
Positive, No. (%)	4 (25.0)	3 (37.5)	7 (43.8)	25 (80.6)
Negative, No. (%)	12 (75.0)	5 (62.5)	9 (56.3)	6 (19.4)

IHC, immunohistochemistry; INSS, International Neuroblastoma Staging System

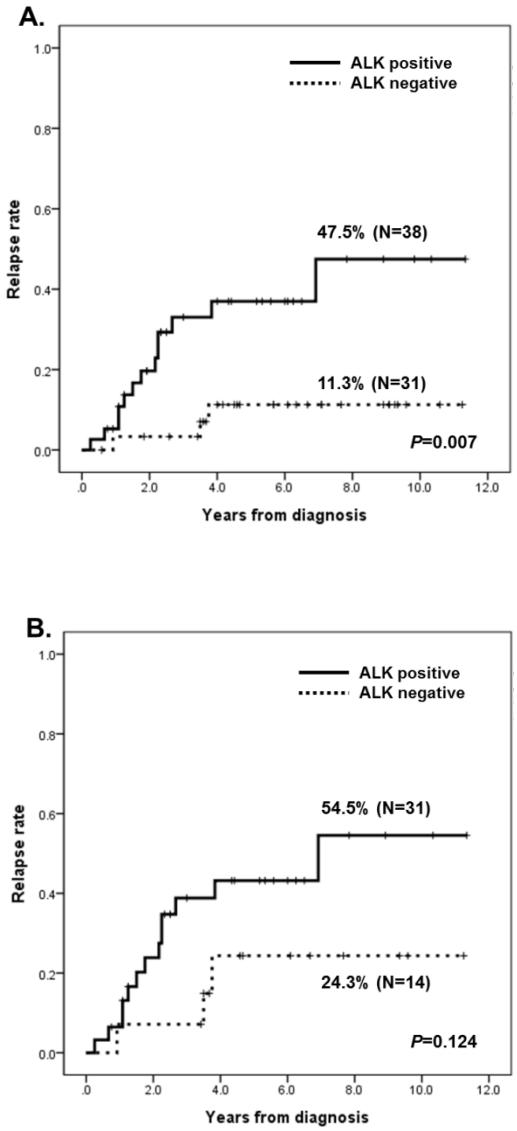

Figure 2. Association of ALK expression with relapse rate. A) Relapse rate was significantly higher in ALK+ patients compared to the ALK- patients. B) Relapse rate of ALK+ patients had tendency to be higher compared to that of ALK- patients in stage III-IV diseases.

ALK amplification and mutation

ALK amplification was evaluable in 65 patients, but amplification was not observed in any of them.

ALK mutation study was done in 49 patients whose tumor samples were sufficient for DNA extraction. DNA sequencing of *ALK* revealed 2 missense mutations (F1174L and R1275Q) in 2 patients (No. 47 and 75) (Figure 3). Sequence analyses of the constitutional DNA from peripheral blood showed that both were somatically acquired mutations.

One patient (No. 47) who carried a R1275Q mutation was a 4.6-year-old male who was diagnosed as INSS stage IV, *MYCN*-non-amplified neuroblastoma originating from the adrenal medulla with metastasis at bone, bone marrow and lymph nodes. ALK IHC result was positive. Even after induction chemotherapy, significant decrease of tumor volume was not achieved showing stable disease. HDCT/aPBSCT could not be done because of persistent bone marrow involvement. Chemotherapy regimen was changed several times, but the patient progressed eventually and died of disease 2.6 years after initial diagnosis.

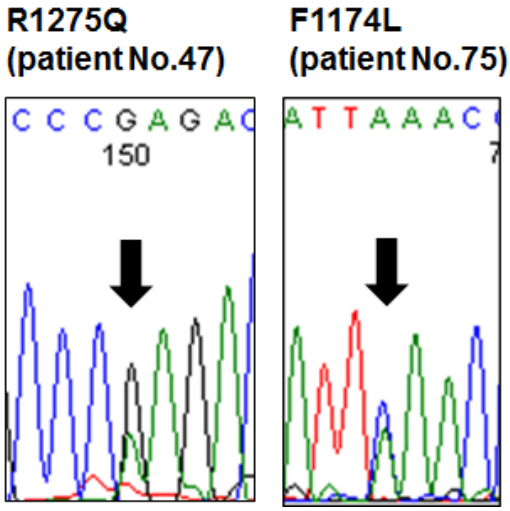

Figure 3. Detection of ALK mutation. DNA sequencing of *ALK* revealed 2 missense mutations (F1174L and R1275Q) in 2 patients (No. 47 and 75).

The other patient who had F1174L (No.75) was a 1.9-year-old girl who was diagnosed as a poorly differentiated neuroblastoma with *MYCN* amplification and ALK+ by IHC. INSS stage was stage IV with metastasis at bone, bone marrow and supraclavicular lymph node. After induction chemotherapy, tandem HDCT/aPBSCT was done, followed by radiotherapy to the primary site and interleukin-2/isotretinoin treatment. The patient relapsed 3 months after second HDCT/aPBSCT and died of disease showing rapid progression despite the salvage chemotherapy.

Loss of nuclear ATRX protein

ATRX expression was evaluable in 69 tumor samples. Nine (13.0%) patients showed loss of nuclear ATRX protein. Clinical and molecular characteristics of these 9 patients were summarized in Table 5. *MYCN* amplification was not observed in any of them.

Among these 9 patients, 5 patients had stage I-III neuroblastoma, and all of them are alive without disease. Except 1 patient (No. 86), the age of the other 4 patients with stage I-III disease were less than 3 years and all showed ALK- by IHC.

Four patients had stage IV diseases, and all of them were older than 3 years, and 2 were older than 10 years. All these 4 patients showed ALK+ by IHC, and 3 of them relapsed. Times to relapse in these 3 patients were 25.9, 27.9 and 46.5 months respectively and 2 of them (No. 23 and No.38) survived more than 4 years even after relapse showing indolent disease course.

One patient (No. 23) was a 12.2-year-old male diagnosed as INSS stage IV, *MYCN*-non-amplified neuroblastoma. HDCT/aPBSCT was planned after induction chemotherapy, but it could not be done because of insufficient stem cell mobilization. He relapsed at 45.6 months after initial diagnosis and underwent salvage chemotherapy. During the salvage chemotherapy he showed stable disease for a long time, but eventually progressed and died at 55.2 months after relapse. The other patient (No. 38) was a 3.6-year-old girl

with INSS stage IV *MYCN*-non-amplified neuroblastoma. After induction chemotherapy, tandem HDCT/aPBSCT was done, followed by radiotherapy to the primary site and interleukin-2/isotretinoin treatment. The patient relapsed 16 months after second HDCT/aPBSCT and underwent salvage chemotherapy. Myelodysplastic syndrome developed concurrently during the salvage chemotherapy, and it made the patient difficult to undergo chemotherapy anymore. Related peripheral stem cell transplantation was done for treatment of myelodysplastic syndrome and neuroblastoma, but the patient relapsed again with metastasis at cerebellum, bone and bone marrow. The patient eventually died after 55.3 months after relapse.

Table 5. Summary of clinical and molecular characteristics of patients with complete or partial loss of nuclear ATRX protein

Patient No.	Age at diagnosis (years)	INSS Stage	*MYCN* amplification	ALK IHC	ATRX IHC	TERT IHC	Outcome
13	1.3	I	-	-	Loss	+	NED
17	1.9	I	-	-	Loss	+	NED
86	12.9	I	-	+	Loss	-	NED
21	0.2	II	-	-	Loss	+	NED
26	2.2	III	-	-	Loss	+	NED
23	12.2	IV	-	+	Loss	+	DOD
38	3.6	IV	-	+	Loss	-	DOD
39	10.8	IV	-	+	Loss	-	NED
47	4.6	IV	-	+	Loss	+	DOD

AWD, alive with disease; DOD, died of disease; IHC, immunohistochemistry; INSS, International Neuroblastoma Staging System; NED, no evidence of disease

TERT protein expression

TERT protein expression was evaluable in 71 tumor samples, and 42 (59.2%) patients had TERT+. Incidence of TERT expression was not different according to the tumor stage. TERT+ patients were younger than the other patients, but there was no statistical significance (2.2±2.7 years vs 4.1±4.3 years, *P*=0.052). TERT+ was not associated with loss of nuclear ATRX protein or ALK protein expression.

Relapse rate was slightly higher in TERT- patients compared to the TERT+ patients without statistical significance (45.5% vs 21.5%, *P*=0.240) (Figure 4-A). When the survival analysis was limited to the stage III-IV disease, the relapse rate of TERT- patients had tendency to be higher compared to that of TERT+ patients (63.8% vs 31.6%, *P*=0.225) (Figure 4-B).

Table 6. TERT expression according to stage

TERT expression by IHC	INSS stage			
	I	II	III	IV
Positive, No. (%)	8 (50.0)	5 (62.5)	11 (68.8)	17 (56.7)
Negative, No. (%)	8 (50.0)	3 (37.5)	5 (31.3)	13 (43.3)

IHC, immunohistochemistry; INSS, International Neuroblastoma Staging System

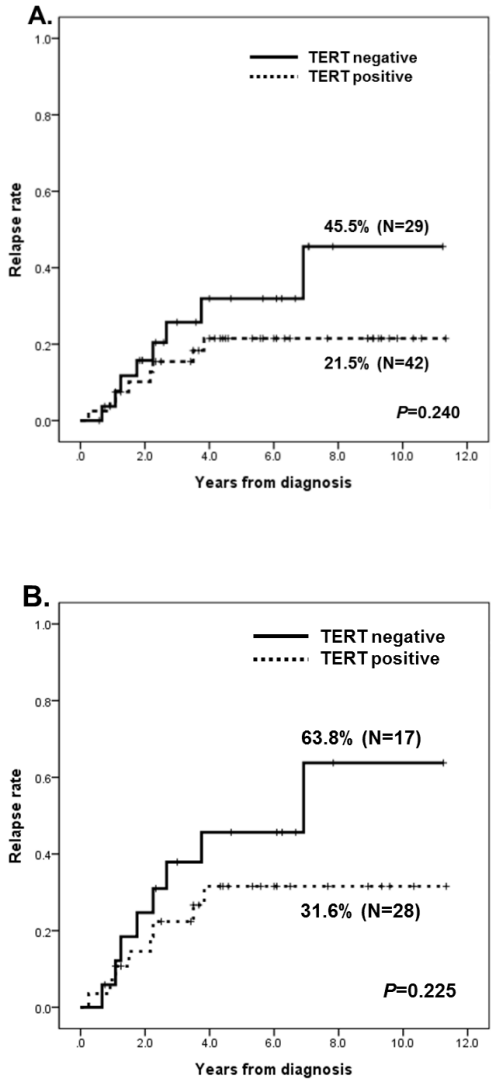

Figure 4. Association of TERT expression with relapse rate. A) Relapse rate was slightly higher in TERT- patients compared to the TERT+ patients without statistical significance. B) When limited to the stage III-IV disease, the relapse rate of TERT- patients had tendency to be higher compared to that of TERT+ patients.

Combination of IHC features and *MYCN* amplification

Among the 69 patients whose survival analysis was possible, all three IHC markers (ALK, ATRX and TERT) and *MYCN* amplification were evaluable in 63 patients. Relapse rate of each group according to the IHC markers and *MYCN* amplification was estimated by Kaplan-Meier analysis and illustrated in figure 5-A. In general, patients with ALK expression showed high relapse rate. Among the ALK+ patients, only patients with TERT+ without *MYCN* amplification or ATRX loss showed good prognosis. When excluding the patients with *MYCN* amplification or ATRX loss, patients with ALK+/TERT+ seemed to have better prognosis compared to the ALK+/TERT- patients, although this difference was not statistically significant in survival graph (15.6% vs 65.0%, P=0.246) (Figure 5-B).

From the point of view of TERT expression, TERT expression itself seemed to have no prognostic significance if the tumor does not have other IHC or molecular markers such as *MYCN* amplification, ATRX loss or ALK expression. Patients with ATRX loss showed poor prognosis in case of ALK+ and patients with ATRX loss without ALK expression showed excellent prognosis.

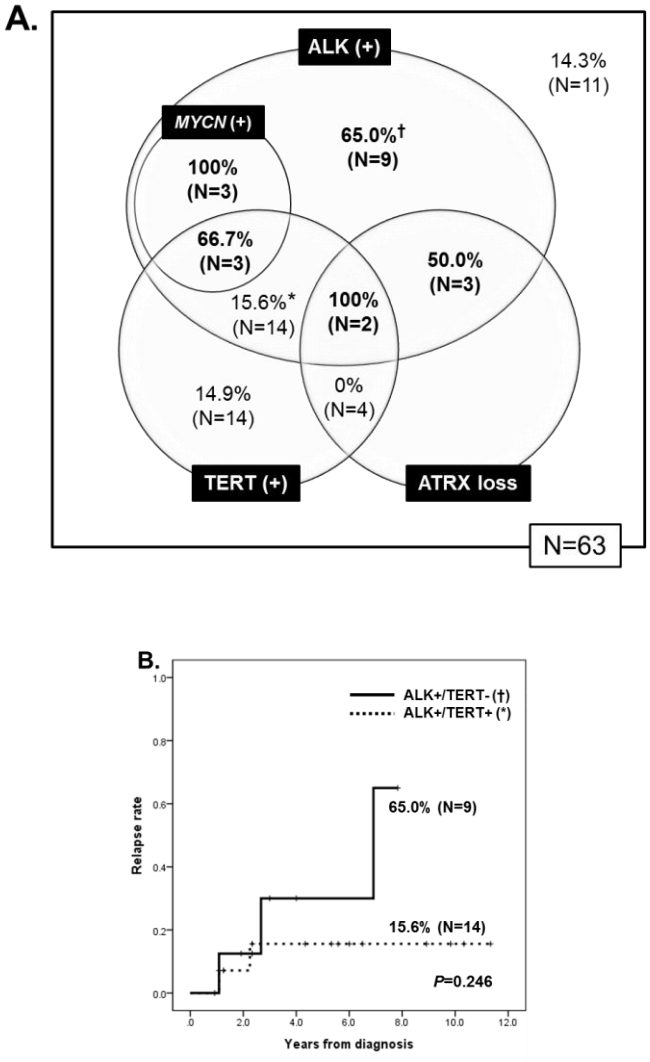

Figure 5. Relapse rate according to the combination of IHC features and *MYCN* amplification. A) Relapse rate of each group was estimated by Kaplan-Meier analysis and the survival rate was illustrated. B) When excluding the patients with *MYCN* amplification or ATRX loss, patients with ALK+/TERT+(*) seemed to have better prognosis compared to the ALK+/TERT-(†) patients.

Discussion

In this study, *MYCN* amplification, *ALK* mutation and amplification, ALK protein expression, loss of the nuclear ATRX protein, and TERT protein expression were studied to investigate if there are any correlations between these molecular characteristics and clinical features or outcomes. *MYCN* amplification was related to high relapse rate and early relapse. *ALK* mutation was found in small population and no amplification was observed in this group, but ALK expression was observed in significant number of patients and ALK expression was correlated with advanced stage neuroblastoma and poor outcome. Loss of ATRX protein was observed in 2 different populations.

MYCN amplification is well known prognostic marker in neuroblastoma. In many studies, *MYCN* amplification has been shown to be strongly associated with rapid tumor progression and poor prognosis in patients of all ages, with any stage of disease [4, 24, 25]. The predictive value of *MYCN* amplification was also confirmed in the INRG cohort of patients showing worse outcome in the *MYCN*-amplified neuroblastoma patients [26]. Along with these previous studies, *MYCN* amplification had strong prognostic significance in this study showing high relapse rate in *MYCN*-amplified neuroblastoma patients. Also, time to relapse was significantly shorter in the *MYCN*-amplified neuroblastoma patients showing the rapid tumor progression. In this study, all the patients with *MYCN* amplification showed ALK+ by IHC.

Correlation between ALK and *MYCN* in neuroblastoma was investigated in a few studies. Schönherr C. *et al* showed that *ALK* activity was important to initiate the transcription of *MYCN* and *MYCN* gene transcription was eliminated by specific ALK inhibitor [27]. This ALK-induced transcription of *MYCN* was found to be mediated by ERK5 (Extracellular signal-regulated kinase 5) in recent published study [28]. These findings support the finding of this study that all of the *MYCN*-amplified neuroblastoma had ALK protein expression.

ALK is a 200 kDa receptor tyrosine kinase that is a member of the insulin receptor superfamily. The normal function of ALK protein is not yet fully identified. ALK is expressed during the early differentiation of neuronal tissues, and high levels of ALK have been measured in fetal neuroblasts [14, 29]. ALK is a dependence receptor that ALK expression enhances apoptosis via its own cleavage by caspases in the absence of ligand, and increased kinase activity of ALK either due to constitutive activation as a chimeric fusion gene or greater ligand availability may lead to increased cell survival and decreased apoptosis [30, 31].

Activating mutation of *ALK* in neuroblastoma has been studied since the first report showing germline *ALK* mutation in neuroblastoma pedigree [13]. Subsequently, somatic *ALK* mutations and *ALK* amplification have been reported in sporadic neuroblastoma [11, 15, 16]. Constitutive *ALK* activation in *ALK*-mutated neuroblastoma has been studied in several studies. Previous

studies showed that neuroblastoma-derived cell lines harboring mutated *ALK* alleles exhibit constitutive ALK phosphorylation [12, 13]. In another study, the mutated kinases were autophosphorylated and displayed increased kinase activity showing the capacity to form tumors in nude mice confirming the oncogenic potential of ALK [10]. They also demonstrated that downregulation of *ALK* through RNA interference suppresses proliferation of neuroblastoma cells harboring mutated ALK, which suggested the possible role of ALK kinase inhibitors in *ALK*-mutated neuroblastoma [10].

ALK expression in neuroblastoma was also investigated in some studies. Overexpression of ALK in cancer has been well known in various cancers such as thyroid carcinoma, non-small cell lung cancer, breast cancer, melanoma, neuroblastoma, glioblastoma, astrocytoma, retinoblastoma, Ewing sarcoma and rhabdomyosarcoma [32, 33], but the mechanisms of high ALK expression for the pathogenesis of these cancers are unclear. In neuroblastoma, germline promotor mutations affecting ALK expression or the presence of activating ligands were studied, but underlying molecular mechanisms of ALK expression still undisclosed [34, 35]. Irrespective of the mechanism, functional consequence of ALK expression was investigated in several studies. Passoni, L. *et al* observed the aberrant ALK phosphorylation not only in cell line carrying the R1275Q mutation but also in high ALK expressing cell line carrying a wild-type *ALK* [35]. More interestingly, they found that small molecule inhibitor targeting ALK exerted potent cytotoxic effect on high-

ALK expressing wild type cells [35]. In one study investigating transcriptomic characteristics, high-ALK expressing neuroblastoma showed a similar global gene expression patterns with neuroblastoma with mutated *ALK* [36]. The molecular mechanism responsible for ALK overexpression need further study including post-transcriptional modification, but these findings suggest a positive correlation between ALK abundance and constitutive *ALK* activation.

Correlation between ALK expression and clinical characteristics or treatment outcome in neuroblastoma has been evaluated in a few studies. In previous 2 studies, *ALK* mRNA levels in neuroblastoma samples were correlated with inferior survival [36, 37]. Schulte *et al*. showed that elevated *ALK* mRNA levels are associated with an unfavorable neuroblastoma phenotype independent of the genomic *ALK* status, indicating a role of elevated ALK expression in the development of aggressive neuroblastoma [36]. Also, high percentages of ALK-positive cells by IHC staining in neuroblastoma were found to be able to predict poor outcome in other studies [35, 37, 38].

ALK oncogene has been considered as a promising therapeutic target in a subset of human malignancy [39-41]. Crizotinib, a small molecule competitive inhibitor of ALK and MET kinase activity has been studied in non-small-cell lung cancer showing high response rate in chemotherapy-refractory patients [42, 43]. In recently reported phase III study, crizotinib

showed superior outcome compared to standard chemotherapy in patients with previous treated, advanced non-small-cell lung cancer with *ALK* rearrangement [44]. Crizotinib has also shown efficacy in preclinical models of neuroblastoma and anaplastic large cell lymphoma that express translocated, mutated, or amplified *ALK* [45, 46]. With these previous studies, phase I study with crizotinib for pediatric patients with refractory solid tumors or anaplastic large cell lymphoma was conducted, and the outcome was recently reported [47]. In this phase I study, 11 patients with *ALK* mutated neuroblastoma were treated, and 1 patient showed complete response and 2 had stable diseases. The other 23 patients with unknown *ALK* status were enrolled in this study, and complete response was achieved in 1 patient and stable disease in 5 patients [47]. This study is still ongoing as a phase I/II study (NCT00939770), and another ALK inhibitor, LDK378, is being studied as a phase I study for pediatric patients with malignancies having genetic alteration in *ALK* (NCT 01742286).

In this study, *ALK* mutation was found in 2 (4.1%) of 49 patients, and *ALK* amplification was not observed. The incidence of *ALK* mutation was relatively lower compared to the previous reports, but it is partly because of the method detecting the mutation. In this study, only 3 exons harboring mutational hotspots were investigated using PCR and direct sequencing. On the other hand, ALK expression was observed in 40 (55.6%) patients, and it was more frequent in advanced stage neuroblastoma. Also, relapse rate was

significantly higher in ALK+ patients compared to the ALK- patients. These finding could suggest the possible role of ALK expression on the pathogenic mechanism of aggressive neuroblastoma along with the previous studies indicating the importance of elevated ALK expression irrespective of genetic *ALK* status such as *ALK* mutation or amplification [35, 36]. Relapsed or refractory neuroblastoma shows dismal prognosis and there is no effective treatment option to date. Molecular targeted therapy could be one option for this group of patients. Mutation at the *ALK* locus has been found in a limited number of cases, and incidence of focal amplification is also very low. Therefore, *ALK* targeted therapy could be justified as a valid therapeutic strategy for neuroblastoma patients having an elevated level of expression of either wild-type or mutated *ALK*.

Association between telomere length and tumor prognosis has been studied in various types of cancers. Cancer cells must maintain telomeres for survival [17], and there are 2 mechanisms to maintain telomere. One is through increased expression of telomerase, a specialized reverse transcriptase that synthesizes the tandem TTAGGG repeat sequence using its own RNA subunit as a template [48, 49]. Reactivation of telomerase is known to be present in up to 90% of human cancers, and it allows cancer cells to maintain telomere length [50]. In neuroblastoma, telomere length itself was studied as a significant prognostic parameter in a study showing that short telomeres were predictive of a favorable prognosis, whereas long or unchanged telomeres

were predictive of a poor outcome. [51]. Telomerase activity in neuroblastoma also has been evaluated in a few studies, and high telomerase activity was found in 30% of neuroblastomas at diagnosis and was predictive of lower event-free survival and overall survival in 2 previous studies [52, 53]. TERT is a catalytic subunit of telomerase, which comprises the most important unit of telomerase complex together with the telomerase RNA component (TERC) [17]. From the beginning of 2013, mutations in *TERT* promotor were found in sporadic and familial melanoma and subsequently in several caners [50]. And the biological assessment of the functional consequence of these mutations revealed that their presence results in a two to fourfold increase in telomerase expression [20, 54].

In this study, TERT was assessed by IHC staining. Although there is no statistical significance, relapse rate was slightly higher in TERT- patients compared to the TERT+ patients. In the analysis considering the IHC markers and *MYCN* amplification together, patients with ALK+/TERT+ seemed to have better prognosis compared to the ALK+/TERT- patients when excluding the patients with *MYCN* amplification or ATRX loss, but still there was no statistical significance. If the tumor does not have other IHC or molecular markers such as *MYCN* amplification, TERT expression itself seemed to have no prognostic significance. TERT protein expression in predicting prognosis has been reported in many malignancies, such as chondrosarcoma, lung cancer and colorectal carcinoma [55-59]. In some studies, TERT expression

was associated with poor prognosis, but in others TERT expression was not exactly correlated with telomerase activity or it was not associated with survival [55, 59]. In this study, TERT expression seemed to be inversely correlated with survival showing better prognosis in TERT+ patients, but this results need to be interpreted carefully because the results were not statistically significant, and further study is needed to draw any conclusion.

One another mechanism of telomere maintenance is through a non-telomerase-dependent mechanism, known as ALT. *ATRX* gene is located on chromosome Xq21.1 and encodes a protein that belongs to the H3.3-ATRX-DAXX chromatin remodeling pathway [60], which functions as a histone chaperone complex for the deposition of the histone variant H3.3 into pericentric, telomeric, and ribosomal repeat sequences [49]. A recent study has documented that ATRX is lost in 90% of *in vitro* immortalized ALT cell lines [61]. Furthermore, mutations in *ATRX* or *DAXX* have been identified in many tumors exhibiting ALT, including pancreatic neuroendocrine tumors, neuroblastomas, pediatric glioblastomas, oligodendrogliomas, and medulloblastomas [19, 61, 62]. These findings suggest that ATRX acts as a suppressor of the ALT pathway, and mutation of *ATRX* is correlated with cancer cell survival through ALT pathway. In the first report about *ATRX* in neuroblastoma [21], *ATRX* mutation was found in 44% of tumors from adolescent and young adult patients with metastatic neuroblastoma, and none of the tumors from infants with metastatic neuroblastoma. The children whose

tumors had *ATRX* mutations were typically older than 5 years or had a chronic or indolent course of disease. The *ATRX* mutations were mutually exclusive of *MYCN* amplification, and associated with loss of the nuclear ATRX protein, longer telomeres, and ALT [21].

In this study, ATRX expression was assessed by IHC staining. Unlike the previous report evaluating ATRX only in metastatic neuroblastoma [21], ATRX expression was studied for stage I-IV patients in this study. Nine (13.0%) patients had loss of nuclear ATRX protein, and none of them had *MYCN* amplification alike the previous study. Among the 4 stage IV patients, 2 patients were older than 10 years at diagnosis, and showed very indolent disease course showing late relapse and survival more than 4 years even after the relapse. Interestingly, loss of ATRX protein was found not only in stage IV patients, but also in 5 patients with stage I-III neuroblastoma, and the age of these lower-stage patients was younger than that of stage IV disease with the youngest patient of 0.2-year old. These lower-stage patients showed ALK- by IHC except 1 patient whose age was older than 10 years. This result was interesting considering that the all of the stage IV patients with loss of ATRX protein had ALK+. This finding suggests that there could be 2 different populations with loss of ATRX protein, one is older patients showing indolent disease course with ALK expression, and the other is young children with lower stage and ALK- neuroblastomas having better prognosis.

Combined analysis considering 3 IHC markers (ALK, ATRX and TERT)

and *MYCN* amplification together was done in this study. In spite of the generally poor prognosis of ALK+ patients, ALK+/TERT+ patients without *MYCN* amplification or ATRX loss showed good prognosis. TERT expression itself seemed to have no prognostic significance if the tumor does not have any other markers. Patients with ATRX loss showed poor prognosis in case of ALK+, but patients with ATRX loss without ALK expression showed excellent prognosis. Any definite conclusion could not be made with these results because of small number of patients per each group, but these findings suggest that more precise prediction of prognosis could be possible with the combination of these IHC markers and *MYCN* amplification.

In summary, *MYCN* amplification had strong prognostic significance in this study showing the correlation with high relapse rate and early relapse. Although only small population of patients had *ALK* mutation and no amplification was observed, ALK protein expression was found in larger group of patients, and ALK expression was correlated with advanced stage neuroblastoma and poor outcome. Loss of ATRX protein was observed in 2 different populations and it was related to ALK expression in this study. With these results, *ALK* targeted therapy could be considered as a valid therapeutic strategy for relapsed/refractory neuroblastoma patients having ALK expression. Regarding the meaning of ATRX and TERT expression in neuroblastoma, further study is required.

References

1. Maris JM, Hogarty MD, Bagatell R, *et al.* Neuroblastoma. *Lancet* 2007;369:2106-2120.

2. Smith MA, Seibel NL, Altekruse SF, *et al.* Outcomes for children and adolescents with cancer: challenges for the twenty-first century. *J Clin Oncol* 2010;28:2625-2634.

3. Ambros PF, Ambros IM, Brodeur GM, *et al.* International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. *Br J Cancer* 2009;100:1471-1482.

4. Brodeur GM, Seeger RC, Schwab M, *et al.* Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984;224:1121-1124.

5. Janoueix-Lerosey I, Schleiermacher G, Michels E, *et al.* Overall genomic pattern is a predictor of outcome in neuroblastoma. *J Clin Oncol* 2009;27:1026-1033.

6. Look AT, Hayes FA, Shuster JJ, *et al.* Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1991;9:581-591.

7. Ladenstein R, Ambros IM, Potschger U, *et al.* Prognostic significance of DNA di-tetraploidy in neuroblastoma. *Med Pediatr Oncol* 2001;36:83-92.

8. Stallings RL. Are chromosomal imbalances important in cancer? *Trends Genet* 2007;23:278-283.

9. Schleiermacher G, Mosseri V, London WB, *et al.* Segmental chromosomal alterations have prognostic impact in neuroblastoma: a report from the INRG project. *Br J Cancer* 2012;107:1418-1422.

10. Chen Y, Takita J, Choi YL, *et al.* Oncogenic mutations of ALK kinase in neuroblastoma. *Nature* 2008;455:971-974.

11. George RE, Sanda T, Hanna M, *et al.* Activating mutations in ALK provide a therapeutic target in neuroblastoma. *Nature* 2008;455:975-978.

12. Janoueix-Lerosey I, Lequin D, Brugieres L, *et al.* Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. *Nature* 2008;455:967-970.

13. Mosse YP, Laudenslager M, Longo L, *et al.* Identification of ALK as a major familial neuroblastoma predisposition gene. *Nature* 2008;455:930-935.

14. Pulford K, Lamant L, Morris SW, *et al.* Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. *Blood* 1997;89:1394-1404.

15. Miyake I, Hakomori Y, Shinohara A, *et al.* Activation of anaplastic lymphoma kinase is responsible for hyperphosphorylation of ShcC in neuroblastoma cell lines. *Oncogene* 2002;21:5823-5834.

16. Osajima-Hakomori Y, Miyake I, Ohira M, *et al.* Biological role of anaplastic lymphoma kinase in neuroblastoma. *Am J Pathol* 2005;167:213-

222.

17. Blackburn EH. Telomeres and telomerase: the means to the end (Nobel lecture). *Angew Chem Int Ed Engl* 2010;49:7405-7421.

18. Cheung NK, Dyer MA. Neuroblastoma: developmental biology, cancer genomics and immunotherapy. *Nat Rev Cancer* 2013;13:397-411.

19. Heaphy CM, de Wilde RF, Jiao Y, *et al.* Altered telomeres in tumors with ATRX and DAXX mutations. *Science* 2011;333:425.

20. Horn S, Figl A, Rachakonda PS, *et al.* TERT promoter mutations in familial and sporadic melanoma. *Science* 2013;339:959-961.

21. Cheung NK, Zhang J, Lu C, *et al.* Association of age at diagnosis and genetic mutations in patients with neuroblastoma. *JAMA* 2012;307:1062-1071.

22. Ambros PF, Ambros IM, Siop Europe Neuroblastoma Pathology B, *et al.* Pathology and biology guidelines for resectable and unresectable neuroblastic tumors and bone marrow examination guidelines. *Med Pediatr Oncol* 2001;37:492-504.

23. Subramaniam MM, Piqueras M, Navarro S, *et al.* Aberrant copy numbers of ALK gene is a frequent genetic alteration in neuroblastomas. *Hum Pathol* 2009;40:1638-1642.

24. Ambros PF, Ambros IM, Strehl S, *et al.* Regression and progression in neuroblastoma. Does genetics predict tumour behaviour? *Eur J Cancer* 1995;31A:510-515.

25. Seeger RC, Brodeur GM, Sather H, *et al.* Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med*

1985;313:1111-1116.

26. Cohn SL, Pearson AD, London WB, *et al.* The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J Clin Oncol* 2009;27:289-297.

27. Schonherr C, Ruuth K, Kamaraj S, *et al.* Anaplastic Lymphoma Kinase (ALK) regulates initiation of transcription of MYCN in neuroblastoma cells. *Oncogene* 2012;31:5193-5200.

28. Umapathy G, El Wakil A, Witek B, *et al.* The kinase ALK stimulates the kinase ERK5 to promote the expression of the oncogene MYCN in neuroblastoma. *Sci Signal* 2014;7:ra102.

29. Iwahara T, Fujimoto J, Wen D, *et al.* Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* 1997;14:439-449.

30. Kelleher FC, McDermott R. The emerging pathogenic and therapeutic importance of the anaplastic lymphoma kinase gene. *Eur J Cancer* 2010;46:2357-2368.

31. Allouche M. ALK is a novel dependence receptor: potential implications in development and cancer. *Cell Cycle* 2007;6:1533-1538.

32. Palmer RH, Vernersson E, Grabbe C, *et al.* Anaplastic lymphoma kinase: signalling in development and disease. *Biochem J* 2009;420:345-361.

33. Dirks WG, Fahnrich S, Lis Y, *et al.* Expression and functional analysis of the anaplastic lymphoma kinase (ALK) gene in tumor cell lines. *Int J Cancer* 2002;100:49-56.

34. Bachetti T, Di Paolo D, Di Lascio S, *et al.* PHOX2B-mediated regulation of ALK expression: in vitro identification of a functional relationship between two genes involved in neuroblastoma. *PLoS One* 2010;5.

35. Passoni L, Longo L, Collini P, *et al.* Mutation-independent anaplastic lymphoma kinase overexpression in poor prognosis neuroblastoma patients. *Cancer Res* 2009;69:7338-7346.

36. Schulte JH, Bachmann HS, Brockmeyer B, *et al.* High ALK receptor tyrosine kinase expression supersedes ALK mutation as a determining factor of an unfavorable phenotype in primary neuroblastoma. *Clin Cancer Res* 2011;17:5082-5092.

37. De Brouwer S, De Preter K, Kumps C, *et al.* Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification. *Clin Cancer Res* 2010;16:4353-4362.

38. Duijkers FA, Gaal J, Meijerink JP, *et al.* High anaplastic lymphoma kinase immunohistochemical staining in neuroblastoma and ganglioneuroblastoma is an independent predictor of poor outcome. *Am J Pathol* 2012;180:1223-1231.

39. Barone G, Anderson J, Pearson AD, *et al.* New Strategies in Neuroblastoma: Therapeutic Targeting of MYCN and ALK. *Clin Cancer Res* 2013;19:5814-5821.

40. Cheng M, Ott GR. Anaplastic lymphoma kinase as a therapeutic target in anaplastic large cell lymphoma, non-small cell lung cancer and neuroblastoma. *Anticancer Agents Med Chem* 2010;10:236-249.

41. Matthay KK, George RE, Yu AL. Promising therapeutic targets in

neuroblastoma. *Clin Cancer Res* 2012;18:2740-2753.

42. Shaw AT, Yeap BY, Solomon BJ, *et al.* Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospective analysis. *Lancet Oncol* 2011;12:1004-1012.

43. Camidge DR, Bang YJ, Kwak EL, *et al.* Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. *Lancet Oncol* 2012;13:1011-1019.

44. Shaw AT, Kim DW, Nakagawa K, *et al.* Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. *N Engl J Med* 2013;368:2385-2394.

45. Christensen JG, Zou HY, Arango ME, *et al.* Cytoreductive antitumor activity of PF-2341066, a novel inhibitor of anaplastic lymphoma kinase and c-Met, in experimental models of anaplastic large-cell lymphoma. *Mol Cancer Ther* 2007;6:3314-3322.

46. Bresler SC, Wood AC, Haglund EA, *et al.* Differential inhibitor sensitivity of anaplastic lymphoma kinase variants found in neuroblastoma. *Sci Transl Med* 2011;3:108ra114.

47. Mosse YP, Lim MS, Voss SD, *et al.* Safety and activity of crizotinib for paediatric patients with refractory solid tumours or anaplastic large-cell lymphoma: a Children's Oncology Group phase 1 consortium study. *Lancet Oncol* 2013;14:472-480.

48. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997;33:787-791.

49. Clynes D, Higgs DR, Gibbons RJ. The chromatin remodeller ATRX: a repeat offender in human disease. *Trends Biochem Sci* 2013;38:461-466.

50. Vinagre J, Pinto V, Celestino R, *et al.* Telomerase promoter mutations in cancer: an emerging molecular biomarker? *Virchows Arch* 2014;465:119-133.

51. Ohali A, Avigad S, Ash S, *et al.* Telomere length is a prognostic factor in neuroblastoma. *Cancer* 2006;107:1391-1399.

52. Coco S, Theissen J, Scaruffi P, *et al.* Age-dependent accumulation of genomic aberrations and deregulation of cell cycle and telomerase genes in metastatic neuroblastoma. *Int J Cancer* 2012;131:1591-1600.

53. Hiyama E, Hiyama K, Yokoyama T, *et al.* Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* 1995;1:249-255.

54. Huang FW, Hodis E, Xu MJ, *et al.* Highly recurrent TERT promoter mutations in human melanoma. *Science* 2013;339:957-959.

55. Malaska J, Kunicka Z, Borsky M, *et al.* Telomerase as a diagnostic and predictive marker in colorectal carcinoma. *Neoplasma* 2004;51:90-96.

56. Lantuejoul S, Soria JC, Moro-Sibilot D, *et al.* Differential expression of telomerase reverse transcriptase (hTERT) in lung tumours. *Br J Cancer* 2004;90:1222-1229.

57. Martin JA, DeYoung BR, Gitelis S, *et al.* Telomerase reverse transcriptase subunit expression is associated with chondrosarcoma malignancy. *Clin Orthop Relat Res* 2004:117-124.

58. Shin JS, Foo T, Hong A, *et al.* Telomerase expression as a predictive marker of radiotherapy response in rectal cancer. *Pathology* 2012;44:209-215.

59. Aras G, Kanmaz D, Urer N, *et al.* Immunohistochemical expression of telomerase in patients with non-small cell lung cancer: prediction of metastasis and prognostic significance. *Anticancer Res* 2013;33:2643-2650.

60. Haberler C, Wohrer A. Clinical Neuropathology practice news 2-2014: ATRX, a new candidate biomarker in gliomas. *Clin Neuropathol* 2014;33:108-111.

61. Lovejoy CA, Li W, Reisenweber S, *et al.* Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway. *PLoS Genet* 2012;8:e1002772.

62. Schwartzentruber J, Korshunov A, Liu XY, *et al.* Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature* 2012;482:226-231.

국문 초록

배경: 신경모세포종은 소아청소년기에 발생하는 흔한 고형종양으로 매우 다양한 임상경과를 보이는 것으로 알려져 있다. 여러 임상적 및 유전적 특성들이 연구되었고, 이러한 특성들이 신경모세포종의 치료를 결정하는 위험군 분류에 사용되고 있다. 본 연구에서는 *MYCN* 유전자의 증폭, anaplastic lymphoma kinase(ALK) 유전자의 변이나 증폭, ALK 단백질의 발현, nuclear ATRX 단백질의 소실 및 TERT 단백질의 발현을 연구하고, 이러한 분자적인 특성이 임상양상이나 치료 결과와 연관되어 있는지 확인하고자 하였다.

재료 및 방법: 2002년 1월부터 2012년 7월까지 서울대학교 어린이병원에서 신경모세포종으로 진단받고 치료받은 104명의 환자 중 초기 종양 검체의 확보 및 평가가 가능하였던 72명의 환자를 대상으로 연구를 시행하였다. 조직은 수술이나 조직검사 시 얻어진 포르말린 고정, 파라핀 내재 (Formalin-fixed, paraffin-embedded) 종양 조직을 사용하였다. *ALK* 유전자의 변이는 23-25번 엑손(exon)의 PCR 증폭과 직접염기서열분석으로 분석하였다. 면역조직화학염색은 4-μm 두께의 조직 마이크로어레이(tissue

microarray) 슬라이드에 ALK 단일클론 항체와 ATRX 다클론성항체, TERT 단일클론 항체를 이용하여 시행하였다. *MYCN* 유전자와 *ALK* 유전자의 증폭을 확인하기 위하여 형광동소보합법(Fluorescence *in situ* hybridization)을 시행하였다.

결과: 7명(10.0%)의 환자가 초기 진단 시 *MYCN* 유전자의 증폭을 보였으며, *MYCN* 유전자의 증폭이 있는 환자의 나이가 *MYCN* 유전자의 증폭이 없는 환자보다 유의하게 낮았다(1.6±0.8세 vs 3.1±3.7세, P=0.010). 40명(55.6%)의 환자에서 ALK 발현이 관찰되었으며, ALK 발현을 보이는 환자 수는 종양의 병기가 증가함에 따라 증가하는 경향을 보였다(P=0.001). ALK 발현을 보인 환자 군에서 통계적으로 유의하게 높은 재발률을 보였다(47.5% vs 11.3%, P=0.007). *ALK* 유전자의 변이는 DNA 추출이 가능하였던 49명의 환자 중에 2명(4.1%)에서만 발견되었으며, *ALK* 유전자의 증폭은 평가가 가능하였던 65명 중 한 명에서도 발견되지 않았다. 9명(13.0%)의 환자가 nuclear ATRX 단백질의 소실을 보였으며, nuclear ATRX 단백질의 소실은 두 개의 다른 그룹에서 관찰되었다. 즉, 나이가 많고 병기 IV인 환자에서 ALK 단백질의 발현을 보이며 indolent한 질병의 경과를 보이는 그룹과 더불어, 나이가 어리고 병기가 낮은 환자에서도 발견되었으며 이 그룹의 환자에서는 ALK 발현이 없으며 예후가 좋은 것으로

확인되었다. TERT 발현이 없는 환자들에서 재발률이 높은 경향을 보였으나 통계적으로 유의한 의미는 없었다.

결론: 본 연구에서 *MYCN* 유전자의 증폭과 ALK 단백질의 발현은 강한 예후인자로 확인되었다. *ALK* 유전자의 변이가 소수의 환자에서만 발견되고 *ALK* 유전자의 증폭은 한 명에서도 발견되지 않았음에도 불구하고, ALK 단백질 발현은 많은 환자들에게서 확인되었으며, ALK 단백질의 발현은 신경모세포종의 진행된 병기 및 불량한 예후와 연관되어 있었다. 이러한 결과를 바탕으로, 재발하거나 불응성인 신경모세포종 환자 중 ALK 단백질의 발현을 보이는 경우, ALK 표적치료를 적용하는 것을 고려해볼 수 있을 것으로 생각된다. 또한 신경모세포종에서 nuclear ATRX의 단백질 소실 및 TERT 단백질의 발현의 의미에 대하여는 추가적인 연구가 필요할 것으로 생각된다.

주요어: 신경모세포종, ALK, ATRX, TERT

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