

# Prognostic significance of *NPM-ALK* fusion transcript overexpression in ALK-positive anaplastic large-cell lymphoma

Chunmei Li<sup>1</sup>, Hisashi Takino<sup>1</sup>, Tadaaki Eimoto<sup>1</sup>, Takashi Ishida<sup>2</sup>, Atsushi Inagaki<sup>2</sup>, Ryuzo Ueda<sup>2</sup>, Ritsuro Suzuki<sup>3</sup>, Tadashi Yoshino<sup>4</sup>, Atsuko Nakagawa<sup>5</sup>, Shigeo Nakamura<sup>6</sup> and Hiroshi Inagaki<sup>1</sup>

<sup>1</sup>Department of Pathology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; <sup>2</sup>Department of Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; <sup>3</sup>Department of HSCT Data Management, Nagoya University School of Medicine, Nagoya, Japan; <sup>4</sup>Department of Pathology, Okayama University, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; <sup>5</sup>Department of Clinical Laboratory, National Center for Child Health and Development, Tokyo, Japan and <sup>6</sup>Department of Pathology and Clinical Laboratories, Nagoya University Hospital, Nagoya, Japan

In anaplastic large-cell lymphomas positive for anaplastic lymphoma kinase (ALK) protein, the *ALK* gene is most commonly fused to the *NPM* gene, and less commonly to *TPM3*, *TFG*, *ATIC*, and other rare genes. Although this lymphoma is generally associated with a favorable clinical outcome, 25% of the patients die of the disease within 5 years. In this study, we developed three assays, all of which can be used with archival formalin-fixed, paraffin-embedded tissues: (1) a sensitive reverse transcription-polymerase chain reaction (RT-PCR) assay for various *X-ALK* fusion genes, (2) a 5' rapid amplification of cDNA ends (RACE) assay to identify unknown fusion partners, and (3) a real-time RT-PCR assay to quantify the amount of the *NPM-ALK* fusion transcript. In 26 cases of ALK<sup>+</sup> anaplastic large-cell lymphoma, the RT-PCR assay showed that the *ALK* was fused to *NPM* in 21 cases, to *TPM3* in three, and to *TFG* in one. The 5' RACE assay detected *ATIC-ALK* fusion in the remaining case. The real-time quantitative RT-PCR assay showed that the *NPM-ALK* transcript was over expressed in four of 20 quantifiable cases. Patients with *NPM-ALK* overexpression showed a significantly unfavorable overall survival compared with those with a low expression of this transcript. The RT-PCR and 5' RACE assays developed here may be useful for identification of known and unknown gene partners fused to the *ALK* gene. Overexpression of the *NPM-ALK* fusion transcript may be associated with a poor prognosis of the patients with ALK<sup>+</sup> anaplastic large-cell lymphomas.

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## Introduction

Anaplastic large-cell lymphoma is a systemic T-cell non-Hodgkin's lymphoma.<sup>1</sup> In more than half of these cases, tumor cells express anaplastic lymphoma kinase (ALK) protein, the expression of which is due to genetic alteration of the *ALK* gene locus on chromosome 2.<sup>2,3</sup> ALK<sup>+</sup> anaplastic large-cell lymphomas are associated with a younger male patient.

Although they often present at an advanced clinical stage, the response to chemotherapy is good, and the clinical outcome is favorable, as compared with ALK<sup>-</sup> anaplastic large-cell lymphomas.<sup>4–6</sup> Nevertheless, one-fourth of patients still die of the disease within 5 years.

The commonest genetic alteration in ALK<sup>+</sup> anaplastic large-cell lymphoma, found in 70–80% of cases, is a t(2;5)(p23;q35) translocation between the *ALK* gene on chromosome 2 and the nucleophosmin (*NPM*) gene on chromosome 5.<sup>7,8</sup> The *ALK* gene encodes a tyrosine kinase receptor belonging to the insulin growth factor receptor superfamily, which is normally expressed in nerve cells<sup>7,9</sup> and in human neuroblastoma cells,<sup>10</sup> but is silent in normal lymphoid cells.<sup>9</sup> It has been demonstrated that constitu-

Correspondence: Dr H Inagaki, MD, PhD, Department of Pathology, Graduate School of Medical Sciences, Nagoya City University, Kawasumi, Mizuho-ku, Nagoya 467-8601, Japan.  
E-mail: hinagaki@med.nagoya-cu.ac.jp  
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tive ALK activity contributes to the malignant transformation of lymphoid cells.<sup>11–13</sup> In the remaining 20–30% of ALK<sup>+</sup> cases, *ALK* gene is fused to various partners including *TPM* (tropomyosin) 3,<sup>14</sup> *TPM4*,<sup>15</sup> *TFG* (TRK fused gene),<sup>16,17</sup> *ATIC* (5-aminimidazole-4-carboxamide ribonucleotide formyltransferase/*IMP* cyclohydrolase),<sup>18,19</sup> *CLTC* (clathrin heavy chain),<sup>20</sup> *MSN* (moesin),<sup>21,22</sup> *ALO17* (*KIAA1618*),<sup>23</sup> and *MYH* (non-muscle myosin heavy chain) 9.<sup>24</sup> The *TPM3* gene has been the second most common fusion partner, and is involved in 10–20% of all ALK<sup>+</sup> anaplastic large-cell lymphomas, while involvement of other genes seems to be rare.<sup>2,3</sup> The *ALK* fusion partners can be estimated from the *ALK* protein subcellular distribution pattern as detected by *ALK* immunohistochemistry.<sup>9,25</sup> In *NPM-ALK* cases, the *ALK* signal is detected in both cytoplasm and nucleus. This distribution may be explained by the functions of *NPM* that shuttles continuously between the cytoplasm to the nucleus.<sup>26</sup> In contrast, in anaplastic large-cell lymphoma cases with fusions other than *NPM-ALK*, the *ALK* signal is detected in the cytoplasm, and rarely in the membrane.<sup>1,21,22</sup> *ALK* immunohistochemistry, although easy to perform, has some shortcomings: non-*NPM-ALK* cases are sometimes misdiagnosed as *NPM-ALK* cases because of non-specific nuclear staining, and further classification of the former cases would be very difficult.

It has been reported that mouse fibroblast clones each stably transfected with respective *ALK* fusion genes showed distinct tumor properties,<sup>27</sup> suggesting that both identification of the *ALK* fusion partners and the quantification of the fusion gene expression may have clinical implications. In this study, we have developed three assays: (1) a sensitive reverse transcription (RT)-polymerase chain reaction (PCR) assay for various *X-ALK* fusion genes, (2) a 5' rapid amplification of cDNA ends (RACE) assay to identify unknown *ALK* fusion partners, and (3) a real-time RT-PCR assay to quantify the amount of the *NPM-ALK* fusion transcript. The advantages of being able to use routinely prepared paraffin materials far outweigh any demerits. All three assays presented here can be used with archival formalin-fixed, paraffin-embedded tissues. For 26 cases of ALK<sup>+</sup> anaplastic large-cell lymphoma, we identified all *ALK* fusion partners using RT-PCR and 5' RACE. In the *NPM-ALK* cases, the amount of the fusion transcript was quantified using real-time RT-PCR,<sup>28,29</sup> and the association between its overexpression and the clinicopathological characteristics was studied.

## Materials and methods

### Clinical Cases and Cell Line

Twenty-six cases of ALK<sup>+</sup> anaplastic large-cell lymphoma were retrieved from the pathology files of Nagoya City University and other hospitals. Specimens were obtained at the initial presentation of the patients, fixed in formalin, and embedded in

paraffin. This study was conducted according to the Declaration of Helsinki. All cases were reviewed carefully, and the histological diagnosis of anaplastic large-cell lymphoma was made according to criteria of the WHO classification of malignant lymphoma.<sup>1</sup> All cases were nodal tumors. Immunohistochemically, CD30 was positive in 26/26 cases, CD3 in 10/26, CD4 in 5/16, CD20 in 0/26, CD43 in 14/20, epithelial membrane antigen in 21/21, and cytotoxic associated antigens (granzyme B or TIA-1) in 20/20. *In situ* hybridization for Epstein-Barr virus-encoded small RNA (EBER) was negative in 26/26 cases. All cases included in this study were positive for *ALK* as determined with anti-*ALK* monoclonal antibody (*ALK-1*, DAKO Cytomation, Kyoto, Japan). As shown in Table 1, *ALK* expression was detected in both nuclei and cytoplasm in 21 cases, and in the remaining five, it was restricted to the cytoplasm. One case (case 4) showed hemophagocytic syndrome, but had no particular history or underlying disorders that would predispose to such a syndrome. Cytogenetic data on the tumors were available in five cases, and translocations were as follows: t(2;5)(p23;q35) for *NPM-ALK* fusion in three cases, t(1;2)(q25;p23) for *TPM3-ALK* fusion in one case, and t(2;3)(p23;q35) for *TFG-ALK* fusion in the remaining case. These cases were used as positive controls for the RT-PCR assay for the respective fusion genes. The Karpas 299 cell line, which was established from an anaplastic large-cell lymphoma cell line carrying the *NPM-ALK* fusion gene, was used as a standard for quantification of the *NPM-ALK* fusion transcript, and normal lymph nodes were used as negative control.

### Histology, MIB-1 Index, and Immunohistochemistry for CD56

Of the 26 anaplastic large-cell lymphoma cases, 20 were histologically classified as the common type, three cases as a lymphohistiocytic variant, and three cases as a small cell variant. To estimate proliferation activity, immunohistochemistry was performed with MIB-1 monoclonal antibody (DAKO Cytomation). The percentage of positive cells (MIB-1 index) was calculated by counting > 500 tumor cells in the most positive areas. Expression of CD56 was detected using anti-CD56 monoclonal antibody (clone 1B6; Novocastra, Newcastle Upon Tyne, UK).

### RNA Extraction

Total RNA was extracted from formalin-fixed, paraffin-embedded sections, as described previously.<sup>30</sup> Briefly, deparaffinized sections were incubated at 56°C overnight in proteinase K digestion buffer. Total RNA was extracted with concentrated phenol/guanidine isothiocyanate (Trizol LS, Invitrogen, Carlsbad, CA, USA), then subjected to RNase-free DNase I treatment, and finally re-suspended in

**Table 1** Clinical, histological, and genetic features of ALK<sup>+</sup> ALCL cases

Case no.	Age (years)	Sex	Histological variant	ALK pattern	Cytogenetic analysis	ALK fusion partner	MIB-1 index (%)	MIB-1 expression	CD56	NPM-ALK <sup>a</sup>	NPM-ALK expression	Clinical stage	B symptoms	Outcome/Follow-up (mo)	Comments
1	46	M	Small	C,N	t(2;5)	NPM	89	High	Neg	1.5	Low	IV	No	AW/36	
2	3	F	Common	C,N	NT	NPM	50	Low	Neg	17.2	High	IV	Yes	DOD/5	
3	12	F	Common	C,N	t(2;5)	NPM	11	Low	Neg	6.4	Low	II	Yes	AW/144	
4	11	M	Common	C,N	NT	NPM	82	High	Neg	31.7	High	IV	Yes	DOD/55	HS
5	13	M	Small	C,N	NT	NPM	NA	Low	Pos	6.6	Low	II	No	AW/53	
6	5	F	Common	C,N	NT	NPM	88	High	Neg	15.5	High	III	Yes	AW/125	
7	28	F	Common	C,N	NT	NPM	91	High	Neg	5.5	Low	III	No	AW/52	
8	17	M	Common	C,N	NT	NPM	51	Low	Neg	3.13	Low	II	Yes	AW/33	
9	10	F	Lymphohistiocytic	C,N	NT	NPM	36	Low	Pos	0.5	Low	IV	No	AW/3	
10	9	F	Common	C,N	NT	NPM	66	High	Neg	5.4	Low	III	No	AW/18	
11	20	M	Common	C,N	NT	NPM	85	High	Neg	4.7	Low	IV	Yes	AW/6	auto PBSCT
12	38	F	Common	C,N	NT	NPM	79	High	Neg	18.3	High	IV	Yes	DOD/3	
13	8	F	Common	C,N	NT	NPM	76	High	Pos	3.8	Low	III	Yes	AW/31	
14	1	F	Small	C,N	t(2;5)	NPM	62	Low	Neg	1.3	Low	II	No	AW/29	
15	10	M	Common	C,N	NT	NPM	66	High	Neg	1	Low	III	No	AW/26	
16	5	M	Lymphohistiocytic	C,N	NT	NPM	26	Low	Neg	1	Low	III	Yes	AW/10	
17	3	M	Common	C,N	NT	NPM	67	High	Neg	2.7	Low	III	No	AW/5	
18	11	F	Common	C,N	NT	NPM	61	Low	Neg	2.2	Low	III	Yes	AW/9	
19	2	M	Common	C,N	NT	NPM	77	High	Neg	2.2	Low	III	Yes	AW/15	
20	3	F	Common	C,N	NT	NPM	92	High	Neg	4.1	Low	III	No	AW/20	
21	30	F	Common	C,N	NT	NPM	77	High	Neg	NA	NA	III	Yes	NA	
22	11	M	Common	C	NT	TPM3	72	High	Neg	NT	NT	I	No	AW/13	
23	22	M	Common	C	t(1;2)	TPM3	57	Low	Neg	NT	NT	III	No	AW/24	
24	40	M	Lymphohistiocytic	C	NT	TPM3	67	High	Neg	NT	NT	II	No	AW/56	
25	3	F	Common	C	t(2;3)	TFG	39	Low	Neg	NT	NT	NA	NA	NA	
26	28	M	Common	C	NT	ATIC <sup>a</sup>	61	Low	Neg	NT	NT	III	Yes	AW/42	

C, cytoplasmic; N, nuclear; NT, not tested; NA, not available; AW, alive and well; DOD, died of disease; HS, hemophagocytic syndrome; autoPBSCT, autologous peripheral blood stem cell transplantation.

<sup>a</sup>Determined by 5' rapid amplification of cDNA ends (5' RACE).

**Table 2** Sequences of primers

Primers	Sequence
NPM-1	TGCATATTAGTGGACAGCAC
TPM3-1	GTAGCCAAGCTGGAAAAGAC
TFG-1	AGCTTGAACACCTGGAGAACC
ATIC-1	CAGCTGTACACACTGCAGC
ALK-1	GTCGAGGTGCGGAGCTTGCTCAGC
ALK-2	GCGGAGCTTGCTCAGCTTGATC
ALK-3	CAGCTTGTACTCAGGGCTCTGC
SMART IIA	AAGCAGTGGTATCAACGCAGAGTACGCCGG
NUP	AAGCAGTGGTATCAACGCAGAGT
Actin-1	GCCAACCGCGAGAAGATGAC
Actin-2	GAGCGCTACAGGGATAGCAC

All sequences are 5'–3'.

RNase-free water. Total RNA was similarly extracted from Karpas 299 tumor cells.

### RT-PCR Detection of Fusion Transcript

The fusion transcripts (*NPM-ALK*, *TPM3-ALK*, and *TFG-ALK*) were detected using a two-step RT-PCR. Briefly, cDNA was synthesized from the total RNA using random hexamers (SuperScript First-Strand Synthesis System kit, Invitrogen, Carlsbad, CA, USA). Forty-five cycles of PCR was performed with ALK-3 primer and the respective primers (NPM-1, TPM3-1, and TFG-1) as shown in Table 2. After amplification, PCR products were electrophoresed in 3% agarose gel, and directly sequenced by means of cycle sequencing with dye-labeled terminators (BigDye Terminator Cycle Sequencing kit, Applied Biosystems, Foster City, CA, USA) and analyzed on an automated DNA sequencer (Model 310, Applied Biosystems). Positive controls for *NPM-ALK*, *TPM3-ALK*, and *TFG-ALK* fusion transcripts consisted of anaplastic large-cell lymphoma cases possessing t(2;5), t(1;2), and t(2;3), respectively. The RNA quality was confirmed by RT-PCR amplification of the  $\beta$ -actin transcript using the primers, Actin-1 and Actin-2 (Table 2).

### 5' Rapid Amplification of cDNA Ends

Identification of unknown ALK partner genes was performed with a 5' RACE technique (SMART™ RACE cDNA Amplification kit, Clontech, Mountain View, CA), using total RNA extracted from formalin-fixed, paraffin-embedded tissues. The first-strand 5' RACE cDNA was synthesized using a gene-specific primer (ALK-1 and SMART IIA, Table 2). The first-round PCR was performed with the primers, ALK-2 primer and the Nested Universal Primer (NUP), followed by seminested PCR, using the primers, ALK-3 and NUP. The final products of 100–200 bp were cloned into pGEM-T easy vector (Promega, Madison, WI, USA), and directly sequenced. Sequence homologies were identified using the BLAST program of the National Center for Biotech-

nology Information available at <http://www.ncbi.nlm.nih.gov/BLAST/>.

### Real-Time Quantitative RT-PCR for the NPM-ALK Fusion Transcript

Real-time quantitative RT-PCR analysis for *NPM-ALK* and  $\beta$ -actin mRNA (an endogenous reference) was carried out with the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). cDNA synthesized with random hexamers was employed. The real-time PCR was performed in a 20  $\mu$ l final reaction mixture that included diluted cDNA and 0.5 pmol/ $\mu$ l of each primer (NPM-1 and ALK-3 for *NPM-ALK* and Actin-1 and Actin-2 for  $\beta$ -actin, Table 2). Amplification conditions were 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, and finally by a dissociation stage (15 s at 95°C, 30 s at 60°C, and 15 s at 95°C).

For construction of the standard curves, 10-fold serial dilutions of cDNA of Karpas 299 were prepared for both target *NPM-ALK* and  $\beta$ -actin. A preliminary study showed that the coefficient of determination ( $R^2$ ) and the slope value for *NPM-ALK* were 0.999 and  $-3.39$ , respectively, and those for  $\beta$ -actin were 0.995 and  $-3.17$ , respectively. A standard curve was created with each run, and the threshold cycle was used for quantification of the input target number. The amount of *NPM-ALK* was divided by that of  $\beta$ -actin. This value was further divided by the tumor cell ratio in the section, which was counted under a microscope using H&E and ALK-immunostained sections as a guide, then a normalized target value was finally obtained. The real-time PCR was performed in triplicate for each case, and the results were averaged. Melting curve analysis was also performed after PCR amplification to confirm that there were no primer dimers in the PCR products.

### Statistical Analysis

Statistical evaluation of data from two groups was performed using Fischer's exact test and Student's *t*-test. All analyses were two-tailed. To identify the parameters significantly associated with an overall survival, the survival rate was calculated by the Kaplan–Meier method, and the statistical difference was estimated using the log-rank and Wilcoxon tests. A value of  $P < 0.05$  for each test was regarded as statistically significant. All of the analyses were performed using the statistical package JMP, version 5 (SAS Institute Inc., Cary, NC, USA).

## Results

### RT-PCR Assay for ALK Fusion Partners

To determine the genes fused to *ALK*, we performed an RT-PCR assay with primer pairs specific to

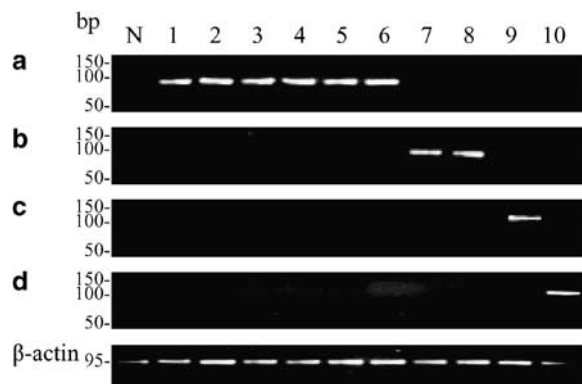
respective fusion types using RNA extracted from paraffin sections (Figure 1). Among the 26 cases that were positive on ALK immunohistochemistry, the *NPM-ALK* fusion transcript (92 bp) was detected in 21, *TPM3-ALK* (105 bp) in three, and *TFG-ALK* (120 bp) in one. In the remaining case, the *ALK* fusion partner was not determined (case 26). All cases positive for the *NPM-ALK* fusion transcript showed a nuclear and cytoplasmic ALK staining pattern (Supplementary Information 1A, 1B). Three cases with the *TPM3-ALK* fusion, one case with the *TFG-ALK* fusion (Supplementary Information 1C), and one partner-unknown case (case 26) showed a cytoplasmic ALK staining pattern.

### 5' RACE Assay

To identify the possible fusion partner in the partner-unknown case (Case 26) by RT-PCR, we performed a 5' RACE assay using total RNA extracted from the paraffin-embedded tissue. Direct sequencing showed that five of six clones obtained from this assay possessed an identical sequence. Comparing of this sequence with those obtained by nucleotide database searches revealed that exon 7 of the *ATIC* gene was fused to exon 20 of the *ALK* gene. To confirm the presence of the *ATIC-ALK* fusion transcript in this case, cDNA synthesized using random hexamers was subjected to PCR using primers, ALK-3 and ATIC-1 (Table 2). The direct sequencing of the RT-PCR product (102 bp) showed that the *ATIC-ALK* fusion transcript (Figure 1, lane 10) had identical break points.

### Clinicopathological Features of ALK<sup>+</sup> Anaplastic Large-Cell Lymphomas

As shown in Table 1, there were 13 males and 13 females with ages ranging from 1 to 46 years (mean,



**Figure 1** An RT-PCR assay for *NPM-ALK*, *TPM3-ALK*, *TFG-ALK*, and *ATIC-ALK* fusion transcripts. (a) *NPM-ALK* (92 bp); (b) *TPM3-ALK* (105 bp); (c) *TFG-ALK* (120 bp); and (d) *ATIC-ALK* (102 bp). N, negative control (normal lymph node); lanes 1–10, ALK<sup>+</sup> anaplastic large-cell lymphomas.  $\beta$ -actin mRNA is amplified in all cases.

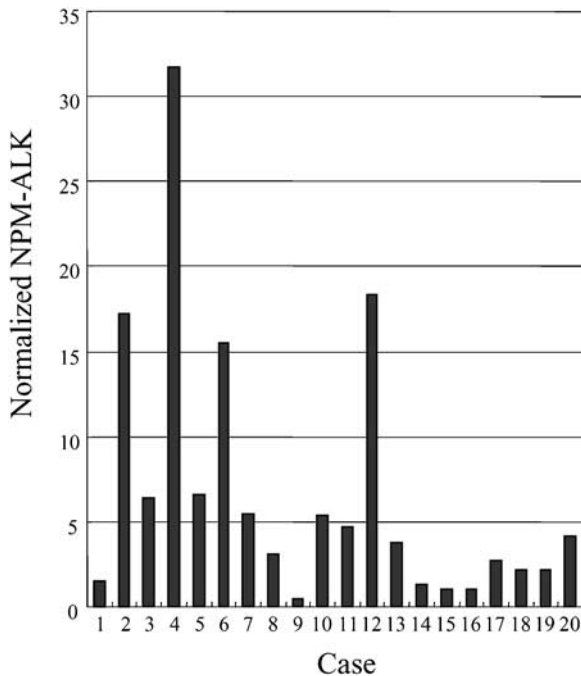
15 years). MIB-1 index, calculated in 25 cases, ranged from 11 to 92% with a mean of 65%. Three cases were immunohistochemically positive for CD56 (Cases 5, 9, and 13) as shown in Supplementary Information 1D. The clinical data were available from 25 patients. Six cases had a less advanced clinical stage (one case at stage I and five at stage II) and 19 cases had an advanced clinical stage (13 cases at stage III and six at stage IV). Thirteen patients exhibited systemic symptoms (B-symptoms) such as night sweats, fever, and weight loss. One patient presented with hemophagocytic syndrome (Case 4). All cases were treated with anthracyclin-based combination chemotherapy. One case underwent autologous peripheral blood stem cell transplantation after induction of complete remission (Case 11). Follow-up data were available in 24 patients. The follow-up periods ranged from 3 to 144 months (mean 34 months). Three of 20 patients with *NPM-ALK* tumors died of the disease while those with non-*NPM-ALK* tumors were alive at the last follow-up. Clinicopathological features and overall survival were not statistically different between patients with *NPM-ALK* and non-*NPM-ALK* tumors.

### Real-Time Quantitative RT-PCR for NPM-ALK Fusion Transcript

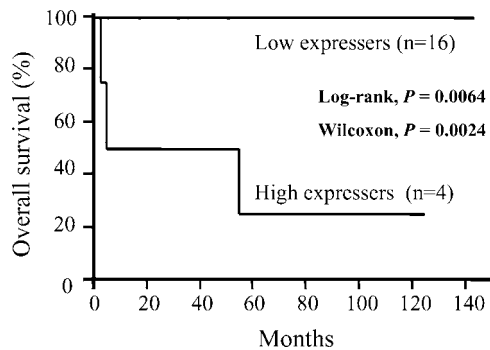
To test the reproducibility of the real-time quantitative RT-PCR using RNA extracted from paraffin sections, we determined normalized target amounts of the transcript in the Karpas 299 cell line and in two clinical cases with the *NPM-ALK* fusion. Reproducible results were obtained in each case, and amounts in the two clinical cases were identical at the corresponding dilutions. These data indicated that the *NPM-ALK* fusion transcript could be reliably quantified in our real-time RT-PCR assay. Among the 21 cases with this transcript, 20 cases were quantifiable (Table 1). The normalized *NPM-ALK* values ranged from 0.5 to 31.7 (mean 6.7). We created a scatter diagram and divided our cases into two groups (Figure 2): high *NPM-ALK* expressers (cases 2, 4, 6, and 12) and low expressers (other cases). Normal lymph nodes showed no detectable *NPM-ALK* fusion transcript. The *NPM-ALK* expression level was not significantly associated with ALK expression or other immunophenotypes of the tumor cells.

### Impact of High Expression of the NPM-ALK Fusion Transcript on Clinicopathological Characteristics

Twenty cases quantifiable of the *NPM-ALK* fusion transcript (Cases 1–20) consisted of nine males and 11 females with a mean age of 12.8 years. None of the seven clinicopathological factors analyzed (age, sex, clinical stage, B-symptoms, histological type, MIB-1 index, and CD56 expression) showed any



**Figure 2** Normalized amount of *NPM-ALK* fusion transcript in *ALK*<sup>+</sup> anaplastic large-cell lymphomas.



**Figure 3** Overall survival of patients with *NPM-ALK*-positive anaplastic large-cell lymphoma relative to expression levels of the fusion transcript.

significant association with the *NPM-ALK* expression level. A survival analysis showed that high *NPM-ALK* expressers presented with significantly reduced overall survivals than did the low expressers with respective 5-year survival rates of 25 and 100% (Figure 3, log-rank  $P = 0.0064$ , Wilcoxon  $P = 0.0024$ ). Other factors had no significant impact on survival.

## Discussion

Among the subgroups of anaplastic large-cell lymphoma, *ALK* expression defines a distinct form, which is associated with a young age and a favorable prognosis compared with *ALK*<sup>-</sup> anaplastic large-cell

lymphoma.<sup>4-6</sup> However, approximately 25% of patients with *ALK*<sup>+</sup> anaplastic large-cell lymphoma still die of the disease within 5 years, thus highlighting the need to find risk factors that identify potential patients who would be better served by risk-adjusted therapies.<sup>4-6</sup> Several risk factors have been reported for *ALK*<sup>+</sup> anaplastic large-cell lymphoma: the International Prognostic Index,<sup>6,31</sup> survivin expression,<sup>32</sup> and CD56 expression.<sup>31</sup> Recently, Armstrong *et al*<sup>27</sup> reported the transforming potential of *X-ALK* fusion proteins using mouse fibroblast clones stably transfected with five different *X-ALK* cDNAs (*NPM-ALK*, *TPM3-ALK*, *TFG-ALK*, *CLTC-ALK* and *ATIC-ALK*). Each clone showed distinct cell properties. The proliferation rate was associated with the level of *X-ALK* expression except for *TPM3-ALK*. The highest invasion capacity was shown by the *TPM3-ALK* clone, and the highest tumorigenicity was observed in the *NPM-ALK* and *TFG-ALK* clones. These observations suggest that identification and quantification of *ALK* fusion partners may have clinical implications in the pathogenesis and prognosis of *ALK*<sup>+</sup> anaplastic large-cell lymphomas.

The most important finding of this study is that in anaplastic large-cell lymphomas with the *NPM-ALK* fusion, the high expression of the fusion transcript was associated with an unfavorable overall survival. We successfully quantified the *NPM-ALK* fusion transcript using RNA extracted from paraffin sections. It is worth noting that *NPM-ALK* expression levels were not associated with any of the clinicopathological factors analyzed such as age, sex, clinical stage, B-symptoms, histological type, MIB-1 index, and CD56 expression. These findings suggest that *NPM-ALK* overexpression may be a novel risk factor independent of known risk factors such as the clinical stage and CD56 expression.<sup>6,31</sup> The direct link between overexpression of the transcript and an unfavorable prognosis is currently unknown. Constitutively active *NPM-ALK* fusion protein, thus far evaluated only *in vitro*, may activate a number of downstream effectors, including phospholipase C- $\gamma$ , RAS, signal transducer and activator of transcription proteins, and phosphoinositol 3'-kinase, and may be associated with both cell growth and apoptosis regulation.<sup>33</sup> In this study, no association was noted between *NPM-ALK* overexpression and tumor cell proliferation (MIB-1 index). Unlike malignant epithelial tumors, cell proliferation is not generally considered as a risk factor in malignant lymphoma. Patients with Burkitt's lymphoma, which is characterized by a high tumor cell proliferation, have a favorable prognosis with the treatment of very intensive chemotherapy.<sup>34</sup> Presumably, *NPM-ALK* overexpression may affect other tumor characteristics such as the invasion capacity, tumorigenicity, and antiapoptosis through differential activation of various signaling pathways.<sup>27,33</sup>

The second important achievement of this study was the successful identification of *ALK*-fusion

partners by RT-PCR using paraffin sections as a source of RNA. Although ALK immunohistochemistry provides a useful means for identifying these partners,<sup>9,25</sup> misinterpretation of the staining pattern (nuclear and cytoplasmic vs cytoplasmic) can sometimes occur, and subclassification of non-*NPM-ALK* tumors is difficult in most cases. Several RT-PCR assays for *X-ALK* fusion transcripts have been reported.<sup>3,7,25</sup> In our preliminary assays, we used RNA extracted from paraffin materials to these assays, but found that sensitivities were not satisfactory (unpublished data). Results may be improved by optimizing RT-PCR assays for use with such degraded RNA, and the RT-PCR assay developed here showed a high degree of sensitivity and specificity in the detection of *ALK* fusion partners. Simple to perform, this assay is expected to become a powerful tool for screening of non-*NPM-ALK* cases. At present, statistical analyses have been incapable of distinguishing prognostically between *NPM-ALK* cases and non-*NPM-ALK* cases. However, the latter cases might have a better overall survival as estimated from our data and those of Falini *et al.*<sup>35</sup> It was also found that non-*NPM-ALK* translocations are common in pediatric anaplastic large-cell lymphomas.<sup>36</sup>

Another important finding of this study is that we identified an *ALK* fusion partner by a 5' RACE technique using paraffin sections as a source of RNA. To the best of our knowledge, this is the first report of a 5' RACE assay that has been successfully performed using paraffin sections. To date, nine different *ALK* partner genes have been reported, and there are likely more to be identified. In determining these partners, the RNA has to be of high quality. Our 5' RACE technique described here will be useful for identification of unknown *ALK* fusion partners when only routinely prepared histological materials are available. Our present success with 5' RACE may be due to the following: one is that the break point of the *ALK* gene has been restricted to a narrow portion around the junction between exons 19 and 20, and (2) the wild-type *ALK* transcript is virtually absent in anaplastic large-cell lymphoma tissue, which helps increase 5' RACE sensitivity. Identification of novel fusion partners is important in clarifying the pathogenesis of *ALK*<sup>+</sup> anaplastic large-cell lymphoma. For example, the recently identified *MYH9-ALK* fusion lacks a functional oligomerization domain,<sup>24</sup> which has been described as critical for the anaplastic large-cell lymphoma tumorigenesis.<sup>37</sup>

Among *ALK*<sup>+</sup> anaplastic large-cell lymphomas, *ALK* most commonly fuses with *NPM*, constituting more than 70% of the total number of cases. We showed that overexpression of the *NPM-ALK* fusion transcript was associated with a poor overall survival. To establish the prognostic significance of this parameter, a large-scale study is needed. Moreover, the accurate selection of *NPM-ALK* cases is essential. The RT-PCR assay presented here would be useful for this selection. Furthermore, it is

expected to be a powerful tool in the collection of non-*NPM-ALK* anaplastic large-cell lymphomas whose precise clinicopathological characteristics remain to be clarified. Lastly, the 5' RACE technique described here should prove to be useful in identifying novel genes that fuse with the *ALK* gene in anaplastic large-cell lymphomas. It should be emphasized that routinely processed paraffin materials can be used in all these assays, which will greatly facilitate future anaplastic large-cell lymphoma studies.

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