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ORIGINAL ARTICLE

Expression of myeloperoxidase and gene mutations in AML patients with normal karyotype: double *CEBPA* mutations are associated with high percentage of MPO positivity in leukemic blasts

Shinya Tominaga-Sato · Hideki Tsushima · Koji Ando · Hidehiro Itonaga · Yoshitaka Imaizumi · Daisuke Imanishi · Masako Iwanaga · Jun Taguchi · Takuya Fukushima · Shinichiro Yoshida · Tomoko Hata · Yukiyoshi Moriuchi · Kazutaka Kuriyama · Hiroyuki Mano · Masao Tomonaga · Yasushi Miyazaki

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Abstract The percentage of myeloperoxidase (MPO)-positive blast cells is a simple and highly significant prognostic factor in AML patients. It has been reported that the high MPO group (MPO-H), in which >50% of blasts are MPO activity positive, is associated with favorable karyotypes, while the low MPO group (≤50% of blasts are MPO activity positive, MPO-L) is associated with adverse karyotypes. The MPO-H group shows better survival even when restricted to patients belonging to the intermediate chromosomal risk group or those with a normal karyotype. It has recently been shown that genotypes defined by the mutational status of *NPM1*, *FLT3*, and *CEBPA* are associated with treatment outcome in patients with cytogenetically normal AML. In this study, we aimed to evaluate the relationship between MPO positivity and gene mutations found in normal karyotypes. Sixty AML patients with normal karyotypes were included in this study. Blast cell MPO positivity was assessed in bone marrow smears stained for MPO. Associated genetic lesions (the *NPM1*, *FLT3*-ITD, and *CEBPA* mutations) were studied using nucleotide sequencing. Thirty-two patients were in the MPO-L group, and 28 patients in the MPO-H group. *FLT3*-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). In patients with *CEBPA* mutations, the carrying two simultaneous mutations ($CEBPA^{double\text{-}mut}$) was associated with high MPO expression, while the mutant *NPM1* without *FLT3*-ITD genotype was not associated with MPO activity. Both higher MPO expression and the $CEBPA^{double\text{-}mut}$ genotype appeared to be associated with improved overall survival after intensive chemotherapy. Further studies are required to determine the importance of blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

S. Tominaga-Sato · H. Itonaga · M. Iwanaga · J. Taguchi · Y. Miyazaki
Department of Hematology and Molecular Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Nagasaki, Japan

H. Tsushima (✉) · Y. Imaizumi · D. Imanishi · T. Fukushima · T. Hata
Department of Hematology, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
e-mail: tsushima@nagasaki-u.ac.jp

K. Ando · S. Yoshida
Department of Internal Medicine, Nagasaki National Medical Center, Ohmura, Nagasaki, Japan

Y. Moriuchi
Division of Hematology, Sasebo City General Hospital, Sasebo, Nagasaki, Japan

K. Kuriyama
School of Health Sciences, University of the Ryukyus, Okinawa, Nishihara, Japan

H. Mano
Division of Functional Genomics, Jichi Medical University, Shimotsuke, Tochigi, Japan

M. Tomonaga
Department of Hematology, Japanese Red-Cross Nagasaki Atomic Bomb Hospital, Nagasaki, Nagasaki, Japan

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1 Introduction

The AML87, -89, and -92 studies conducted by Japan Adult Leukemia Study Group (JALSG) revealed that patient age, ECOG performance status, leukocyte count, FAB subclass, the number of induction courses required to achieve complete remission (CR), the presence of good prognostic chromosomal abnormalities [t(8;21) or inv(16)], and percentage of myeloperoxidase (MPO)-stained positive blast cells at diagnosis were significant risk factors for overall survival (OS) of patients with acute myeloid leukemia (AML) [1]. In more recent AML201 study, it was shown that significant unfavorable prognostic features for OS were adverse cytogenetic risk group [2], age of more than 50 years, WBC more than 20×10^9/L, FAB classification of either M0, M6, or M7, and MPO-positive blasts less than 50% [3]. These observations imply that the percentage of MPO-positive blast cells is one of the important prognostic markers along with cytogenetics and molecular genetic information.

MPO, a microbicidal protein, is considered to be a golden marker for the diagnosis of AML in the French–American–British (FAB) and WHO classifications [4, 5]. In our previous reports [6–8], AML patients with a high percentage of MPO-positive blasts (>50% of blasts are MPO activity positive, MPO-H) had a significantly better complete remission (CR) rate, disease-free survival, and overall survival compared with the low MPO activity positive blast group (≤50% of blasts are MPO activity positive, MPO-L). Most patients with a favorable chromosomal risk profile were in the MPO-H group, and most of the patients with an adverse chromosomal risk profile were in the MPO-L group. The difference in OS between the low and high MPO groups was still observed in a cohort of patients with normal karyotypes, suggesting that MPO is highly expressed in the leukemic blasts of AML patients with a favorable prognosis. To fully understand this phenomenon, it would be important to analyze genetic factors associated with MPO expression, especially in patients with a normal karyotype.

In the WHO classification, mutations of *FLT3*, *NPM1* and *CEBPA* have been emphasized to have prognostic significance in AML patients with normal karyotype. The nucleophosmin 1 gene (*NPM1*) has been shown to be mutated in 45–64% of AML cases with a normal karyotype [9, 10], and *NPM1* mutations are associated with a favorable prognosis in the absence of the internal tandem duplication (ITD) type of fms-related tyrosine kinase-3 gene (*FLT3*) mutation, a known adverse prognostic factor [11]. The CCAAT/enhancer binding protein-alpha gene (*CEBPA*) is another gene that has been shown to be mutated in AML patients with a normal karyotype [12, 13]. Mutations in the *CEBPA* gene are found in 5–14% of all AML cases and are associated with a relatively favorable outcome, and hence, have gained interest as a prognostic marker [14]. Recently, it has been shown that most AML patients with *CEBPA* mutations carry 2 simultaneous mutations ($CEBPA^{\text{double-mut}}$), whereas single mutations ($CEBPA^{\text{single-mut}}$) are less common. In addition it was found that the $CEBPA^{\text{double-mut}}$ genotype is associated with a favorable overall and event-free survival [15, 16]. It is still unclear why $CEBPA^{\text{double-mut}}$ AML patients have better outcomes than those with a single heterozygous mutation.

In this study, we retrospectively examined 60 de novo adult AML patients with normal karyotypes in order to obtain a better insight into the relationships between MPO positivity and other prognostic factors (*NPM1*, *FLT3*, and *CEBPA* mutations). In line with previous reports, both high MPO positivity in AML blasts and the $CEBPA^{\text{double-mut}}$ genotype appeared to be associated with a favorable outcome, and it appeared that it was the $CEBPA^{\text{double-mut}}$ genotype that associated with high blast MPO activity.

2 Materials and methods

2.1 Patients and treatments

The study population included 60 patients with newly diagnosed de novo AML that had been treated at the Department of Internal Medicine, Nagasaki National Medical Center, between 1990 and 2010. All patients had normal karyotype AML. AML was diagnosed according to the FAB classification. Two members independently assessed the percentage of MPO-positive blast cells in MPO-stained bone marrow smears. The main biological and clinical features of the patients are shown in Table 1. Excluding the 25 patients who did not receive conventional induction chemotherapy, all patients were treated according to the Japan Adult Leukemia Study Group (JALSG) protocols (AML89, -92, -95, -97, and -201 studies) [3, 17–19]. CR was determined as when blasts accounted for less than 5% of the cells in normocellular bone marrow with normal peripheral neutrophil and platelet counts. This study was approved by the Ethical Committees of the participating hospitals.

2.2 Analysis of the *FLT3*, *NPM1*, and *CEBPA* genes

High molecular weight genomic DNA was extracted from bone marrow and peripheral blood samples after Ficoll

Table 1 Characteristics of de novo AML patients with a normal karyotype

	All patients ($n = 60$)	Patients who received intensive chemotherapy ($n = 36$)
Median age (range) (year)	59.5 (15–81)	49 (15–67)
Male/female	32/28	18/18
FAB type		
M0	5	3
M1	10	5
M2	21	14
M4	18	11
M5	3	1
M6	3	2
M7	0	0
WBC ($\times 10^9$/L), median (range)	14.9 (0.7–556)	13.0 (0.7–246)
Performance status		
0–2	55	34
3–4	5	2
LDH (IU/L), median (range)	296 (120–5,325)	291 (140–2,606)
MPO		
Low (≤50%)	32	20
High (>50%)	28	16

FAB French–American–British, *WBC* white blood cells, *LDH* lactate dehydrogenase, *MPO* myeloperoxidase

separation of mononucleated cells (35 and 4 patients, respectively) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). In addition, we isolated genomic DNA from the BM smears of the AML patients (21 samples) using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany).

Mutations in the *FLT3*, *NPM1*, and *CEBPA* genes were detected by genomic DNA PCR and direct sequencing. Exons 14 and 15 and the intervening intron of the *FLT3* gene were amplified from DNA using the previously described primers FLT3-11F and FLT3-12R [20]. PCR for NPM1 exon 12 was performed with genomic DNA, the same reagent, and the published primer molecules NPM1-F and NPM1-R [21]. PCR for *CEBPA* was performed using 2 overlapping primer pairs: CEBPA-CT3F (5′-TGCCGGGTATAAAA-GCT GGG-3′) and CT3R (5′-CTCGTTGCTGTTCTTGTCCA -3′), CEBPA-PP2F (5′-TGCCGGGT-ATAAAAGCTGG G-3′) and PP2R (5′-CACGGTCTGGGCAAGCCTCG AGAT-3′). The PCR reactions were run in a final volume of 50 μL containing 10 ng DNA, 5× buffer, 0.2 mmol/L of each deoxynucleotide triphosphate, primers (0.3 μmol/L of each), nucleotides (0.2 mmol/L of each), and 1 U of KOD-Plus-Neo polymerase (TOYOBO, Osaka, Japan). The mixture was initially heated at 94°C for 2 min, before being subjected to 35 cycles of denaturation at 94°C for 10 s and annealing and extension at 68°C for 1 min. The amplified products were cut out from a 1.2% agarose gel and purified with the MinElute Gel extraction kit (QIAGEN, Germany). To screen for mutations, the PCR products were sequenced in both directions with the following primers: FLT3-11F, FLT3-12R, NPM1-F, NPM-R, CEBPA-CT1F, CEBPA-1R, CEBPA-PP2F, CEBPA-PP2R, CEBPA-2F (5′-GCTG GGCGGCATCTGCG-A-3′), and CEBPA-1R (5′-TGT-GC TGGAACAGGTCGGCCA-3′) using a BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3100 ×l Genetic Analyzer (Applied Biosystems, CA, USA). In the case of *NPM1* and *CEBPA* genes, when heterozygous data were identified by sequence screening, mutations were confirmed by cloning with the StrataClone Blunt PCR Cloning Kit (Stratagene, CA, USA) according to the manufacturer's recommendations. Four to ten recombinant colonies were chosen and cultured in LB medium. Plasmid DNA was prepared using a QIAprep spin plasmid miniprep kit (Qiagen, Hilden, Germany), and both strands were sequenced using the T3 and T7 primers and the CEBPA-2F and CEBPA-1R primers.

2.3 Statistical methods

To evaluate the relationship between the frequency of mutations status and clinical characteristics, the following variables were included in the analysis: age, FAB classification, peripheral WBC count, MPO-positivity rate, JALSG score [1], and CR achievement. A comparison of frequencies was performed using Fisher's exact test. Differences in percentage of MPO-positive blasts among patients with different mutational status of genes were compared using the non-parametric Kruskal–Wallis test and followed by Dunn's multiple comparison post-test. Overall survival (OS) was calculated using the Kaplan–Meier method [22], and the group differences were compared using the log-rank test. Thirteen patients who underwent allogeneic or autologous hematopoietic stem cell transplantation were not censored at the time of transplantation. For all analyses, statistical significance was considered at the level of two-tailed 0.05.

3 Results

3.1 Patients' characteristics

As shown in Table 1, the series included 60 patients. Their median age was 59.5 (15–81 years), and there were 32 males (53.3%) and 28 females (46.7%). All patients had normal cytogenetics. Using the percentage of MPO-positive leukemic blasts, as judged from bone marrow slides, the cases

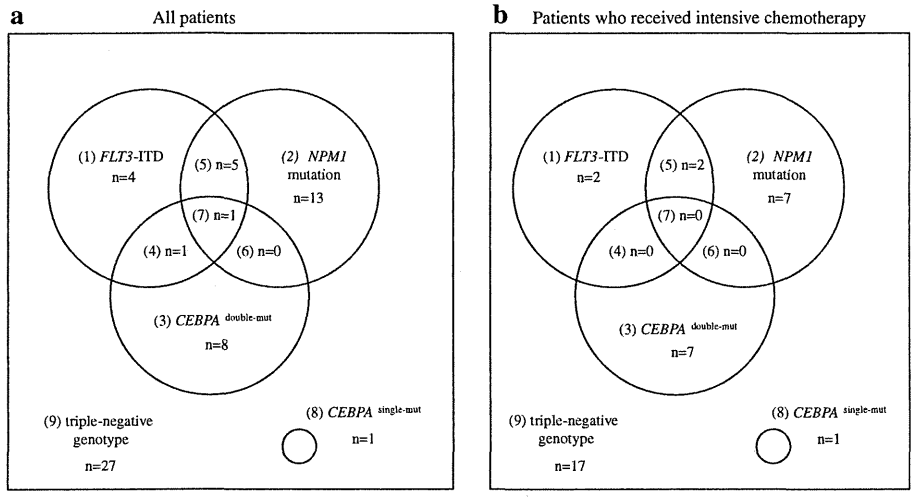

Fig. 1 Frequency and overlapping patterns of AML patients with a normal karyotype. Data are shown for all patients (**a**) and for patients who received intensive chemotherapy (**b**). **a** (*1*) *FLT3*-ITD + wt *NPM1* + wt *CEBPA* ($n = 4$, 6.7%), (*2*) wt *FLT3* + *NPM1* mutation + wt *CEBPA* ($n = 13$, 21.7%), (*3*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 8$, 13.3%), (*4*) *FLT3*-ITD + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 1$, 1.7%), (*5*) *FLT3*-ITD + *NPM1* mutation + wt *CEBPA* ($n = 5$, 8.3%), (*6*) wt *FLT3* + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*7*) *FLT3*-ITD + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 1$, 1.7%), (*8*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{single-mut}}$ ($n = 1$, 1.7%), (*9*) triple-negative genotype ($n = 27$, 45%). **b** (*1*) *FLT3*-ITD + wt *NPM1* + wt *CEBPA* ($n = 2$, 5.6%), (*2*) wt *FLT3* + *NPM1* mutation + wt *CEBPA* ($n = 7$, 19.4%), (*3*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 7$, 19.4%), (*4*) *FLT3*-ITD + wt *NPM1* + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*5*) *FLT3*-ITD + *NPM1* mutation + wt *CEBPA* ($n = 2$, 5.6%), (*6*) wt *FLT3* + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*7*) *FLT3*-ITD + *NPM1* mutation + $CEBPA^{\text{double-mut}}$ ($n = 0$, 0%), (*8*) wt *FLT3* + wt *NPM1* + $CEBPA^{\text{single-mut}}$ ($n = 0$, 0%), (*9*) triple-negative genotype ($n = 17$, 47.2%). *wt* wild-type

were divided into the High group (MPO-positive blasts > 50%) and Low group (MPO-positive blasts ≤ 50%). Thirty-two patients were classified into the Low group, and 28 patients were classified into the High group.

3.2 Mutational analysis

FLT3-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). Frequency and an overlapping pattern of mutations are shown in Fig. 1. Among the patients with *CEBPA* mutations, approximately 90% (10 of 11 patients) of the patients had two *CEBPA* mutations ($CEBPA^{\text{double-mut}}$), whereas 10% (1 of 11 patients) had a single mutation. As previously reported, the mutations in the $CEBPA^{\text{double-mut}}$ patients were clustered in the N- and C-terminal hotspots (Table 2; Fig. 2). *FLT3*-ITD mutation was associated with a higher WBC at the time of diagnosis, as reported previously. Neither *NPM1* nor *CEBPA* mutation status displayed a significant association with age, PS, WBC, FAB subtype, JALSG score, or CR achievement (Table 3).

3.3 Clinical outcome

OS was analyzed only in patients who received intensive chemotherapy ($n = 36$). They received chemotherapy based on the treatment protocol described in the JALSG AML89, -92, -95, -97, and -201 studies. As reported previously [6], we observed an association between the percentage of MPO-positive blasts and the survival rate in the normal karyotype patients treated with intensive chemotherapy, although the significance in this cohort was rather low ($P = 0.10$) (Fig. 3). Figure 4 shows Kaplan–Meier curves according to genotype. 'Other genotypes' included the *FLT3*-ITD genotype, the $CEBPA^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. In line with previous reports [14], the patients with the $CEBPA^{\text{double-mut}}$ genotype tended to show higher survival rate compared with patients displaying other genotypes ($P = 0.07$). In this study, the mutant *NPM1* without *FLT3*-ITD genotype was not significantly associated with treatment outcome, possibly due to the small number of patients.

3.4 Difference of MPO-positivity rate by gene mutation status

Figure 5 shows the level of the percentage of MPO-positive blasts by gene mutational status of the *CEBPA*, *FLT3*-ITD, and *NPM1*. The MPO-positivity rate was very high, over 50% (median 96, range 71–100), in all $CEBPA^{\text{double-mut}}$ cases, but it was 20% in one case displaying the

Table 2 Genetic findings of the patients with *CEBPA* mutations

Patient	Category	Nucleotide changes	Amino acid changes	Comments
4	Double	218_219insC	P23fsX107	Produces N-terminal stop codon
		1129_1130insATGTGGAGACGCAGCAGAAGGTGCTGGAGCTG ACCAGTGACAATGACCGCCTGCGCAAGC	K326_327insHVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
6	Double	200_218delinsCT	S16fsX101	Produces N-terminal stop codon
		1087_1089dup	K313dup	In-frame duplication in bZIP
7	Double	368_369insA	A72fsX107	Produces N-terminal stop codon
		1080_1082del	T310_Q311del	In-frame deletion in bZIP
13	Double	303_316del	P50fsX102	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
19	Double	215_225del	P21fsX103	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCAGA AGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
22	Double	213del	P22fsX159	Produces N-terminal stop codon
		1064_1129dup	K304_Q305insQRNVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
27	Double	324_328dup	E59fsX161	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
39	Double	213del	P22fsX159	Produces N-terminal stop codon
		1081_1086dup	Q311_Q312dup	In-frame duplication in bZIP
47	Double	397del	F82fsX159	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCA GAAGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
49	Double	297_304del	A48fsX104	Produces N-terminal stop codon
		758del	A202fsX317	Frameshift between TAD2 and bZIP; produces stop codon in bZIP
35	Single	1087_1089dup	K313dup	In-frame duplication in bZIP

Nucleotide numbering was performed according to NCBI Entrez accession no. XM_009180.3, in which the major translational start codon starts at nucleotide position 151. The locations of functional domains are derived from Mueller and Pabst.1

bZIP basic leucine zipper region, *TAD2* second transactivation domain

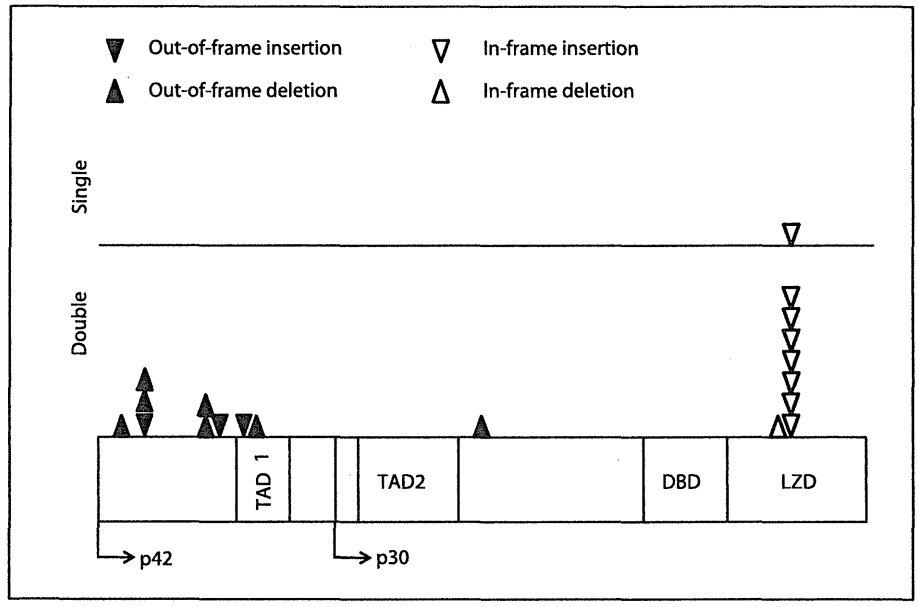

Fig. 2 Location of mutations detected in the *CEBPA*$^{single\text{-}mut}$ and *CEBPA*$^{double\text{-}mut}$ patients. Transactivation domain (TAD) 1, amino acids (AA) 70–97; p30 ATG, AA120; TAD2, AA 126–200; DNA-binding domain (DBD), AA 278–306; leucine zipper domain (LZD), AA 307–358

CEBPA$^{single\text{-}mut}$ genotype (data not shown). The MPO-positivity rate was widely distributed in patients who had mutant *NPM1* without *FLT3*-ITD genotype (median 26, range 0–100) and other genotypes (median 31, range 0–100). Kruskal–Wallis test showed that a significant difference of the MPO-positivity rate among three groups ($P = 0.005$). When comparing the individual groups by Dunn's Multiple Comparisons post hoc test for each group, there was a significant difference only for patients with *CEBPA*$^{double\text{-}mut}$ versus patients with other genotypes.

4 Discussion

While cytogenetic group is considered to be the primary prognostic indicator in AML, the percentage of MPO-positive blast cells could be used to predict the prognosis of patients with normal karyotypes [6]. In this study, we found that *CEBPA* gene mutational status has impact on the frequency of MPO expression: the patients with the *CEBPA* mutation genotype displayed a significantly higher percentage of cells expressing MPO than those with other genotypes ($P < 0.01$). The association was even more significant when analyzed without the *CEBPA*$^{single\text{-}mut}$ carrying patient, suggesting that high blast MPO activity is related to double *CEBPA* mutations. Although the mutant *NPM1* without *FLT3*-ITD genotype has been reported to be associated with a favorable prognosis in AML patients, there was no relationship between this type of mutation and the percentage of blasts showing MPO expression.

It is not clear how the *CEBPA*$^{double\text{-}mut}$ genotype enhances MPO activity in AML blasts. It has been shown that the MPO enhancer contains a *CEBPA* site contributing to its functional activity [23, 24], suggesting that the MPO gene is a major target of C/EBPα. Since it has been shown that both N-terminal frame-shift mutant and C-terminal mutant do not show transcriptional activity [25], we first speculated that mutations of the *CEBPA* gene might lead to decreased MPO activity, which turned out to be wrong. AML1 is another gene that has been reported to participate in up-regulation of MPO gene [26]. An AML1 site was identified in upstream enhancer of the human MPO gene, which appears to be necessary for maximal stimulation of MPO promoter activity. In patients with AML with t(8;21), the translocation results in an in-frame fusion between 5 exons of the AM1 gene and essentially all of the ETO gene producing a chimeric protein [27]. This protein, AML1-ETO, acts as a negative dominant inhibitor of wild-type AML1 [28], which theoretically could lead to down-regulation of AML1 target genes, such as MPO gene. However, blasts with t(8;21) have been shown to display higher levels of MPO expression both in clinical samples and in vitro experiments [29, 30], suggesting that the transcriptional alterations caused by these mutations are complex. The upregulation of blast MPO activity seen in CEBP/$\alpha^{double\text{-}mut}$ cases may be due to alterations in the gene expression profile, rather than a simple dominant negative effect of mutated CEBP/α. Further experiments including investigation of transactivation potential of CEBP/α mutants on MPO promoter is necessary to clarify this mechanism.

CEBPA mutations are associated with a relatively favorable outcome, and it was recently shown in a multivariable analysis including cytogenetic risk and the

Table 3 Frequency *of FLT3*-ITD, *NPM1*, and *CEBPA* mutations by clinical characteristics in de novo AML cases with a normal karyotype

	FLT3		*P*	*NPM1*		*P*	*CEBPA*		*P*
	ITD ($n = 11$)	Other type ($n = 49$)		Mutation without *FLT3*-ITD ($n = 13$)	Other type ($n = 47$)		Double mutation without *FLT3*-ITD ($n = 8$)	Other type ($n = 52$)	
Age			0.08			0.74			0.10
≤50	1	19		5	15		5	15	
>50	10	30		8	32		3	37	
PS			1.00			0.20			0.52
0–2	10	45		11	45		7	48	
3–4	1	4		2	2		1	4	
WBC			0.02			1.00			1.00
≤20,000	2	30		7	25		4	28	
>20,000	9	19		6	22		4	24	
FAB subtype			0.33			0.18			0.58
M1, M2, M4, M5	11	41		13	39		8	44	
M0, M6, M7	0	8		0	8		0	8	
JALSG score[a]			0.79			0.72			0.09
Favorable	0	5		0	5		2	3	
Intermediate	2	18		5	15		5	15	
Adverse	2	9		2	9		0	11	
CR[a]			1.00			0.56			0.56
Achievement	4	27		7	24		7	24	
Failure	0	5		0	5		0	5	

[a] Analysis was carried in 36 patients with intensive chemotherapy

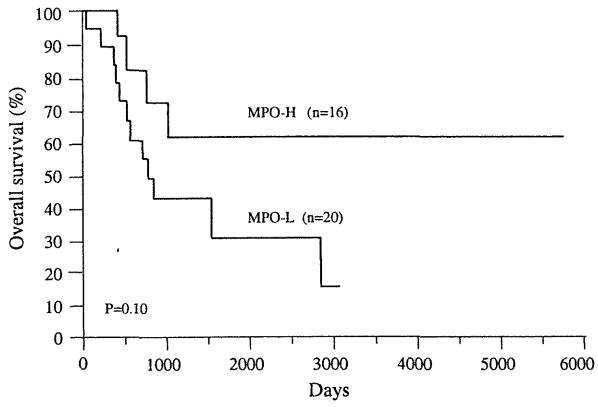

Fig. 3 Kaplan–Meier estimates of the probability of overall survival in 36 patients who received intensive chemotherapy, according to the percentage of myeloperoxidase-positive blasts. MPO-H (MPO-positive blasts: >50%) tended to have a positive effect on overall survival compared with MPO-L (MPO-positive blasts: ≤50%), although the difference was not statistically significant. The statistical significance of differences was evaluated with the log-rank test

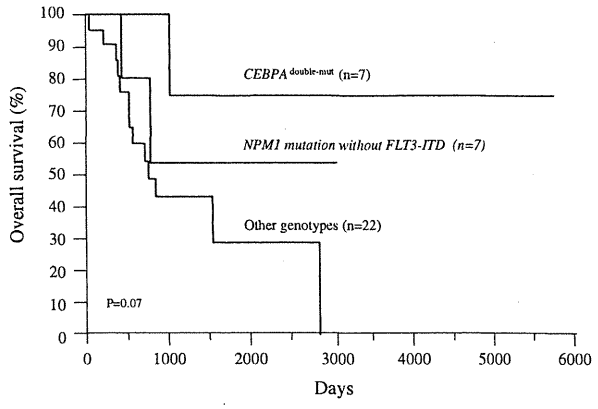

Fig. 4 Overall survival according to genotype in patients administered intensive chemotherapy. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA*$^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The patients with the *CEBPA*$^{\text{double-mut}}$ genotype tended to show higher overall survival compared with the patients with 'other genotypes' ($P = 0.07$)

FLT3-ITD and *NPM1* mutations that the *CEBPA*$^{\text{double-mut}}$ genotype is associated with favorable overall and event-free survival [15, 16]. In a cohort of 60 cases of adult de novo AML, we identified 1 *CEBPA*$^{\text{single-mut}}$ case and 10 *CEBPA*$^{\text{double-mut}}$ cases, and in line with previous reports, our study tended to show better overall survival in *CEBPA*$^{\text{double-mut}}$ cases compared to cases with wild-type *CEBPA* in patients treated with intensive chemotherapy. We failed to find a prognostic effect in relation to the *CEBPA*$^{\text{double-mut}}$ in patients treated with low dose

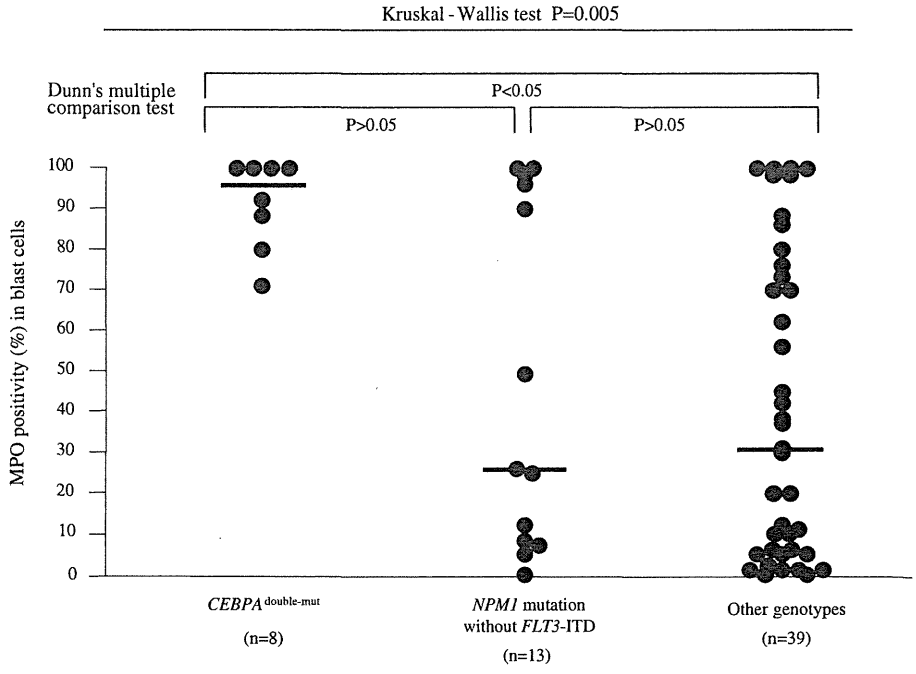

Fig. 5 MPO-positivity rate in blast according to genetic abnormalities in de novo AML patients with a normal karyotype. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA*$^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The median MPO-positivity rate (*horizontal line*) was significantly different between the *CEBPA*$^{\text{double-mut}}$ genotype and 'other genotypes' (Kruskal–Wallis test followed by Dunn's multiple comparisons test: $P < 0.05$)

chemotherapy (data not shown), suggesting that the standard chemotherapy dose is necessary to improve the outcome of *CEBPA*$^{\text{double-mut}}$ cases.

It is unclear why *CEBPA*$^{\text{double-mut}}$ AML patients have a better outcome than those with *CEBPA* wild-type AML. One explanation is that high MPO expression leads to increased sensitivity to chemotherapeutic agents, such as to Ara-C [8]. To test this hypothesis, we also examined the association between blast MPO positivity and overall survival in *CEBPA* wild-type cases. Unexpectedly, when the patients were treated with intensive chemotherapy, the percentage of MPO-positive blasts was not significantly associated with overall survival in this group (data not shown), suggesting that the level of MPO expression itself is not responsible for the improvement in overall survival. However, as this analysis only involved 28 cases, we need to increase the number of cases in order to draw a definitive conclusion.

In summary, the data presented here suggested that the *CEBPA*$^{\text{double-mut}}$ genotype was associated with high MPO blast activity in patients with a normal karyotype. Although the results were obtained from a single institution, the presence of *CEBPA*$^{\text{double-mut}}$ genotype in high MPO group could explain, at least in part, why high MPO blast activity is associated with better overall survival. Further studies in a larger cohort of patients are necessary to assess blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

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Conflict of interest All authors have no conflict of interest to report.

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Clinical Cancer Research

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Clinical Cancer Research

Imaging, Diagnosis, Prognosis

Pulmonary Inflammatory Myofibroblastic Tumor Expressing a Novel Fusion, PPFIBP1–ALK: Reappraisal of Anti-ALK Immunohistochemistry as a Tool for Novel ALK Fusion Identification

Kengo Takeuchi[1,2], Manabu Soda[4], Yuki Togashi[1,2], Emiko Sugawara[1,5], Satoko Hatano[1,2], Reimi Asaka[1,2], Sakae Okumura[3], Ken Nakagawa[3], Hiroyuki Mano[4,6], and Yuichi Ishikawa[2]

Abstract

Purpose: The anaplastic lymphoma kinase (ALK) inhibitor crizotinib has been used in patients with lung cancer or inflammatory myofibroblastic tumor (IMT), both types harboring ALK fusions. However, detection of some ALK fusions is problematic with conventional anti-ALK immunohistochemistry because of their low expression. By using sensitive immunohistochemistry, therefore, we reassessed "ALK-negative" IMT cases defined with conventional immunohistochemistry (approximately 50% of all examined cases).

Experimental Design: Two cases of ALK-negative IMT defined with conventional anti-ALK immunohistochemistry were further analyzed with sensitive immunohistochemistry [the intercalated antibody-enhanced polymer (iAEP) method].

Results: The two "ALK-negative" IMTs were found positive for anti-ALK immunohistochemistry with the iAEP method. 5′-rapid amplification of cDNA ends identified a novel partner of ALK fusion, protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein-binding protein 1 (PPFIBP1) in one case. The presence of PPFIBP1–ALK fusion was confirmed with reverse transcriptase PCR, genomic PCR, and FISH. We confirmed the transforming activities of PPFIBP1–ALK with a focus formation assay and an *in vivo* tumorigenicity assay by using 3T3 fibroblasts infected with a recombinant retrovirus encoding PPFIBP1–ALK. Surprisingly, the fusion was also detected by FISH in the other case.

Conclusions: Sensitive immunohistochemical methods such as iAEP will broaden the potential value of immunohistochemistry. The current ALK positivity rate in IMT should be reassessed with a more highly sensitive method such as iAEP to accurately identify those patients who might benefit from ALK-inhibitor therapies. Novel ALK fusions are being identified in various tumors in addition to IMT, and thus a reassessment of other "ALK-negative" cancers may be required in the forthcoming era of ALK-inhibitor therapy. *Clin Cancer Res; 17(10); 3341–8. ©2011 AACR.*

Introduction

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase that was discovered in anaplastic large cell lymphoma (ALCL) in the form of a fusion protein, NPM–ALK. (1, 2). In addition to ALCL (fused to NPM, TPM3, TPM4, ATIC, TFG, CLTC, MSN, MYH9, or ALO17; refs. 1–10), ALK has further been found to generate fusions in inflammatory myofibroblastic tumor (IMT; TPM3, TPM4, CLTC, CARS, RANBP2, ATIC, or SEC31L1; refs. 10–15), ALK-positive large B-cell lymphoma (CLTC, NPM, SEC31L1, or SQSTM1; 16–19), lung cancer (EML4 or KIF5B; refs. 20, 21), and ALK-positive histiocytosis (TPM3; ref. 22) Besides, some ALK fusions have been reported without showing histopathologic evidence: TPM4–ALK in esophageal squamous cell carcinoma (23, 24), TFG–ALK in lung adenocarcinoma (25), and EML4–ALK in colon and breast carcinomas (26). The wild-type ALK is mainly expressed in the developing nervous system, and is usually not expressed in other normal tissues (27). A fusion protein formation with a partner through chromosomal translocations is the most common mechanism of ALK overexpression and ALK kinase domain activation. These features render ALK fusion oncokinase an ideal molecular target.

Recently, the ALK inhibitor crizotinib has been used in patients with lung cancer or IMT, both types harboring ALK fusions (28, 29). The compound showed a 57% response rate in lung cancers (28), and a strong response for several months in IMT (29). Crizotinib and other ALK inhibitors

Authors' Affiliations: [1]Pathology Project for Molecular Targets; [2]Division of Pathology; and [3]Department of Thoracic Surgical Oncology, Thoracic Center, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo; [4]Division of Functional Genomics, Jichi Medical University, Tochigi; [5]Department of Comprehensive Pathology, Graduate School, Tokyo Medical and Dental University; and [6]Department of Medical Genomics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

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Corresponding Author: Kengo Takeuchi, Pathology Project for Molecular Targets, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan. Phone: +8133-520-0111; Fax: +8133-570-0230; E-mail: kentakeuchi-tky@umin.net

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Translational Relevance

Anaplastic lymphoma kinase (ALK) inhibitors have become one of the most promising groups of molecularly targeted drugs. Therefore, ALK is no longer a mere research target or simply a diagnostic marker, but is directly linked to the therapeutic benefit of patients harboring the fusions.

Pathologic diagnoses for ALK fusion-positive tumors have been made reliably with anti-ALK immunohistochemistry. Since the discovery of EML4–ALK, however, an unexpected problem in anti-ALK immunohistochemistry has become apparent, that is, the inability to detect a low level of EML4–ALK expression. To overcome this, we developed the intercalated antibody-enhanced polymer immunohistochemistry, which successfully detected EML4–ALK.

In other words, this indicates that unknown ALK fusions, particularly those expressed at a low level, may wait to be discovered in "ALK-negative" tumors defined with conventional immunohistochemistry. In the forthcoming era of ALK-inhibitor therapy, "ALK-negative" tumors should be reassessed with a high sensitive immunohistochemistry and, if positive, be further examined with appropriate molecular method(s).

have thus become one of the most promising groups of molecularly targeted drugs. Therefore, the sensitive and accurate identification of ALK fusion in tumors has also become clinically relevant, because it is no longer a mere research target or simply a diagnostic marker, but is directly linked to the therapeutic benefit of patients harboring the fusions.

Identification of such ALK fusions, especially within ALCL, has been prompted by the immunohistochemical staining pattern with antibodies to ALK. In ALCL, the most common ALK fusion is NPM–ALK (comprising approximately 80% of all cases), and its immunohistochemical staining pattern is both nuclear and cytoplasmic. NPM has a nuclear localization signal in the C-terminal region, and therefore the heterodimers of wild-type NPM with NPM–ALK fusion protein are transported to the nucleus whereas NPM–ALK homodimers remain within the cytoplasm (30). In contrast, other fusions do not localize in the nucleus and do not show a nuclear staining pattern in anti-ALK immunohistochemistry. Interestingly, each ALK fusion usually has its own characteristic anti-ALK immunohistochemical staining pattern, because the subcellular localization of ALK fusions is dependent on the corresponding fusion partners. Anti-ALK immunohistochemistry has thus become a highly useful tool for both research and diagnostic purposes.

Since the discovery of EML4–ALK fusion in lung cancer (20), however, an unexpected problem in anti-ALK immunohistochemistry has become apparent, that is, the inability to detect a low level of fusion expression. To overcome this, we developed the intercalated antibody-enhanced polymer (iAEP) method, which moderately raises sensitivity in the immunohistochemical detection system (21). With this very simple method, anti-ALK immunohistochemistry has become a potent weapon in the diagnosis of EML4–ALK-positive lung cancer (21, 31–33). Other researchers used an anti-ALK rabbit monoclonal antibody, which is usually more sensitive than mouse monoclonal antibody, which can stain EML4–ALK (34). However, most EML4–ALK-positive lung cancer tissues do not stain well with conventional anti-ALK immunohistochemical methods because of the low message/protein level of EML4–ALK (21, 35). The expression level of a fusion gene depends on the promoter activity of the 5′-side gene, and that of EML4 is likely to be lower than that of the other ALK fusion partner genes, which may explain why EML4–ALK had not been discovered until 12 years after the development of the first anti-ALK antibody became available for immunohistochemistry (36). In other words, a tumor that immunostains for ALK only by a sensitive immunohistochemistry method may harbor a novel ALK fusion. Interestingly, in this study, we detected 2 IMT cases positive for ALK immunohistochemistry only when stained by iAEP method (21), and successfully identified a novel fusion gene, protein-tyrosine phosphatase, receptor-type, F polypeptide-interacting protein-binding protein 1 (PPFIBP1)–ALK.

Materials and Methods

Materials

Pathologic specimens from 2 pulmonary IMT cases, originally diagnosed as fibrous histiocytoma (1988: case 1, 45-year-old male; 1998: case 2, 34-year-old female), were reassessed morphologically and immunohistochemically. Surgically removed tumor specimens were routinely fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathologic examination. For case 2, total RNA was extracted from the corresponding snap-frozen specimen and purified with the use of an RNeasy Mini kit (Qiagen). The study was approved by the institutional review board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was sliced at a thickness of 4 μm, and the sections were placed on silane-coated slides. For antigen retrieval, the slides were heated for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako). For the conventional staining procedure, the slides were incubated at room temperature with Protein Block Serum-free Ready-to-Use solution (Dako) for 10 minutes and then with primary antibodies against ALK (5A4), smooth muscle actin, muscle-specific actin (HHF35), CD34, cytokeratins (AE1/AE3), S100, or desmin for 30 minutes. The immune complexes were then detected with dextran polymer reagent (EnVision + DAB system; Dako) and an AutoStainer instrument (Dako). The iAEP method was also used for the sensitive detection of ALK, as described previously (21).

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Isolation of PPFIBP1–ALK fusion

To obtain cDNA fragments corresponding to a novel *ALK* fusion gene, we used a 5′-RACE method with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions, with a minor modification: the ALK2458R primer (5′-GTAGTTGGGGTTGTAGTCGGT-CATGATGGT-3′) was used as the gene-specific reverse primer.

From the oligo(dT)-primed cDNA obtained from case 2 RNA, a 471bp cDNA fragment containing the fusion point was specifically amplified with the primers PPFIBP1-592F (5′-AGAGACACAGAGGGGCTGATT-3′) and ALK3078RR (5′-ATCCAGTTCGTCCTGTTCAGAGC-3′).

PCR analysis of genomic DNA for *PPFIBP1–ALK* in case 2 was carried out with a pair of primers flanking the putative fusion point, PPFIBP1-607F (5′-CTGATTCAGGAGATCA-ATGATTTGAGGT-3′) and Fusion-RT-AS (5′-TCTTGCCAG-CAAAGCAGTAGTTGG-3′).

From the cDNA, a full-length cDNA for *PPFIBP1–ALK* was amplified by PCR with the PA-w-cDNA-in-S primer (5′-TATCTGGGTTGGAATTTGCCCCTG-3′) and the KA-w-cDNA-in-AS primer (5′-TGAGTGTGCGACCGAGCTCAGG-3′) and PrimeSTAR HS DNA polymerase (TakaraBio).

FISH

FISH analysis of gene fusion was carried out with bacterial artificial chromosome (BAC) clone-derived DNA probes for *ALK* and *PPFIBP1*. Unstained sections (4 μm thick) were subjected to hybridization with an *ALK*-split probe set (Abbott) or BAC clone-derived probes for *ALK* (RP11-984I21, RP11-62B19) and *PPFIBP1* (RP11-1060J15). Hybridized slides were then stained with DAPI and examined with the fluorescence microscope BX51 (Olympus).

Transformation assay for ALK fusion proteins

Analysis of the transforming activity of PPFIBP1–ALK was carried out as described previously (20, 37, 38). Briefly, the pMXS-based expression plasmid for PPFIBP1–ALK, EML4–ALK variant 1, or NPM–ALK was used to generate recombinant ecotropic retrovirus, followed by individual infection of mouse 3T3 fibroblasts (39). Formation of the transformed foci was evaluated after culturing the cells for 14 days. The same set of 3T3 cells was subcutaneously injected into nu/nu mice, and tumor formation was examined after 20 days. The animal experiments were approved by the animal ethics committee of Jichi Medical University.

Results

Morphology and immunophenotype of PPFIBP1–ALK-positive IMT

Histopathologic analysis of the 2 IMT cases revealed a marked proliferation of cells composed of somewhat histiocytoid spindle cells showing a fascicular or storiform pattern. The tumor cells were uniform and had pale eosinophilic cytoplasm and an oval vesicular nucleus, within which a small nucleolus was centrally located. Mild inflammatory infiltrate containing lymphocytes, plasma cells, foamy histiocytes, and multinucleated giant cells was observed (Fig. 1A and 1D). The immunophenotype of the 2 cases was negative for smooth muscle actin,

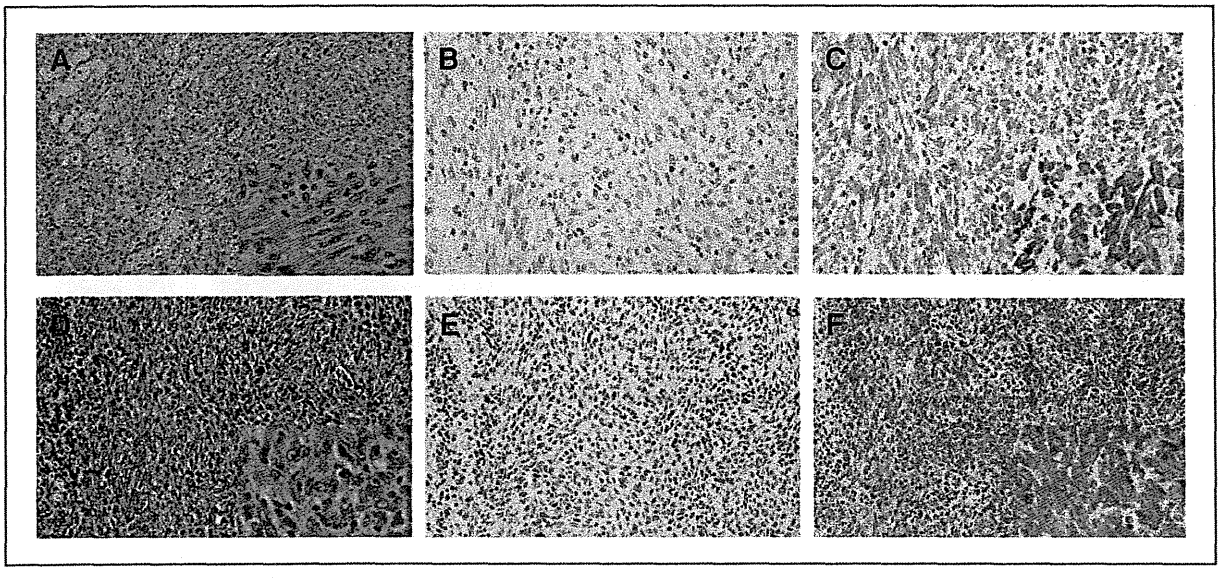

Figure 1. Histopathology of PPFIBP1–ALK-positive IMT. Diffuse proliferation of histiocytoid spindle cells showing a fascicular or storiform pattern. The tumor cells were uniform and had pale eosinophilic cytoplasm and an oval vesicular nucleus, within which a small nucleolus was centrally located. Mild inflammatory infiltrate containing lymphocytes, plasma cells, and foamy histiocytes is observed (A and D). The tumor cells were negative for ALK with conventional anti-ALK immunohistochemistry (B and E), but were clearly positive for ALK when the iAEP method was used. The staining pattern is diffuse cytoplasmic (C and F). Case 1 (A–C), Case 2 (D–F).

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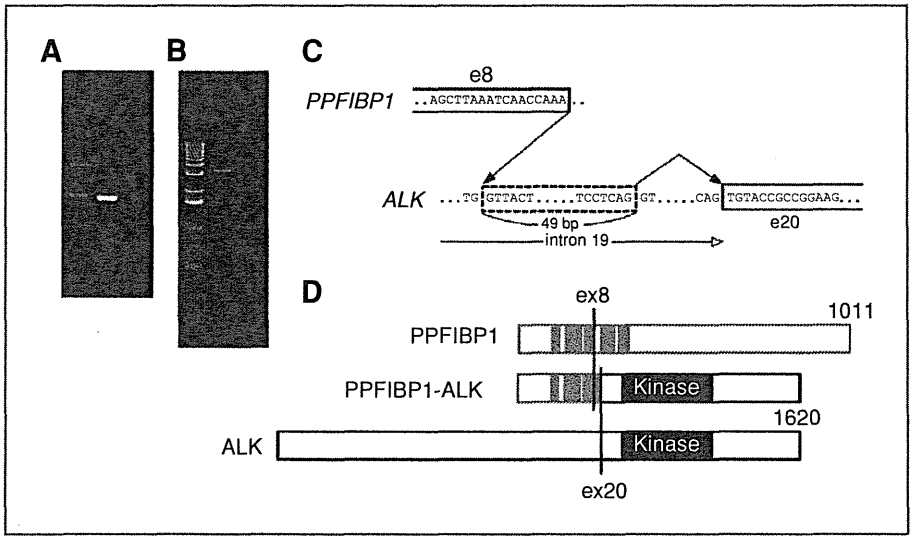

Figure 2. Identification of PPFIBP1–ALK: a PCR product of 471 bp covering the fusion point of *PPFIBP1–ALK* cDNA was specifically amplified from the tumor cells of case 2. The left lane contains DNA size standards (100 bp ladder). The right lane represents no template control (A). A PCR product of approximately 3 kbp covering the genomic fusion point of *PPFIBP1–ALK* was specifically amplified from the tumor cells of case 2. The left lane contains DNA size standards (1 kbp ladder). The right lane represents no template control (B). In our 5′-RACE products, exon 8 of *PPFIBP1* cDNA was fused to a 49 bp sequence in intron 19 of *ALK*, followed by exon 20 of *ALK* (C). PPFIBP1 contains 5 coiled-coil domains. A chromosome translocation, t(2;10)(p23;p11), generates a fusion protein in which the top 3 coiled-coil domains of PPFIBP1 and the intracellular region of ALK (containing the tyrosine kinase domain) are conserved. Numbers indicate amino acid positions of each protein (D).

HHF35, CD34, AE1/AE3, and S100. Desmin was focally positive in case 1, but was negative in case 2.

Identification of PPFIBP1–ALK as a novel ALK fusion gene

We conducted anti-ALK immunohistochemistry on 2 morphologically typical pulmonary IMT cases, originally diagnosed as fibrous histiocytoma. Immunostaining for ALK with the conventional polymer method led to the revised diagnosis of "ALK-negative" IMT (Fig. 1B and E). In the present study, anti-ALK immunohistochemistry with the iAEP method, however, showed a diffuse positive cytoplasmic staining (Fig. 1C and F), indicating the possibility of ALK fusion to a novel partner gene, the expression level of which is modest. To address this issue, in case 2 we conducted 5′-RACE assay for the isolation of an upstream cDNA to the ALK kinase domain cDNA, for which snap-frozen material was available.

Interestingly, we isolated a cDNA fragment containing exon 8 of *PPFIBP1* followed by a 49 bp-sequence within intron 19 of *ALK* and coupled to exon 20 of *ALK* (Fig. 2), suggesting the presence of a novel fusion between *PPFIBP1* and *ALK* genes. Because insertion of the intronic 49 bp allows an in-frame fusion between the 2 genes, this rearrangement likely produces a novel fusion-type tyrosine kinase. To confirm the genomic rearrangement responsible for the *PPFIBP1–ALK* fusion, a genomic PCR assay (Fig. 2B) and both *ALK* split and *PPFIBP1–ALK* fusion FISH assays (Fig. 3) were carried out. All results were consistent with the presence of t(2;12)(p23;p11) leading to the generation of *PPFIBP1–ALK*. Owing to the limited material available in case 1, only the FISH analyses were carried out. Surprisingly, these results also indicate the presence of *PPFIBP1–ALK* (Fig. 3, Supplementary Fig. 2A–C).

Transforming activities of PPFIBP1–ALK

To prove that the t(2;12)(p23;p11) rearrangement leads to the production of PPFIBP1–ALK kinase, in case 2 we attempted to amplify from the cDNA a full-length cDNA encoding the protein. By using a sense primer at the 5′-untranslated region of *PPFIBP1* mRNA (GenBank accession no. NM_003622) and an antisense primer at

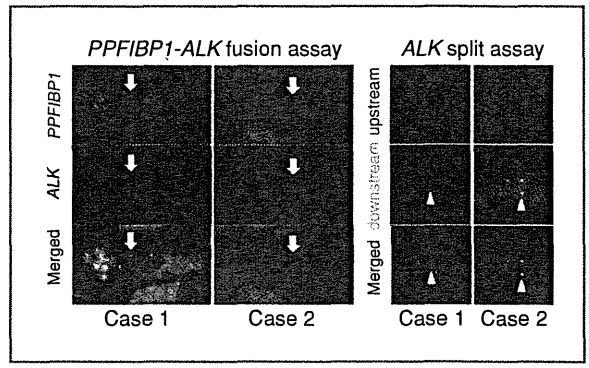

Figure 3. FISH analyses for *PPFIBP1–ALK*: sections of tumors positive for PPFIBP1–ALK were subjected to FISH analyses. In *PPFIBP1–ALK* fusion assays (left) the fusion genes are indicated by arrows. In *ALK* split assays (right) the 3′-sides of *ALK* are indicated by arrowheads. The color of fluorescence for the BAC clones and the case numbers in each hybridization are indicated. Nuclei are stained blue with DAPI.

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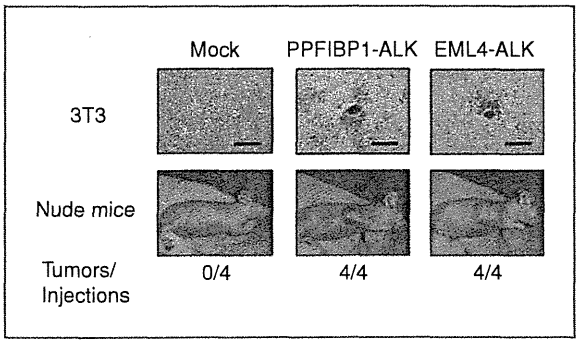

Figure 4. Transforming potential of PPFIBP1–ALK. Top, mouse 3T3 fibroblasts were infected with retroviruses encoding PPFIBP1–ALK or EML4–ALK or with the corresponding empty virus (Mock). The cells were photographed after 14 days of culture. Scale bars, 400 μm. Bottom, Nude mice were injected subcutaneously with the corresponding 3T3 cells, and tumor formation was examined after 14 days. The number of tumors formed per 4 injections is indicated at the bottom.

the 3′-untranslated region of *ALK* mRNA (GenBank accession no. NM_004304), a full-length *PPFIBP1–ALK* cDNA of 2488 bp was successfully amplified, which should have produced a fusion kinase of 811 amino acids with a predicted molecular weight of 90,740 Da (Supplementary Fig. 1).

To examine the transforming potential of PPFIBP1–ALK, a recombinant ecotropic retrovirus was generated to express PPFIBP1–ALK, which was used to infect mouse 3T3 fibroblasts. As shown in Figure 4, PPFIBP1–ALK produced hundreds of transformed foci over 14 days of culture, which was comparable with the observation with EML4–ALK. Furthermore, subcutaneous injection of the infected 3T3 cells into the shoulder of nude mice revealed that those expressing either PPFIBP1–ALK or EML4–ALK formed large tumors *in vivo*.

Discussion

Since their discovery in 1994, appropriate diagnosis of ALK fusion-positive tumors with conventional anti-ALK immunohistochemistry methods has been accepted. However, EML4–ALK in lung adenocarcinoma, identified in 2007, did not stain positive for ALK with conventional immunohistochemistry methods (21, 35). We developed a sensitive immunohistochemistry method, the iAEP method, and successfully stained EML4–ALK with ordinary anti-ALK mouse monoclonal antibodies (21, 31–33). Such observation further indicates a possibility that staining cancer specimens with sensitive immunohistochemical methods (such as iAEP) may detect novel ALK fusions in the "ALK-negative" tumors defined by conventional anti-ALK immunohistochemistry methods. On the basis of this hypothesis, we have identified a novel ALK fusion in "ALK-negative" IMT.

Caution is needed in practical settings. For example, rhabdomyosarcoma, especially of the alveolar type, often expresses wild-type ALK at a detectable level with conventional anti-ALK immunohistochemistry (40). Moreover, in our experience, a small portion of small cell carcinoma and large cell endocrine carcinoma of the lung, and some sarcomas, may be positive for ALK by iAEP immunohistochemistry, expressing wild-type ALK. Therefore, in order to specifically detect ALK fusions with sensitive anti-ALK immunohistochemistry, a confirmatory test by using FISH, RT-PCR, or similar is usually required. If a tumor is positive for a confirmatory test and the suspected partner gene is not a reported one, 5′-RACE or inverse reverse transcriptase PCR methods can be used for the identification of the suspected partner. Even if overexpressed, wild-type ALK may not be oncogenic (20, 21, 37, 38), although some investigators have suggested that wild-type ALK overexpression above a certain threshold level drives the growth of neuroblastoma (41). Further investigation will be required to clarify if wild-type ALK overexpression is a target for ALK inhibitor therapy.

IMT is a rare mesenchymal tumor that has been referred to as inflammatory pseudotumor, plasma cell granuloma, fibroxanthoma, fibrous histiocytoma, pseudosarcomatous myofibroblastic tumor, and invasive fibrous tumor of the tracheobronchial tree (42). It occurs in the soft tissues as well as in the viscera and the lung, and is more likely to occur in children and young adults. Histologically, IMT is composed of a variable admixture of bland, spindle-shaped myofibroblast-like cells and an inflammatory component of lymphocytes, eosinophils, plasma cells, and macrophages. Recent genetic studies have elucidated clonal chromosomal abnormality involving 2p23, at which ALK is located, in a subset of IMT. The expression of ALK fusion proteins is detected by anti-ALK immunohistochemistry in approximately 50% of IMT cases (42), in which various ALK fusion genes have been reported (Table 1). Collectively, these lines of evidence support ALK-positive IMT being a distinct neoplastic entity. However, the other 50% of IMT cases are negative for anti-ALK immunohistochemistry, and thus in terms of pathogenesis it remains unknown whether these ALK-negative IMTs should be included in the same entity or not. In fact, 1 ALK-negative IMT case did not respond to crizotinib therapy (29). However, we have detected a novel ALK-fusion in "ALK-negative" IMT that subsequently proved positive for ALK with the iAEP immunohistochemistry method. Therefore, unexpectedly lowly expressed ALK fusions may explain the pathogenesis of a portion of "ALK-negative" IMT cases. PPFIBP1–ALK represents such an ALK fusion, although we do not yet know what proportion of "ALK-negative" IMTs can be attributed to this novel subtype. "ALK-negative" IMT warrants screening with the iAEP method to detect this fusion or other, unrecognized, ALK fusions.

PPFIBP1 codes liprin beta 1 (also called PTPRF-interacting protein-binding protein 1). This 114 kDa protein is a member of the leukocyte common antigen–related (LAR) transmembrane tyrosine phosphatase-interacting protein family that may regulate LAR protein properties via interaction with another member of the family, liprin alpha1 (43). Liprin beta 1 expresses in intestinal lymphatic endothelial cells *in vitro* and lymphatic vasculature *in vivo*,

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Table 1. ALK fusion partners in well-documented IMT cases

Partner	Locus	Age	Sex	Site	Year, First author
TPM3	1p23	30	F	Lung	2000, Lawrence
		23	F	Abdomen	2000, Lawrence
		4	M	Lung	2006, Yamamoto
		29	F	Ileum	2006, Milne
		4	M	Lung	2007, Kinoshita
TPM4	19p13	1	M	Abdomen	2000, Lawrence
		6	M	Mesentery	2003, Hisaoka
		25	M	Prostate	2003, Hisaoka
		5	M	Mesentery	2006, Yamamoto
		5	F	Urinary bladder	2006, Yamamoto
CLTC	17q23	3	F	Neck	2001, Bridge
		37	M	Pelvis	2001, Bridge
		2	M	Thoracic cavity	2006, Yamamoto
		6	M	Mesentery	2006, Yamamoto
		0	F	Mediastinum	2007, Patel
CARS	11p15	0	M	Abdomen	2002, Cools
		10	M	Neck	2003, Debelenko
RANBP2	2q13	7	M	Abdomen	2003, Ma
		0	M	Abdomen	2003, Ma
		2	M	Abdomen	2007, Patel
		34	M	Liver	2008, Chen
		44	M	Abdomen	2010, Butrynski
ATIC	2q35	46	M	Urinary bladder	2003, Debiec-Rychter
SEC31L1	4q21	23	M	Abdomen	2006, Panagopoulos
PPFIBP1	12p11	45	M	Lung	Present case 1
		34	F	Lung	Present case 2

and plays an important role in the maintenance of lymphatic vessel integrity in *Xenopus* tadpoles (44). PPFIBP1 has 5 coiled-coil domains in exons 5 through 12, and the upper 3 domains are conserved in fusion form with ALK (Fig. 2D). The coiled-coil domain is shared in all ALK fusion partners (except for NPM, MSN, and SQSTM1), with which the ALK fusion proteins homodimerize leading to constitutive activation of ALK kinase domains (8, 19). As expected, in the present study, the oncogenicity of PPFIBP1–ALK was clearly confirmed with an *in vitro* focus formation assay and an *in vivo* tumorigenicity assay.

The difference in subcellular localization has contributed to the discovery/identification of various ALK fusions. Likewise, the difference in the expression level found is here proved important in the accurate detection of fusion proteins. Sensitive immunohistochemical methods such as iAEP will broaden the potential value of immunohistochemistry, which is a simple and long-established histopathologic technique in the fields of research and diagnosis. The ALK positivity rate (approximately 50%) in IMT should be reassessed with these more sensitive methods, possibly leading to the identification of novel ALK fusions and more candidates for ALK inhibitor therapy. A novel ALK fusion, VCL–ALK, has recently been identified in renal cancers (45, 46). In addition to IMT, therefore, a reassessment of diverse "ALK-negative" human cancers may be required in the forthcoming era of ALK inhibitor therapy.

Disclosure of Potential Conflicts of Interest

K. Takeuchi, scientific advisor for developing an anti-ALK iAEP immunohistochemistry kit (ALK Detection Kit, Nichirei Bioscience, Japan) and in charge of pathology screening for ALK fusions using the immunohistochemistry kit and an original probe set for ALK split FISH assay in a clinical trial of an ALK inhibitor (AF802, Chugai, Japan). The other authors disclosed no potential conflicts of interest.

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Identification of a novel fusion, SQSTM1-ALK, in ALK-positive large B-cell lymphoma

Kengo Takeuchi,[1] Manabu Soda,[2] Yuki Togashi,[1] Yasunori Ota,[3] Yasunobu Sekiguchi,[4] Satoko Hatano,[1] Reimi Asaka,[1] Masaaki Noguchi,[4] and Hiroyuki Mano[2,5]

[1]Pathology Project for Molecular Targets, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo; [2]Division of Functional Genomics, Jichi Medical University, Tochigi; [3]Department of Pathology, Toranomon Hospital, Tokyo; [4]Department of Hematology, Urayasu Hospital, Juntendo University, Chiba; [5]CREST, Japan Science and Technology Agency, Saitama, Japan

ABSTRACT

ALK-positive large B-cell lymphoma is a rare subtype of lymphoma, and most cases follow an aggressive clinical course with a poor prognosis. We examined an ALK-positive large B-cell lymphoma case showing an anti-ALK immunohistochemistry pattern distinct from those of 2 known ALK fusions, CLTC-ALK and NPM-ALK, for the presence of a novel ALK fusion; this led to the identification of SQSTM1-ALK. SQSTM1 is an ubiquitin binding protein that is associated with oxidative stress, cell signaling, and autophagy. We showed transforming activities of SQSTM1-ALK with a focus formation assay and an *in vivo* tumorigenicity assay using 3T3 fibroblasts infected with a recombinant retrovirus encoding SQSTM1-ALK. ALK-inhibitor therapies are promising for treating ALK-positive large B-cell lymphoma, especially for refractory cases. SQSTM1-ALK may be a rare fusion, but our data provide novel biological insights and serve as a key for the accurate diagnosis of this rare lymphoma.

Key words: ALK-positive, large B-cell lymphoma, fusion.

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Introduction

Anaplastic lymphoma kinase-positive large B-cell lymphoma (ALK+LBCL) is a rare subtype of lymphoma that was first described in 1997.[1] Approximately 50 cases have been reported to date,[2] with most cases (60%) following an aggressive clinical course.[3] In well-characterized cases, 3 genes have been reported as a fusion partner of *ALK*: *clathrin* (*CLTC-ALK*),[4-6] *nucleophosmin* (*NPM-ALK*),[7-8] and *SEC31A* (*SEC31A-ALK*).[9] In this paper, we report a case of ALK+LBCL that harbored a novel ALK fusion partner, sequestosome1 (SQSTM1).

Design and Methods

Materials

Biopsied specimens were fixed in 20% neutralized formalin and embedded in paraffin for conventional histopathological examination. We extracted DNA and total RNA from the snap-frozen specimens and subsequently purified the samples. Written informed consent was obtained from the patient. The study was approved by the Institutional Review Board of the Japanese Foundation for Cancer Research.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was used. For antigen retrieval, we heated the slides for 40 min at 97°C in Target Retrieval Solution (pH 9.0; Dako), and subsequently detected the immune complexes with a dextran polymer reagent (EnVision+DAB system, Dako) and an AutoStainer instrument (Dako).

Isolation of ALK fusion cDNA

To obtain cDNA fragments corresponding to novel ALK fusion genes, we used an inverse reverse transcription-polymerase chain reaction (RT-PCR) method slightly modified from one previously reported.[10] Double-stranded cDNA was synthesized from 2 μg of total RNA with 1 pM of the primer ALKREVex22-23 (5′-TGGTTGAATTTGCTGATGATC-3′) and a cDNA Synthesis System (Roche), and was self-ligated by incubation overnight with T4 DNA ligase (TaKaRa Bio). We subjected the resulting circular cDNA to PCR (35 cycles of 94°C for 15 sec, 62°C for 30 sec, and 72°C for 1 min) with primers ALKREV3T (5′-CTGATGGAGGAGGTCTTGCC-3′) and ALKFWDex20-21 (5′-ATTCGGGGTCTGGGCCAT-3′) in a final volume of 20 μL. We subjected 1 μL of the 1:100 diluted reaction products to a second PCR step (the same settings as above), with primers ALKREV4T (5′-GGTTGTAGTCGGTCATGATGGTC-3′) and ALKFWDex21-22 (5′-AGTGGCTGTGAAGACGCTGC-3′) in a final volume of 20 μL. The resulting products were purified by gel extraction and directly sequenced in both directions with primers ALKFWDex20-21 and ALKREV4T.

The fusion point of *SQSTM1-ALK* cDNA was amplified by RT-

The online version of this article has a Supplementary Appendix.

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Correspondence: Kengo Takeuchi, M.D., Ph.D. Pathology Project for Molecular Targets, Cancer Institute, Japanese Foundation for Cancer Research. 3-8-31 Ariake, Koto, Tokyo 135-8550, Japan. E-mail: kentakeuchi-tky@umin.net; Phone: international +81-3-3520-0111; Fax: international +81-3-3570-0558.

PCR with primers SQSTM1 565F (5′-AAACACGGACACTTCGGGT-3′) and ALK3078RR (5′-ATCCAGTTCGTCCTGTTCAGAGC-3′).

Full-length *SQSTM1-ALK* cDNA was obtained from the specimen by RT-PCR with primers SQSTM1v1-F90 (5′-CTCGCTATGGCGTCGCTCACCGTGAA-3′) and KA-W-cDNA-out-AS (5′-CCACGGTCTTAGGGATCCCAAGG-3′).

Fluorescence in situ hybridization (FISH)

We performed FISH analysis of the gene fusion for unstained slides (4 μm thick) with bacterial artificial chromosome (BAC) clone-derived DNA probes for *ALK* (RP11-984I21, RP11-62B19) and *SQSTM1* (RP11-55M16).

Transformation assay for ALK fusion protein

We analyzed the transforming activity of SQSTM1-ALK as described previously.[11-13] Briefly, cDNA for SQSTM1-ALK was inserted into the retroviral expression plasmid pMXS.[14] The resulting plasmid and similar pMXS-based expression plasmids for EML4-ALK variant 1 or NPM-ALK were used to generate recombinant ecotropic retroviruses, which were then used to infect mouse 3T3 fibroblasts. We evaluated formation of transformed foci after culturing the cells for 14 days. We subcutaneously injected the same set of 3T3 cells into nu/nu mice and examined tumor formation after 20 days.

PCR for IGH gene rearrangement

Genomic PCR was used for amplification of the rearranged *IGH* gene using the primers FR2A 5′-TGG(A/G)TCCG(A/C)CAG(C/G)C(C/T)(C/T)CNGG-3′ and LJH 5′-ACCTGAGGAGACGGTGACC-3′. Several clones were sequenced after subcloning the PCR product into pGEM-T-Easy Vector (Promega).

Results and Discussion

Case presentation

A 67-year old man was admitted with a tumor in the left side of his neck. A systemic workup revealed swelling of cervical, mediastinal, and hilar lymph nodes. Blood counts were within normal ranges. Lactose dehydrogenase was slightly elevated (223 IU/L) in peripheral blood with high IgG (2,425 mg/dL), normal IgA (157 mg/dL) and low IgM (32 mg/dL) levels.

Histopathological examination of the biopsied specimen from the cervical lymph node showed a diffuse infiltrate of tumor cells with a round, vesicular nucleus containing a centrally located large nucleolus. The cytoplasm was abundant (Figure 1A). These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was large compared with typical immunoblasts and plasmablasts. Immunophenotypically, the tumor cells were negative for CD3, CD4, CD5, CD10, CD20, CD57, CD79a, and most cytokeratins (CK5/6, CK8, CK19, CK20); focally positive for CD30 and cytokeratins (AE1/AE3, CAM5.2, CK7, CK18) (Figure 1B); weakly pos-

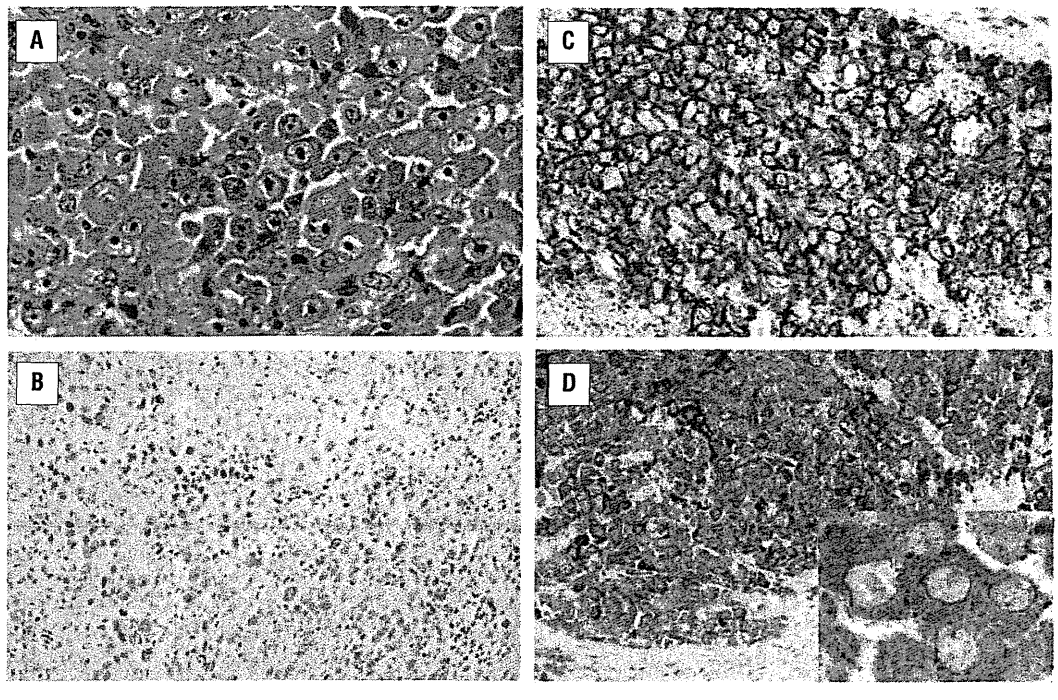

Figure 1. Histopathology of SQSTM1-ALK-positive large B-cell lymphoma. (A) The pattern of tumor infiltration was diffuse. The lymphoma cells were large with abundant cytoplasm and had round, vesicular nuclei, each containing a centrally located large nucleolus. These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was extremely large compared with these typical cell types (Magnification 40×). (B) Some lymphoma cells expressed cytokeratin (AE1/AE3) (Magnification 20×). (C) Syndecan1/CD138 was strongly expressed (Magnification 20×). (D) In anti-ALK immunohistochemistry, a diffuse cytoplasmic staining pattern with ill-demarcated spots was clearly shown (Magnification 20×).