Short Communication

Granulocytic Sarcoma in *MLL*-Positive Infant Acute Myelogenous Leukemia

*Fluorescence in Situ Hybridization Study of Childhood Acute Myelogenous Leukemia for Detecting *MLL* Rearrangement*

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Granulocytic sarcoma is considered to be rare and its frequent occurrence is associated with specific genetic changes such as t(8;21). To investigate an association between *MLL* (mixed lineage leukemia or myeloid-lymphoid leukemia) rearrangement and granulocytic sarcoma, we applied fluorescence *in situ* hybridization for detection of the 11q23/*MLL* rearrangements on the bone marrow cells of 40 patients with childhood acute myelogenous leukemia (AML). Nine (22.5%) of 40 patients exhibited *MLL* rearrangements. Three (33.3%) of these nine patients had granulocytic sarcoma and were younger than 12 months of age. Of these three patients one presented as granulocytic sarcoma of both testes with cerebrospinal fluid involvement, the second case presented in the form of an abdominal mass, and the third as a periorbital granulocytic sarcoma. On the other hand, no granulocytic sarcomas were found among *MLL*-negative patients. It is likely that *MLL*-positive infant AML may predispose granulocytic sarcoma. Regarding the findings of our study and those of other reports, we would guess that the incidence of granulocytic sarcoma in pediatric *MLL*-positive AML may be equal to or greater than the 18 to 24% described in AML with t(8;21). Further investigations designed to identify 11q23/*MLL* abnormalities of leukemic cells or extramedullary tumor may be helpful for the precise diagnosis of granulocytic sarcoma. (*Am J Pathol* 2001, 159:2011–2016)

Granulocytic sarcoma is an extramedullary tumor composed of immature granulocytic cells, and is also called

myelosarcoma or myeloblastoma. The tumor masses are most frequently located in close proximity to bone and are often present in perineural and epidural structures, but they may occur anywhere in the body. Granulocytic sarcoma is considered to be rare. In one study of 478 patients with myelogenous leukemia, 15 cases (3.1%) of localized myeloblastic tumors were detected.¹ However, the true incidence depends on the percentage and extent of the postmortem examinations because at least one-half of granulocytic sarcomas are asymptomatic. The majority of granulocytic sarcomas are diagnosed during the course of, or concurrent with, the diagnosis of acute myelogenous leukemia (AML). Rarely, granulocytic sarcoma may precede the onset of AML by several months, or even years, and such a tumor often poses a diagnostic problem and may well be misinterpreted. Of 154 published cases of primary extramedullary leukemia, 71 cases (46.1%) were initially misdiagnosed.² Although little is known about the factors that influence the development of granulocytic sarcoma, AMLs with t(8;21)(q22;q22) are known to predispose granulocytic sarcoma.^{2,3}

The *MLL* (mixed lineage leukemia or myeloid-lymphoid leukemia) gene is located on chromosome 11q23. The 11q23/*MLL* rearrangement occurs in both AML and acute lymphoblastic leukemia (ALL) and is among the most common cytogenetic abnormalities observed in hematopoietic malignancies.⁴ Rearrangements of the *MLL* gene have been reported in 5 to 10% of acute leukemias. Although more than 30 chromosome partners have been reported in *MLL* rearrangements, the commonest abnormalities are t(4;11)(q21;q23), t(9;11)(p22;q23), and t(11;19)(q23;p13). Translocation (4;11) is associated with pre-B malignancies co-expressing myeloid antigens,

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whereas t(9;11) is associated with AML M5. For searching an association between *MLL* rearrangement and granulocytic sarcoma, we applied fluorescence *in situ* hybridization (FISH) to evaluate the bone marrow aspirate in pediatric AML for detection of the *MLL* rearrangements, and investigated the incidence of granulocytic sarcoma.

Materials and Methods

Patient Characterization

Forty pediatric patients in our department with newly diagnosed AML were included in this study. Leukemic blasts from the bone marrow aspirates of all 40 patients were analyzed for conventional cytogenetics and FISH to detect the 11q23/*MLL* rearrangements. To determine the cut-off value of *MLL* rearrangement, 20 bone marrow specimens of patients without malignant hematological disorders were also analyzed by FISH. Physical examination and systemic review were performed for investigation of granulocytic sarcoma.

Conventional Cytogenetics

Cell culture and chromosome preparation were performed according to two different protocols, synchronized or unsynchronized techniques. At least 20 metaphases were analyzed by Giemsa-trypsin staining. Chromosomes are described in accord with the International System for Human Cytogenetic Nomenclature.⁵ Cells from every specimen were dropped onto positively charged microscopic slides, and after air drying, were stored at -70°C for FISH.

Fluorescence In Situ Hybridization

Previously prepared slides were pretreated with $2\times$ standard saline citrate (300 mmol/L sodium chloride and 30 mmol/L sodium citrate) for 60 minutes at 37°C , and dehydrated with cold 70%, 85%, and 100% ethanol for 1 minute each. Thereafter, FISH studies were performed according to the manufacturer's instructions. Approximately 200 nuclei were scored for *MLL* rearrangements. Nuclei with ambiguous signals and cells with poor morphology were excluded from the scoring.

MLL Probe

A directly labeled FISH probe (LSI *MLL* Dual Color Rearrangement Probe; Vysis Inc., Downers Grove, IL), designed for detecting the 11q23 rearrangements, was applied in this study. The probe consists of a centromeric portion labeled in SpectrumGreen and a 190-kb telomeric portion labeled in SpectrumOrange. The centromeric probe begins between *MLL* exons 6 and 8 and extends 350 kb toward the centromere on chromosome 11, and thus covers the region centromeric of the breakpoint cluster region. The telomeric probe begins between

Figure 1. Representative patterns of *MLL* rearrangement FISH. **A:** Normal interphase nucleus lacking the *MLL* rearrangement (green/orange fusion signals). **B:** Interphase nucleus showing the results of *MLL* rearrangement (separate green and orange signals).

exons 4 and 6 and covers a region primarily telomeric of the breakpoint cluster region.

Interphase nuclei lacking the *MLL* rearrangement can be expected to contain two green/orange fusion signals. In the interphase nucleus showing the *MLL* rearrangement, the telomeric orange signal may move to the partner chromosome and the centromeric green signal may remain on the long arm of chromosome 11. Consequently, separate green and orange signals represent the *MLL* rearrangement (Figure 1).

Results

Based on 200 nuclei from each of the 20 normal bone marrow specimens, the mean percentage and SD of the *MLL* rearrangement was 0.6 ± 0.53 , and the cut-off value was determined on 2.19%. *MLL* rearrangements were found in 9 (22.5%) of the 40 patients with childhood AML. Clinical and genetic findings of the 40 patients are summarized in Table 1. Among the nine AML cases with 11q23/*MLL* abnormalities, FISH detected one case (patient 7), that had not been detected by conventional cytogenetics. Of the eight infants aged younger than 1 year with AML, six (75%) exhibited *MLL* rearrangement (Table 2).

We detected 3 patients with granulocytic sarcoma, all of whom were *MLL*-positive and younger than 12 months of age, among the 40 childhood AML cases. All three had granulocytic sarcoma in the earliest stages of AML. Patient 7 had an abdominal granulocytic sarcoma and FISH study detected *MLL* rearrangements in 41.5% of the 200 nuclei. Patient 10 had granulocytic sarcoma in both testes and we found *MLL* rearrangements in 54.5% of the 200 nuclei examined. In patient 28, granulocytic sarcoma appeared in the periorbital region and *MLL* rearrangements were found in 54.5% of the 200 nuclei (Table 1).

Discussion

Granulocytic sarcoma may rarely be the first manifestation of AML.^{2,6,7} The presence of granulocytic sarcoma is

Table 1. Clinical and Genetic Features of 40 Childhood Acute Myelogenous Leukemia

Patient	Age	Sex	GS*	WBC ($\times 10^6/L$)	FAB	Karyotype	MLL [†]
1	6 years	M	—	6,530	M4	46,XY[25]	2/200
2	14 years	M	—	72,000	M2	46,XY,t(8;21)(q22;q22)[12]/43~45,idem, X[3],-12[3],-15[3][cp7]	0/200
3	2 years	F	—	87,000	M5	46,XX,t(1;11)(p32;q23)[17]/46,XX[3]	146/200
4	5 years	F	—	4,070	M3	46,XX,t(15;17)(q22;q11)[2]/46,XX[38]	0/200
5	6 years	M	—	104,980	M2	47,XY,+21[18]/46,XY[2]	0/200
6	3 years	F	—	1,600	M4	46,XX,del(14)(q13q24)[14]/46,idem,del(9)(q12q12)[7]	0/200
7 [‡]	11 months	M	+	10,320	M5	46,XY,der(3)t(1;3)(q25;q29)[10]/47,XY,+8[8]/46,XY[4]	83/200
8	3 years	F	—	101,820	M2	46,XX,der(8)t(8;11)(q10;q10),del(11)(q21)[6]/46,idem, inv(9)(p11q13)[3]	0/200
9	7 months	F	—	73,210	M5	46,XX,add(11)(q23),13ph+[4]/46,XX[17]	81/200
10 [§]	8 months	M	+	7,500	M5	50,XY,t(2;11)(p21;q23),+6,+8,+16,+19[10]/46,XY[16]	109/200
11	15 years	M	—	24,160	M5	46,XY[20]	1/200
12	3 years	M	—	2,800	M4	46,XY,inv(9)(p11q12)[3]/46,idem,del(12)(q21q23)[7]/ 46,idem,del(12)(q21q23),inv(16)(p13q22)[7]	0/200
13	10 months	M	—	9,060	M6	50,XY,t(5;10)(q35;q24),+6,t(6;7)(q23;q?),+17,del(17) (q24),+19,del(20)(q11.2),+21[16]/46,XY[4]	3/200
14	6 years	F	—	1,380	M3	46,XX,t(15;17)(q22;q12)[5]/46,idem,del(9)(q22)[10]/ 46,idem,i(21)(q10)[5]	0/200
15	1 month	M	—	3,770	M4	46,XY,del(5)(q15),t(10;11)(p12;q23)[19]/46,XY[2]	39/200
16	15 years	F	—	1,200	M7	46,XX,t(9;11)(p22;q23)[6]/47,idem,+6[7]/92,idem $\times$ 2[2]/94,idem $\times$ 2,+6,+6[5]	175/200
17	12 years	M	—	71,240	M4	46,XY,inv(16)(p13q22)[13]	3/200
18	3 years	M	—	820	M5	46,XY,t(9;11)(p22;q23)[17]/45,idem,-12[3]	76/200
19	10 years	M	—	56,890	M5	46,XY,del(5)(q31)[20]	0/200
20	14 years	M	—	47,890	M2	46,XY,t(8;21)(q22;q22)[13]/45,idem,-Y[6]	0/200
21	2 years	M	—	16,000	M7	46,XY,der(14;21)(q10;q10)c,+21c[9]/42~46,idem, del(1)(p32)[11],t(4;7)(q31;q33)[11],-5[5], -10[4],-13[3],-18[5],-21[3][cp24]	0/200
22	11 years	M	—	10,470	M2	45,X,-Y,t(8;21)(q22;q22)[13]	0/197
23	6 years	M	—	13,670	M4	46,XY,add(7)(q32),t(8;21)(q22;q22)[20]	0/200
24	15 years	F	—	23,990	M4	47,XX,+der(8)idic(8)(q11)[8]/47,XX,+4[2]/46,XX[6]	0/200
25	1 year	F	—	23,470	M7	43~54,XX,+2,+6,+7,+8,13cenh+,+19[cp10]/46,XX[3]	1/200
26	2 years	M	—	44,380	M4	46,XY,inv(16)(p13q22)[2]/46,XY[23]	0/200
27	5 years	M	—	79,850	M4	47,XY,inv(16)(p13q22),i(17)(q10),+22[21]	0/200
28 [¶]	9 months	F	+	40,240	M1	46,XX,t(9;11)(p22;q23)[21]	109/200
29	8 years	M	—	5,610	M6	44~45,XY,-7,-19,-21/46,XY[16]	0/200
30	10 years	M	—	92,000	M2	45,XY,t(10;11)(p14;q13),-20[4]	0/200
31	9 years	M	—	2,900	M4	46,XY[20]	3/200
32	9 years	F	—	48,710	M2	46,XX,t(7;11)(p15;p15)[25]	0/199
33	12 years	F	—	117,600	M4	46,XX,inv(16)(p13q22)[16]/47,idem,+22[4]	4/200
34	8 years	M	—	10,320	M4	45,X,-Y,t(8;21)(q22;q22),del(9)(q21q21)[20]	0/200
35	4 years	F	—	78,000	M2	46,XX,t(8;21)(q22;q22)[20]	0/200
36	7 months	F	—	3,980	M5	46,XX,+5,del(5)(p15),-6,del(7)(q22),t(19;21) (q31;q22)[18]/46,XX[3]	0/200
37	4 months	M	—	218,000	M4	46,XY,t(11;17)(q23;q21)[20]	41/200
38	10 years	F	—	4,840	M4	46,XX,t(8;21)(q22;p?),del(9)(q22)[7]/45,idem,-X[4]	1/200
39	13 years	M	—	3,700	M4	46,XY,t(6;9)(p23;q34)[17]	0/200
40	8 years	M	—	63,700	M2	45,X,-Y,t(8;21)(q22;q22)[20]	0/200

WBC, white blood cell count; FAB, French-American-British classification; M, male; F, female.

*Granulocytic sarcoma; +, present; —, absent.

[†]MLL rearrangements by fluorescent *in situ* hybridization; as the number of MLL-positive nuclei out of total scored nuclei.[‡]Patient 7 had an abdominal granulocytic sarcoma.[§]Patient 10 presented as granulocytic sarcoma of both testes with cerebrospinal fluid involvement.[¶]Patient 28 had a periorbital granulocytic sarcoma.

associated with a generally poorer outcome and a shorter overall survival.⁸ The incidence of granulocytic sarcoma, in a study on 478 patients with myelogenous leukemia, was 3.1%.¹ It should be emphasized that the great majority of granulocytic sarcomas were discovered at autopsy and, in the study, postmortem examinations were performed on only 52% of the myelogenous leukemia cases. The true incidence of granulocytic sarcoma might be underestimated. The diagnosis of granulocytic sarcoma during life has frequently been a problem for pathologists because of the relatively immature nature of the

tumor cells. Non-Hodgkin's lymphoma is the most frequent misdiagnosis.⁹ Granulocytic sarcomas can be mistaken for large-cell lymphomas because of their similar histopathologies in soft tissue biopsy specimens. Granulocytic sarcoma may present as isolated subcutaneous masses and may therefore be confused with a primary or metastatic carcinoma. Chloroacetate esterase stains, anti-lysozyme immunoperoxidase reaction or anti-myeloblast monoclonal antibodies may be required to establish granulocytic nature when biopsy specimens are studied.⁶ Some AML-associated genetic changes seem to

Table 2. Characteristics of 40 Childhood Acute Myelogenous Leukemia by FAB Type

FAB type	Cases (infants)	MLL (infants)	GS (infants)
M1	1 (1)	1 (1)	1 (1)
M2	9 (0)	0 (0)	0 (0)
M3	2 (0)	0 (0)	0 (0)
M4	14 (2)	2 (2)	0 (0)
M5	7 (4)	5 (3)	2 (2)
M6	1 (1)	0 (0)	0 (0)
M7	3 (0)	1 (0)	0 (0)
Unclassified	3 (0)	0 (0)	0 (0)
Total	40 (8)	9 (6)	3 (3)

FAB, French-American-British classification; *MLL*, cases with *MLL* abnormalities; GS, cases with granulocytic sarcoma.

predispose granulocytic sarcoma. AML with t(8;21) has some inclination toward granulocytic sarcoma. Granulocytic sarcomas may occur in up to 18 to 24% of t(8;21) AML.^{2,3,10,11} Tetrasomy 8 may be specifically associated with the occurrence of extramedullary tumors.¹²

The *MLL* gene located on 11q23 is one of the most frequently disrupted genes in acute leukemia. Alterations in this gene are found in ~5 to 10% of primary acute leukemias.¹³ In a previous study¹⁴ on a series of 126 patients with AML, *MLL* rearrangements were detected in 17 of the 74 cases with AML M4 or AML M5 and in 2 of the 52 cases with other leukemic subtypes. Of the 74 cases with AML M4 or AML M5, all of the 5 patients younger than 1.5 years, 6 of the 19 patients between 1.5 and 18 years, and 6 of the 50 patients older than 18 years showed *MLL* rearrangements. *MLL* rearrangements are present in 70 to 80% of acute lymphoblastic leukemia and 50 to 60% of AML that occurs in infants younger than 1 year of age.¹⁵ Clinically, *MLL* rearrangements have aroused interest because of their prognostic significance. *MLL* rearrangements are associated with poor prognosis,¹⁵⁻¹⁷ and therefore, the accurate detection of such abnormalities may be of clinical value in assigning individual cases to risk categories at presentation. Infants with acute leukemias and *MLL* rearrangements have extremely poor prognoses.^{15,16} Older children and adults with acute lymphoblastic leukemia carrying *MLL* rearrangements also tend to fare poorly.¹⁷

The methods of detecting *MLL* rearrangement in hematological malignancies include conventional cytogenetics, Southern blot analysis, and FISH. The detection of *MLL* rearrangement by FISH represents a rapid and cheap alternative to Southern blot analysis, the most reliable means of detecting *MLL* rearrangements.^{4,18,19} Southern blot analysis directly examines the integrity of the breakpoint cluster region of the gene, within which all reported *MLL* rearrangements occur. However, the assay is technically demanding, labor-intensive, limitedly available, and time-consuming, taking several days to perform. On the other hand, conventional cytogenetics is widely available but does not detect all *MLL* rearrangements.²⁰ The FISH technique allows the detection of *MLL* rearrangements when abnormal cells do not divide in culture or when translocations are too subtle to be detected by conventional cytogenetic analysis. FISH can also detect specific genetic changes within a morpholog-

ical context. The evaluation of the FISH results is very analogous to immunohistochemical evaluation and requires experience in histopathology. In a previous study²¹ of 33 cases, all confirmed as having *MLL* rearrangements by an abnormal *MLL* Southern blot result, all 33 cases also demonstrated abnormal *MLL* FISH results, when the Vysis probe was used.

In the present study, we applied FISH using *MLL* dual-color probes to detect the 11q23/*MLL* rearrangements associated with the various translocations involving the *MLL* gene. Of the 40 patients with childhood AML in our department, 9 (22.5%) exhibited the *MLL* rearrangement, and 3 (33.3%) of these 9 have had granulocytic sarcoma. All of three were younger than 12 months of age. However, no granulocytic sarcomas were found among the *MLL*-negative patients. The incidence of granulocytic sarcoma was 7.5% among 40 pediatric AML patients.

Raimondi and colleagues²² reported that 88 (18.4%) of 478 children diagnosed with acute myeloid leukemia had 11q23 abnormalities. This is similar to the results in our department. In a review by Johansson and colleagues,²³ 14 (1.9%) of 752 published AML cases with 11q23 aberrations have had granulocytic sarcoma, either as a presenting feature or during disease progression. The incidence of granulocytic sarcoma has varied significantly between children (3.8%) and adults (0.8%), and the most common AML subtype has been AML M5 (71.4%) in the review.²³ In our study, the incidence (33.3%) of granulocytic sarcoma among patients with 11q23/*MLL*-positive AML is higher than that found in a review of 752 published cases. The difference in the observed frequencies may be because of the relatively small number of patients analyzed in our department. On the other hand, the authors of the review suggested that the presence of granulocytic sarcoma might be underreported because of limited clinical data in most publications. Actually, the incidence of granulocytic sarcoma is notably higher in reported series that include such information.²³⁻²⁶ According to a report on six children below the age of 16 years with AML and 11q23 abnormalities, three (50.0%) had granulocytic sarcoma.²³ In the report, no granulocytic sarcomas were detected among 11 adults of AML with 11q23 abnormalities.

MLL-positive infant AML seems to have a predisposition to granulocytic sarcoma, according to the findings of our study. Along with other reports, it is likely that pediatric 11q23/*MLL*-positive AML may predispose granulocytic sarcoma. But, the apparent association between 11q23/*MLL* rearrangements and granulocytic sarcoma may simply reflect an association between *MLL* rearrangements and AML M5, which is known to be associated with a greater frequency of extramedullary disease. However, a study including 65 adults with *de novo* AML M4 and AML M5 revealed no significant differences in clinical features, including the presence of central nervous system involvement between the 15 patients with *MLL* rearrangements and the 50 patients with germline configurations.²⁷ Nevertheless, it is unclear whether extramedullary infiltrates at locations other than the central nervous system were characterized in that study. The association of *MLL* rearrangements with granulocytic sar-

coma is further supported by our case (patient 28) in AML M1. In our present study, five cases of *MLL*-positive AML M5 were included and granulocytic sarcoma was found in two cases. Prospective studies are needed to clarify any clinicogenetic associations between *MLL* rearrangements and granulocytic sarcoma, and the mechanism underlying the localization and expansion of myeloid cells in extramedullary tissues remains to be elucidated.

Patient 10 had both testicular granulocytic sarcomas identified by immunohistochemical staining and also showed positive cerebrospinal cytology. Reports of granulocytic sarcoma in the testis are very rare.^{12,28-31} The central nervous system involvement in AML is an uncommon presenting finding, but 5 to 7% of patients with AML have asymptomatic central nervous system involvement, as determined by positive cerebrospinal fluid cytology.³² The finding of asymptomatic central nervous system disease does not alone seem to predict a poor prognosis.³³ Prominent abdominal presentation as seen in our patient 7 is not usual. Gastrointestinal involvement has been reported in 6.6% of granulocytic sarcomas.⁶

In conclusion, it is likely that *MLL*-positive infant AML may predispose granulocytic sarcoma, and that the incidence of granulocytic sarcoma in 11q23/*MLL*-positive childhood AML, according to the findings of our study and those of other reports, may be equal to or greater than the 18 to 24% described in AML with t(8;21). Therefore, in the case of childhood AML, further investigations of leukemic cells or extramedullary tumor for the detection of 11q23/*MLL* abnormalities may be helpful to diagnose granulocytic sarcoma precisely.

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