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A RELATION BETWEEN *C-MYC* EXPRESSION AND PROGNOSIS OF LEUKEMIA PATIENTS IN INDONESIA

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INDEXING WORDS

c-myc; *c-myb*; *c-fos*; leukemia; Northern blot

SYNOPSIS

Overexpression of *c-myc*, *c-myb* and *c-fos* proto-oncogene has been observed in many leukemia cells. However, a relation between those oncogene expressions and prognosis of the leukemia is not known in Indonesia. In order to elucidate the relation, expression of those oncogenes in leukemia cells from 52 patients in Indonesia was examined by Northern blot analysis and was compared with prognosis of the disease. The leukemia patients who survived for more than 2 years were 37 % of the 52 patients. Many of the samples expressed *c-myc* mRNA (92 %). Although strong expression of *c-myb* and *c-fos* mRNA was detected in the samples (*c-myb* expression in 35 % of the leukemia cells and *c-fos* in 52 %), those coexpressions with *c-myc* mRNA did not alter the prognosis. On the contrary, all of the 4 patients suffering from leukemia which did not express *c-myc* mRNA survived for more than 2 years. Therefore, *c-myc* expression in leukemia cells may be used as an indicator for deciding prognosis of the leukemia patients in Indonesia.

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INTRODUCTION

Recent molecular analysis of human cancer cells demonstrates multiple genetic lesions in the cell such as chromosomal translocations, gene amplification and point mutations.[25,27] Those genetic alterations result in overexpression of proto-oncogenes and/or loss of tumor suppressor genes.[2,32] This abnormal expression of those oncogenes is a primary event for tumorigenesis and may affect prognosis and therapy of the patients. Recent study demonstrated that tumor cells which lost the function of the p53 tumor suppressor gene were resistant against radiation and chemo-therapy and made prognosis of the patients poor.[13] Therefore, it is very important to examine expression of proto-oncogenes and tumor suppressor genes in tumors in order to decide therapy and prognosis of the patients.

The products of *c-myc*, *c-myb* and *c-fos* genes are nuclear transcription factors which regulate cell growth and diferentiation.[2] Overexpression of those proto-oncogenes has been reported in many leukemia cells.[24,26,29] Although a role of the overexpression in the pathogenesis of leukemia cells is not clearly resolved, overexpression profile of those genes in leukemia cells may affect prognosis of the disease. However, a relation between the overexpression profile and prognosis of the disease has not been carefully studied in Indonesia. This study analyzed expression of those genes in leukemia cells from patients in Indonesia and compared the expression profile with prognosis of the disease. Coexpression of those genes did not change prognosis of the disease. On the other hand, undetectable expression of *c-myc* in leukemia cells made better prognosis of the patients. A possibility that *c-myc* expression in leukemia cells is used as an indicator for deciding prognosis of the patients is discussed.

MATERIALS AND METHODS

Leukemia cells

Leukemia cells were obtained from patients in Yogyakarta, Semarang and surrounding areas of the Central Java, Indonesia, between 1991-1993, when they were diagnosed as primary leukemia without any treatments. Mononulear cells were isolated from heparinized peripheral blood using Ficoll Hypaque (Sigma, USA) density gradient centrifugation. Cells were cryopreserved at -140°C in the liquid nitrogen for subsequent molecular genetic studies. Informed consent was given in each instance before acquisition of peripheral blood samples. All studies were approved by the institution board review.

Northern analysis

Levels of *c-myc*, *c-myb* and *c-fos* mRNA were determined by Northern blot analysis, as described.[21] Briefly, total RNA from leukemia cells and normal cells were extracted by the acid-guanidine method, as described.[5] Total RNA (10μg) was electrophoresed in a 1 % agarose gel with formaldehyde. The RNA in the gel was blotted onto a Hybond N-nylon membranes (Amersham) by capillary blotting. A baked filter was prehybridized and then hybridized overnight with Digoxigenin (Dig)-labelled cDNA probes (*c-myc*, *c-myb* and *c-fos*) (Boehringer Mannheim). After washing the filter, the remaining probes were detected by chemiluminescent detection system using anti-Dig antibody. The filter was exposed to Kodak X-Omat AR films at room temperature using an intensifying screen (Dupont).

The Dig-labelled cDNA probes for human *c-myc*, *c-myb* and *c-fos* were prepared by polymerase chain reaction (PCR). Fragments of 250 bp of

PstI/Pvu-II fragment from the exon 2 of the human genomic *c-myc*,[31] 532 bp of EcoRI/BamHI fragment of the human *c-myb* cDNA,[14] and 550 bp of ApaI fragment from the exon 3 of the human genomic *c-fos*[8] were amplified with specific primer in the presence of Dig-labelled dCTP by PCR. An ethidium bromide stained gel containing 28S and 18S RNA bands was used as an amount index of RNA.

RESULTS

Leukemia samples

Mononuclear cells were isolated from peripheral blood of leukemia patients before any treatments. More than 10% of those cells were leukemia (blast) cells. Type of leukemia cells was diagnosed by morphological examination based on the FAB classification. Leukemia cells used in this study were summarized in Table 1. In acute lymphocytic leukemia (ALL), more than 90 % of patients were under 20 years of age.

Table 1. A list of leukemia patients.

Leukemia[a]	Cases	Age		Sex	
		<20	>20	M	F
ALL	22	19	3	15	7
AML	23	8	15	9	14
CML	7	4	3	4	3

[a] Leukemia was classified according to the FAB classification.

c-myc, c-myb and c-fos proto-oncogene expression in leukemia cells

Expression of the *c-myc*, *c-myb* and *c-fos* proto-oncogenes were analyzed in total RNA isolated from leukemia cells by Northern blot. Fig. 1 shows that the 2.3 Kb band for *c-myc* mRNA, 3.8 Kb band for *c-myb* mRNA and 2.2 Kb band for *c-fos* mRNA were detected in some samples on the filter. Those strong expressions on the samples were determined as positive expression although those three mRNAs were not detected in samples from healthy persons (data not shown).

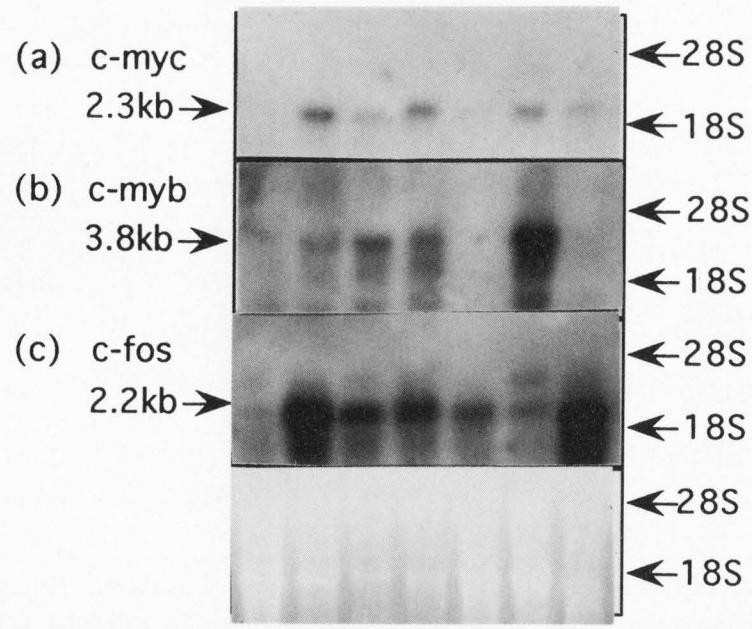

Fig. 1: Oncogene expressions in seven leukemia cells. *c-myc* (a), *c-myb* (b), and *c-fos* (c) mRNA expression were detected in 10 μg of total RNA from leukemia cells by Northern blot analysis. Lane 1-4; ALL, lane 5-7; AML. Ethidium bromide staining of the gel is shown at the bottom of the figure.

In summary shown in Table 2, expression of *c-myc* mRNA was detected in total RNA from many of ALL (95 %), acute myelogenous leukemia (AML) (96 %) and chronic myelogenous leukemia (CML) (71 %). Although about 40 % of ALL and AML leukemia cells expressed *c-myb* mRNA, *c-myb* mRNA was detected in only 14 % of CML. Expression of *c-fos* mRNA was detected in about 50 % of leukemia cells (45 % in ALL, 60 % in AML and 43 % in CML). Almost all of the leukemia cells which expressed *c-myb* or *c-fos* mRNA also expressed *c-myc* mRNA (96 %). These results indicate that *c-myc* mRNA is dominantly expressed in acute type of leukemia.

Table 2. Oncogene expression in leukemia cells.

Leukemia	Patients	mRNA Expression		
		c-myc	*c-myb*	*c-fos*
ALL	22	21[a](95)[b]	8 (36)	10 (45)
AML	23	22 (96)	9 (39)	14 (60)
CML	7	5 (71)	1 (14)	3 (43)
Total	52	48 (92)	18 (35)	27 (52)

[a] Number of the oncogene expression positive leukemia patients.
[b] Percentage of the oncogene expression positive leukemia patients.

A relation between the proto-oncogene expression and prognosis of the disease

Since expression of the *c-myc*, *c-myb* and *c-fos* genes was involved in the proliferation and differentiation of hematopoietic cells [1,11,12],

overexpression of those genes might affect growth and differentiation of leukemia cells and prognosis of the disease. To examine whether expression profile of these genes affected the prognosis, the proto-oncogene expression profile shown in Table 2 was compared with prognosis of the patients. All of the patients were given standard regimen therapy after they were diagnosed. The patients who survived for more than 2 years after the disease was diagnosed were described as good prognosis in this study. The results were summarized in Table 3. The patients with good prognosis were 37 % of total leukemia patients examined.

Table 3. Relation between oncogene expression and good prognosis[a].

Leukemia		*c-myc*		*c-myb*		*c-fos*	
		+	-	+	-	+	-
ALL	7/22[b]	6/21	1/1	1/8	6/14	4/10	3/12
	(32)[c]	(29)	(100)	(13)	(45)	(40)	(25)
AML	8/23	7/22	1/1	4/9	4/14	4/14	4/9
	(34)	(30)	(100)	(44)	(29)	(29)	(44)
CML	4/7	2/5	2/2	1/1	3/6	1/3	3/4
	(57)	(40)	(100)	(100)	(40)	(33)	(75)
Total	19/52	15/48	4/4	6/18	13/34	9/27	10/25
	(37)	(32)	(100)	(33)	(38)	(33)	(36)

[a] Patients who achieved remission and survived for more than 2 years.
[b] Number of patients with good prognosis per total number of leukemia patients examined.
[c] Percentage of the patients with good prognosis.

Table 4. Relation between multiple oncogene expressions and good prognosis[a].

Leukemia		*myc* +			*c-myb* +
		c-myb+*c-fos*	*c-myb*	*c-fos*	*c-fos*
ALL	3/11[b] (27)[c]	1/6 (17)	0/2 (0)	2/3 (66)	-
AML	5/15 (33)	2/7 (26)	1/1 (100)	1/6 (17)	1/1 (100)
CML	1/3 (33)	-	1/1 (100)	0/2 (0)	-
Total	9/29 (31)	3/13 (23)	2/4 (50)	3/11 (27)	1/1 (100)

[a] Patients who achieved remission and survived for more than 2 years.
[b] Number of patients with good prognosis per total number of leukemia patients expressed several oncogenes.
[c] Percentage of the patients with good prognosis.

In ALL, expression of *c-myb* mRNA made prognosis very poor (13 %). Expression of *c-myc* (29 %) or *c-fos* mRNA (40 %) did not affect prognosis. Negative expression of *c-myc* mRNA in ALL was detected only in one patient who showed good prognosis. In AML, expression or no-expression of those proto-oncogenes did not change the prognosis except in a case with negative expression of *c-myc* mRNA. No-expression of *c-myc* mRNA in AML was detected only in one patient who showed good prognosis. In CML, percentage of the good prognosis was rather high (57 %). Expression of *c-myc* or *c-fos* mRNA did not change the prognosis. No-expression of *c-myc* mRNA demonstrated high percentage (100 %) of the good prognosis. In total, all of the 4 patients with leukemia cells which did not express *c-*

myc mRNA survived for more than 2 years. Logistic regression analysis indicated that the probability of death in patients who expressed *c-myc* was about 400 times more than that in patients who showed negative *c-myc* expression. These findings suggest that *c-myc* expression indeed increase the level of aggressiveness. The relation in the patients with leukemia cells which coexpressed more than 2 of those three proto-oncogenes was shown in Table 4. In generalization, there was no significant difference of the prognosis in leukemia cells between expression of *c-myc* mRNA only and coexpression of *c-myc* mRNA and the others. More data were needed to confirm these results.

DISCUSSION

Overexpression of *c-myc* mRNA was found in many leukemia cells, suggesting that c-Myc plays a critical role in multistage of leukemogenesis.[6,10] Although constitutive activation of the *c-myc* gene disrupts the growth regulation of cells[15] and has been proposed as an essential factor for carcinogenesis, mechanisms of leukemogenesis by the *c-myc* overexpression are still unknown.[6] Evidence showed that expression of *c-myc* mRNA in leukemia cells correlated with poor responsiveness to therapy[30] and with shorter duration of remission.[18] This phenomenon was also observed in the level of c-Myc protein in bone marrow cells from AML and CML patients.[33] Our study also support the notion since a rate of the good prognosis of the patients who suffered from *c-myc* overexpressed leukemia cells was poor and 4 out of 4 leukemia patients who did not express *c-myc* mRNA survived for more than 2 years. Therefore, expression of *c-myc* mRNA may affect the level of aggressiveness of the disease. Evan *et al*[9] revealed that constitutive c-Myc expression disrupted the regulation of cell cyle progression.

Elevated *c-myc* expression had been observed in human multiple myeloma by the *c-myc* gene translocation.[10,11,23] However, there was no translocation of the *c-myc* gene in leukemia cells examined in this study by karyotype analysis (data not shown). Baer *et al* revealed abnormal prolongation of the *c-myc* mRNA turnover in some of the AML since there was a truncation of the 3' end of the mRNA which enhanced stability of the mRNA.[1] They also reported that enhanced *c-myc* stability was not due to cytogenetic abnormality. Brewer reported that destabilizing element was a protein that bind to the AU rich element in 3' untranslated *c-myc* RNA.[4] Further molecular analysis is required to elucidate mechanisms of *c-myc* overexpression in those leukemia cells to extend its biologic outcome.

c-Myb is required for normal hematopoiesis at the proliferation of progenitor cells. Inactivation of the *c-myb* gene results in defective hematopoiesis.[17] During the differentiation of hematopoietic cells expression of *c-myb* mRNA is down regulated.[22] Constitutive expression of *c-myb* mRNA can prevent terminal differentiation in certain cell types[7] or enhance competence for cell cycle progression.[28] Indeed, our study revealed that expression of *c-myb* mRNA demonstrated poor prognosis of the ALL. Recent data demonstrated that c-Myb can activate *c-myc* gene expression.[6,34] These data were repeated by our results in that 17 out of 18 leukemia cells which expressed *c-myb* mRNA expressed *c-myc* mRNA. Since there was almost no difference of a rate of the good prognosis of the patients with leukemia cells expressed between *c-myc* mRNA alone and *c-myc* plus *c-myb* mRNA, *c-myc* expression may affect the aggressiveness of the disease.

Oncogenic potential of the deregulated c-Fos in leukemia cells is still controversial.[12] Overexpression of c-Fos transforms fibroblasts *in vitro*.[16]

Many *c-fos* transgenic mice were generated to examine oncogenic activity of the deregulated c-Fos in various organs and cell lineages *in vivo*.[19,20] Several lines of those mice developed bone hyperplasia and bone tumors. However, no malignant transformation of lymphocytes was induced in the mice. In our study 2 out of the 2 patients with leukemia cells that expressed *c-fos* mRNA alone survived well, suggesting that *c-fos* expression does not affect the agressiveness of the disease.

In conclusion, this study shows that *c-myc* overexpression at the onset of the disease in leukemia may increase aggressiveness of the disease and therefore might be used as an indicator of prognosis.

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