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Level of *HOXA5* Hypermethylation in Acute Myeloid Leukemia is Associated with Short-term Outcome

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Hypermethylation of the homeobox (*HOX*) gene promoter leads to decreased expression of the gene during tumor development and is thought to be correlated with the clinical outcome in leukemia. In this study, we performed pyrosequencing to quantify the methylation level of *HOXA5* genes in the bone marrow samples obtained from 50 patients with AML and 19 normal controls. The methylation percentage of *HOXA5* in AML patients (median=65.4%, interquartile range=35.9-72.3%) was higher than that of *HOXA5* in control patients (median=43.1%, interquartile range=36.7-49.6%, Mann-Whitney U test, *P*=0.012). The patients of the AML group who had a high methylation percentage (>70%) had a good prognosis with a 3-yr overall survival (OS) of 82.5%, whereas the patients with a low methylation percentage (≤70%) showed a 3-yr OS of 40.5% (*P*=0.048). Cox proportional hazards regression showed that the methylation percentages of *HOXA5* were independently associated with the 3-yr OS of AML patients, regardless of their karyotypes. We propose that the quantification of *HOXA5* methylation by pyrosequencing may be useful for predicting short-term prognosis in AML. However, the limitations of our study are the small sample size and its preliminary nature. Thus, a larger study should be performed to clearly determine the relationships among *HOXA5* methylation levels, cytogenetics, and prognosis in AML patients. *(Korean J Lab Med 2010;30:469-73)*

Key Words : *AML, Methylation, HOXA5*

Homeobox (*HOX*) genes are members of a transcription factor family and play a crucial role in embryonic development and in the control of differentiation of adult hematopoietic cells [1, 2]. DNA methylation in the 5′ region of the genes, particularly of the CpG islands, has been clearly linked to the repression of gene transcription and is known to play an important role in tumor development [3, 4]. Concerning hematologic malignancies, recent studies have shown that hypermethylation of the *HOXA4* gene is associated with transcriptional repression in chronic lymphocytic leukemia and that extremely high methylation levels of *HOXA5* are observed in AML, far greater than that seen in normal hematopoietic cells [5, 6]. The *HOXA* genes are associated not only with the development of hematologic malignancies but also with the prognosis of these conditions. The inactivation of *HOXA4* and *HOXA5* genes by hypermethylation is strongly correlated with progression to blast crisis in CML [6], and some recent studies have shown that increased expression of *HOXA* genes is correlated to the cytogenetic findings associated with poor prognosis in AML and mixed lineage leukemia [7, 8]. Nevertheless, the clear-cut correlation of *HOXA5* with prognosis in AML has not been reported. In this study, we quantified the level of *HOXA5* methylation by pyrosequencing and examined its correlation with the clinical outcome. We

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also considered other factors influencing the prognosis of AML patients.

DNA was isolated from bone marrow samples obtained at diagnosis from 50 consecutive AML patients who were admitted at the Pusan National University Hospital, Busan, Korea, between October 2003 and January 2007. Peripheral blood samples were also obtained from 17 healthy male and 2 healthy female volunteers (controls). The Human Studies Committee at our institution approved this study. The patients were divided into the following 3 prognostic groups according to their cytogenetic findings as described by Grimwade et al. [9]: favorable karyotype (N=13), intermediate karyotype (N=29), and unfavorable karyotype (N=7). Cytogenetic studies failed for one of the cases.

DNA was extracted from EDTA-decalcified bone marrow samples by using a DNeasy kit (Qiagen Ltd, Hamburg, Germany). The DNA concentration was measured using NanoDrop (Labtech International Ltd, Ringmer, UK). Bisulphite treatment of 2 μg of each sample was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA), and the modified DNA was eluted in 50 μL of 0.1×Tris-EDTA (TE) buffer.

PCR and pyrosequencing analysis were performed according to the established protocols [10]. The forward PCR primer was 5′-GGAAATGTAATAATTTTGTTATAATGGGTTG-3′, and the reverse PCR primer was 5′-CTAAAACATATACTTAATTCCCTCCTAC-3′, with a 5′-biotin label on the reverse primer. Hot-start PCR was performed with 3 μL of bisulphite-treated DNA by using a HotStar Taq Master Mix Kit (Qiagen Ltd.). PCR was performed with 1 cycle of 95℃ for 6 min; 45 cycles of 95℃ for 30 sec, 58℃ for 30 sec, and 72℃ for 30 sec; and finally, 1 cycle of 72℃ for 5 min. Pyrosequencing with single-strand binding proteins (PyroGold reagents, Biotage AB, Uppsala, Sweden) was performed using the PSQ96MA System (Biotage AB), according to the manufacturer's protocol. The sequencing primer was 5′-GATGAGTTTTGTTTTTAG-3′. Pyrosequencing primers were subsequently designed to focus on a series of 8 "target" CpG dinucleotides and thereby avoid CpGs within the primer sequence. In sequencing, the assay was validated using an internal control (non-CpG cytosine in target methylation sequence region).

The "mean *HOXA5* methylation percentage" for each sample was calculated as the mean of the methylation percentages of 8 CpGs (^{m}C/total C) of the promoter CpG islands. The patients were classified into 2 groups (high and low *HOXA5* methylation percentage) according to a 70% cut-off, which was derived from the ROC curve analysis of the *HOXA5* methylation levels of AML patients and healthy controls. Statistical correlations between methylation percentages and clinical variables were calculated using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

The 50 AML patients showed significantly higher percentages of methylation (Mann-Whitney U test, P=0.012) (median=65.4%, interquartile range=35.9-72.3%) than the 19 healthy control individuals (median=43.1%, interquartile range=36.7-49.6%). The ROC curve analysis showed that a cut-off of 70% *HOXA5* methylation yielded sensitivity and specificity of 60% and 100%, respectively (Fig 1). We divided the AML patients into 2 subgroups on the basis of the 70% cut-off for *HOXA5* methylation derived from the ROC curve analysis. The patients whose *HOXA5* methylation percentage was greater than 70% showed more favorable karyotypes at the time of diagnosis than those whose *HOXA5* methylation percentage was less than 70% (Table 1). The other clinical variables were not

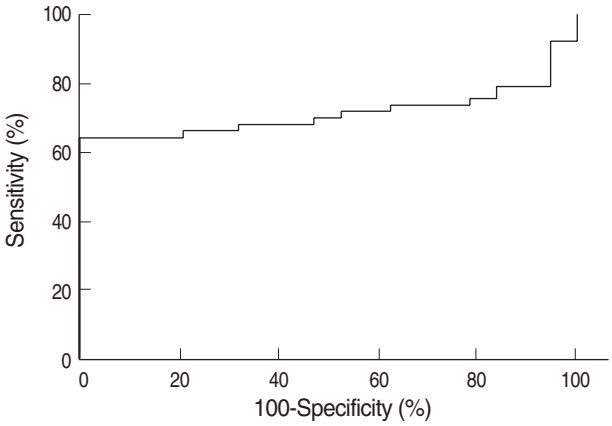

Fig 1. ROC curve analysis showed that a cut-off of 70% *HOXA5* methylation yielded sensitivity and specificity of 60% and 100%, respectively.

statistically significant.

Among AML patients, the patients with high *HOXA5* methylation percentage (>70%) showed a better 3-yr overall survival than those with low methylation percentage (*HOXA5* methylation percentage ≤70%) (82.5% vs. 40.5%, respectively, *P*=0.048, Fig. 2). Multivariate Cox proportional hazards regression showed that *HOXA5* methylation was an independent factor associated with the 3-yr overall survival of AML patients and with the karyotypic findings of the leukemic cells (Table 2).

In this study, the level of *HOXA5* methylation showed significant association with short-term mortality in AML patients. Interestingly, *HOXA5* methylation showed correlation with the 3-yr overall survival of AML patients, regardless of other clinical variables considered at diagnosis, such as age, lactate dehydrogenase level, cytogenetic findings, and white blood cell count. However, the rate of disease-free survival (DFS) was not statistically different between the patients showing higher methylation percentages and those showing lower methylation percentages (data not shown). Because most patients in this study belonged to the intermediate and favorable groups, the mean *HOXA5* methylation percentage had a limited significance.

HOX genes are known to be involved in the maintenance and expansion of hematopoietic stem cells [11, 12], and *HOXA5-11* genes are expressed throughout the $CD34^+$ compartment. High methylation levels of *HOXA5* are frequently observed in AML patients; in contrast, low methylation levels are observed in patients with solid tumors. *HOXA5* methylation may play an important role in the

Table 1. Characteristics of AML patients according to *HOXA5* methylation

	Mean of MtP		*P* value
	Less than 70% (N=32)	More than 70% (N=18)	
Age (mean±SD)	51.9±14.2	47.1±16.5	0.201
Sex (%)			
Male	15 (46.9)	13 (72.2)	
Female	17 (53.1)	5 (27.8)	
FAB classification (%)			0.452
AML, unclassified	3 (9.3)	0 (0.0)	
M0	2 (6.3)	0 (0.0)	
M1	7 (21.9)	7 (38.9)	
M2	9 (28.1)	3 (16.7)	
M3	4 (12.5)	6 (33.3)	
M4	5 (15.6)	1 (5.6)	
M5	1 (3.1)	1 (5.6)	
M6	1 (3.1)	0 (0.0)	
Karyotyping (%)			0.004
Unfavorable	4 (12.5)	3 (17.6)	
Intermediate	24 (75.0)	5 (29.4)	
Favorable	4 (12.5)	9 (52.9)	
Normal karyotype (%) among intermediate group	17 (70.8)	4 (80.0)	1.000
Chemotherapy regimens (%)			0.114
AI	25 (78.1)	9 (50.0)	
AIDA	4 (12.5)	6 (33.3)	
Other	3 (9.4)	3 (16.7)	
LDH (mean±SD)*	1,597.5±1,464.6	1,458.9±1,505.3	0.303
Hb (mean±SD)*	8.5±1.5	8.5±1.2	0.973
WBC ($\times 10^3/\mu$L, mean±SD)*	29.5±60.6	38.0±67.7	0.412

*each value was determined at the time of diagnosis.
Abbreviations: MtP, methylation percentage; AI, Ara-C+Idarubicin; AIDA, ATRA+Idarubicin; LDH, lactate dehydrogenase; FAB, French-American-British; WBC, white blood cells.

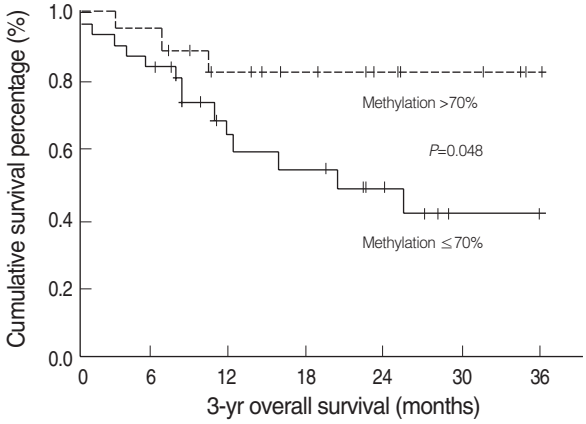

Fig 2. Percentage of 3-yr overall survival of patients with "mean *HOXA5* methylation percentage greater than 70%" and of those with "mean *HOXA5* methylation less than 70%".

Table 2. Cox proportional hazard regression analysis for 3-yr overall survival of AML patients

Variables	Hazard ratio	95% confidence interval		*P* value
HOXA5 methylation ≤70% vs. >70%	5.685	1.308	24.714	0.020
Karyotype (favorable)	-			
Karyotype (intermediate)	0.635	0.111	3.651	0.611
Karyotype (unfavorable)	10.455	1.809	60.427	0.009

arrest of normal differentiation during the development of AML [10]. Even though in this study, the proportion of favorable AML karyotypes was greater in patients showing high methylation percentage (>70%) than in those with low methylation percentage (≤70%), hypermethylation proved to be an independent factor for the overall survival. There have been conflicting data on the prognostic impact of *HOXA5* methylation. One study showed that hypermethylation of both *HOXA5* and *HOXA4* was strongly correlated with progression to blast crisis in CML and that hypermethylation was more strongly correlated to normal karyotype than favorable karyotype in AML patients [6]. However, Strathdee et al. [10] also reported that hypermethylation was observed in all cytogenetic-risk groups. Recently, several researches consistently reported that low HOX expression levels were correlated with favorable AML. Andreeff et al. [13] showed that downregulated *HOX* expression was a consistent feature in favorable AML patients. Debernardi et al. [14] showed similar results in their study. Since gene methylation downregulates its expression, low *HOXA5* expression in favorable AML would be directly related to hypermethylation of *HOXA5*. In our study, *HOXA5* was hypermethylated in the favorable group rather than in the intermediate or unfavorable groups. Our results indicate that hypermethylation shows a good correlation with prognosis in AML patients, thereby corroborating the findings of Andreeff et al. [13]. Other *HOX* genes (*HOXA7-11* genes) are "upregulated together" in AML, and their increased expression is also correlated with poor prognosis in AML [15]. Because there is no surrogate marker other than cytogenetic classification for prognosis of AML patients, *HOX* gene methylation can be considered as a surrogate predictor of survival or response to therapy.

In conclusion, the quantification of *HOXA5* methylation level by pyrosequencing was found to be useful for predicting short-term prognosis in AML patients. This finding indicates that downregulation of *HOXA5* by methylation could be related to favorable AML. However, the small sample size and the preliminary nature of this study are the limitations of our study. Thus, a larger study should be performed to clearly determine the relationships among *HOXA5* methylation levels, cytogenetics, and prognosis of AML patients.

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