RESEARCH ARTICLE

FLT3-ITD, *NPM1*, and *DNMT3A* Gene Mutations and Risk Factors in Normal Karyotype Acute Myeloid Leukemia and Myelodysplastic Syndrome Patients in Upper Northern Thailand

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Abstract

Objective: Approximately 40-45% of AML and MDS patients have a cytogenetically normal karyotype (CN-AML and CN-MDS). The frequency and types of gene mutations in these cases may differ among various populations. The objective of this study was to identify frequencies and types of *FLT3-ITD*, *NPM1*, and *DNMT3A* mutations, and associations of them with clinical data and risk factors in CN-AML and CN-MDS cases in upper Northern Thailand. **Methods:** Bone marrow samples of 40 CN-AML and 60 CN-MDS patients were analyzed for gene mutations by direct sequencing. In addition, data for potential risk factors were obtained for comparison. **Results:** Frequencies of *FLT3-ITD*, *NPM1*, and *DNMT3A* mutations were 25.0%, 17.5%, and 10.0%, respectively in CN-AML, but all zero in CN-MDS cases. *NPM1* mutations were found at a median age older than the wild type (58 vs 47 years) while *DNMT3A* mutations were associated with an increase in the white blood cell count. In all patients, factors for the mutations of these three genes included age ≤ 60 years, and a history of hypertension. **Conclusion:** When considering mutations in only normal karyotype patients, the frequency of *FLT3-ITD*, *NPM1*, *DNMT3A* mutations in CN-AML patients in upper Northern Thailand were found to occur at lower rates than in Western patients and to differ from other Asian populations including parts of Thailand. No mutations were observed in CN-MDS cases. Some types of gene mutations differed from previous studies, possibly attributable to differences in geography, lifestyle and genetic backgrounds. Links with age ≤ 60 years and history of hypertension were found. Investigation of these three genes in an intermediate risk group with a normal karyotype is useful for a better understanding of molecular leukemogenetic steps in CN-AML and CN-MDS patients and may be beneficial for planning treatment and prevention in the population of upper Northern Thailand.

Keywords: Cytogenetically normal- acute myeloid leukemia- myelodysplastic syndrome- upper Northern Thailand

Introduction

Myeloid malignancies are described as a clonal disorder occurring in hematopoietic stem cells (HSCs) and myeloid progenitor cells. Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) are also included in the group of myeloid malignancies (Murati et al., 2012). The disorder in proliferation and differentiation of hematopoiesis leads to the accumulation of blast cells in bone marrow (BM) and peripheral blood (PB). Approximately 31% of MDS patients eventually developed an AML (Bains et al., 2011). In AML and MDS patients, 40-45% have CN-AML and CN-MDS (Gregory et al., 2009; Grossmann et al., 2012). These patients are classified as intermediate risk AML by the Medical Research Council (MRC) and Southwest

Oncology Group (SWOG) (Grimwade et al., 1998; Slovak et al., 2000). Nowadays, cytogenetic results are important data for treatment. However, the heterogeneity among the intermediate risk CN-AML patients has a range of five years for an overall survival rate between 24-42% (Gregory et al., 2009). In addition the treatment plan remains controversial. Many molecular markers in CN-AML have been identified in various research studies and can be used to make an accurate prognosis. These markers may be employed to decide treatment and identify novel target therapies for normal karyotype patients in the future (Gregory et al., 2009). Previous studies have reported that various gene mutations play a role in leukemogenesis and the prognostic factors in CN-AML and CN-MDS patients. Fms-like tyrosine kinase-3 internal tandem duplication (*FLT3-ITD*), Nucleophosmin (*NPM1*),

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and DNA methyltransferase 3A (*DNMT3A*) mutations were frequently found in CN-AML cases (Lin et al., 2011). *FLT3-ITD* mutation (*FLT3-ITD*^mut) was frequently found in exon 14-15 in 22.4-38.0% of the CN-AML patients (Kim et al., 2010; Park et al., 2012), whereas CN-MDS patients revealed the occurrence of *FLT3-ITD* mutations at only 2.2% (Bains et al., 2011). *NPM1* mutation (*NPM1*^mut) primarily presented in exon 12 in 38.3-53.1% of CN-AML and 10.8% of CN-MDS (Schlenk et al., 2008; Bains et al., 2011; Park et al., 2012). *DNMT3A* mutation (*DNMT3A*^mut) was frequently detected in exon 23 in 13.3-34.2% of CN-AML (Marcucci et al., 2012; Ahmad et al., 2014) and 6.3% of CN-MDS patients (El Ghannam et al., 2014). These gene mutations were associated with demographic and clinical parameters, for example gender, white blood cell (WBC) count, platelet count, and BM blast cells in the patients (Dohner et al., 2005; Marcucci et al., 2012). The frequency and type of gene mutations were different among various populations. Many risk factors for AML and MDS have been identified (Nisse et al., 2001; Ma et al., 2010). However, an association of risk factors with gene mutations is not yet well established, particularly in CN-AML and CN-MDS of Thai population. From the cancer registry of Thailand in the period of 2010-2012, leukemia was ranked as the tenth leading form of cancer among both male and female patients (Imsamran et al., 2015). The provinces in upper Northern Thailand are situated in valleys that are surrounded by high mountains. People in upper Northern Thailand are exposed to smoke haze caused by forest fires or burning of agricultural fields. Moreover, the people in Northern Thailand have a different genetic backgrounds from the other parts of Thailand (Wangkumhang et al., 2013). Therefore, this study aimed to investigate the frequency and type of *FLT3-ITD*, *NPM1*, and *DNMT3A* mutations that are present in this region, and to evaluate the risk factors that influence the mutations of these three genes for a useful and better understanding of the molecular leukemogenetic steps and with a hope in the prevention of carcinogenesis in upper Northern Thailand.

Materials and Methods

Patients and Samples

Bone marrow specimens of adult newly diagnosed AML and MDS patients were obtained from the Division of Hematology, Department of Internal Medicine, Maharaj Nakorn Chiang Mai Hospital, Thailand. The specimens were cultured for karyotyping. The 100 BM specimens of AML (n = 40) and MDS (n = 60) patients who had normal karyotype were examined to identify gene mutations. The informed consents forms were signed by all patients who participated in this study according to the protocols of the Ethics and Research Committee of the Faculty of Medicine, Chiang Mai University.

Cytogenetics

Karyotyping was conducted by following the modifying method (Sun NC, 1974). BM specimens were cultured in RPMI 1640 medium containing fetal bovine serum, antibiotics, and colchicine at 37 ºC overnight.

After being treated with colcemid at 37 ºC for 30 minutes, metaphase chromosomes were harvested by being washed with phosphate buffer saline (PBS), treating them with 0.075 M KCl, fixing cell membranes with methanol: acetic acid (3:1), and then spreading the cells on slides and staining them with the G- or Q-banding methods. Metaphase chromosomes were analyzed under a light or fluorescence microscope and chromosomes were karyotyped according to the International System for Human Cytogenetic Nomenclature (2016).

Identification of Gene Mutations

DNA was extracted from BM specimens by modifying a standard inorganic salting out protocol (Seielstad et al., 1999). Briefly, specimens were treated three times with red cell lysis buffer, and lysed white cell pellets with white cell lysis buffer. After that, lysate was treated with proteinase K solution (20 mg/ml) at 56°C. Protein was then precipitated using 6M NaCl. The supernatant was collected and DNA was precipitated using cold isopropanol. The DNA pellets were washed twice with a solution of 70% cold ethanol. Then, 50-100 µl of Tris EDTA (TE) buffer was resuspended into DNA pellets. Polymerase chain reaction (PCR) was performed on exon 14-15 for *FLT3-ITD*, exon 12 for *NPM1*, and exon 23 for *DNMT3A* (currently, those were exon 14-15, exon 11, and exon 26, respectively in NCBI). The sequence of primers included forward-5'-CTGCAGAACTGCCTATTCCTA-3', reverse-5'-AGAAGGCATGGGTGGGAAAC-3' for *FLT3-ITD*; forward-5'-TTAACTCTCTGGTGGTAGAATGAA-3', reverse-5'-CAAGACTATTTGCCATTCCTAAC-3' for *NPM1* (Dohner et al., 2005); and forward-5'-TCCTGCTGTGTGGTTAGACG-3', reverse-5'-ATGATGTCCAACCCTTTTCG-3' for *DNMT3A* (Thol et al., 2011). PCR cycle was run according to MyTaq HS polymerase condition (Bioline, London, UK), with an initial denaturation step at 95°C for one minute, followed by 35 cycles at 95°C for 15 seconds, 58°C for 15 seconds, 72°C for one minute on a thermal cycler (Eppendorf Mastercycler, USA). The PCR products were purified using GeneJET PCR Purification Kits (Thermo scientific, USA), and were labeled with 0.5 µl of BigDye Terminator Version 3.1 (Applied Biosystems, Foster City, California, USA) in a final volume of 10 µl of the sequencing reaction. Sequencing was conducted in both directions, while only reverse sequence was performed for *NPM1* by using the primers as PCR. Sequencing reactions were purified using the Ethanol/EDTA/Sodium acetate precipitation method and capillary electrophoresis was run on an ABI Prism® 3130 DNA Analyzer (Applied Biosystems, Foster City, California, USA) or Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA), and the electropherograms were analyzed for gene mutations by comparing with the reference DNA sequence using the SeqScape program V2.5 (Applied Biosystems, Foster City, California, USA). For all mutations, PCR and sequencing were performed again for confirmation. The reported mutation sequences were based on coding DNA reference sequences of *FLT3* (NM_004119.2), *NPM1* (NM_002520.6), and *DNMT3A* (NM_022552.4) from GenBank.

Risk Factors

The risk factors were studied by interviewing the patients or their relatives using questionnaires at a follow-up appointment. The clinical characteristics of the patients were collected from the electronic medical records of Maharaj Nakorn Chiang Mai Hospital.

Statistical Analysis

For all patients, characteristics and clinical data were presented as medians and interquartile ranges (IQRs) for continuous variables and as counts and percentages for categorical variables. For continuous variables were used and dichotomized as follows: age ≤ 60 years and BMI <25 kg/m2. Fisher's exact test and Wilcoxon-Mann-Whitney tests were performed to compare characteristics between groups. The association between gene mutation and risk factors were analyzed by logistic regression test. Factors associated with a P-value of lower than 0.10 through the univariate analysis were included in the multivariate analysis. All reported P-values were 2-sided and P-values <0.05 were considered statistically significant. Statistical analysis was performed using SPSS Version 17.0.

Results

Characteristics and Clinical Data of CN-AML and CN-MDS Patients

There were 40 (40.0%) CN-AML and 60 (60.0%) CN-MDS of a total of 100 newly diagnosed AML and MDS patients. The general characteristics of the patients are presented in Table 1.

Frequency and Type of Gene Mutations

The most frequently mutated genes were *FLT3-ITD* (10.0%), followed by *NPM1* (7.0%), and *DNMT3A* (4.0%) of all 100 patients. Since, these gene mutations were not found in CN-MDS, the frequency of the mutations in CN-AML patients were 25.0%, 17.5%, and 10.0% for *FLT3-ITD*, *NPM1*, and *DNMT3A*, respectively.

FLT3-ITD Mutation

FLT3-ITD^{mut} was found in 10 out of 40 of the CN-AML cases (25.0%). The patterns of *FLT3-ITD*^{mut} were shown in three patterns (Figure 1). The pure single duplicated ITD pattern was presented in five cases (case 3, 5, 8, 9, and 10) with the duplicated sequence in 60, 90, 36, 69, and 72 nucleotides, respectively. The insertion before duplicated ITD pattern was found in four cases (case 1, case 4, case 6, and case 7) that showed an insertion of nucleotides with a length of 3, 9, 2, and 2 nucleotides before the duplicated sequence in 21, 150, 106, and 34 nucleotides, respectively. The double duplicated ITD pattern was presented in one case (case 2) with the duplicated sequence in 18 and 78 nucleotides. Therefore, these three patterns were illustrated for 11 types of *FLT3-ITD*^{mut} with the sizes of mutant ITD sequence varying from 18 to 159 nucleotides.

NPM1 Mutation

NPM1^{mut} was found in seven cases (17.5%) of CN-AML, of which, there were three types of *NPM1*^{mut} (Figure 2).

Table 1. Characteristics and Clinical Data of CN-AML and CN-MDS Patients (N = 100)

| Characteristics	Total	CN-AML	CN-MDS
	N = 100 (%)	N = 40 (%)	N = 60 (%)
1. Gender			
Male	35 (35.0%)	12 (30.0%)	23 (38.3%)
Female	65 (65.0%)	28 (70.0%)	37 (61.7%)
2. Age (years), Median (IQR)	55.1 (20.0)	48.5 (21.0)	61.5 (20.0)
3. Occupation			
Armed Forces	2 (2.0%)	1 (2.5%)	1 (1.7%)
Professional	6 (6.0%)	1 (2.5%)	5 (8.3%)
Technician	7 (7.0%)	5 (12.5%)	2 (3.3%)
Clerk	3 (3.0%)	3 (7.5%)	0 (0%)
Service Worker	1 (1.0%)	1 (2.5%)	0 (0%)
Agriculture	22 (22.0%)	7 (17.5%)	15 (25.0%)
Trade Worker	18 (18.0%)	7 (17.5%)	11 (18.3%)
Elementary	29 (29.0%)	12 (30.0%)	17 (28.3%)
Student	3 (3.0%)	3 (7.5%)	0 (0%)
Housewife	6 (6.0%)	0 (0%)	6 (10.0%)
Retired Worker	3 (3.0%)	0 (0%)	3 (5.0%)
4. BMI (kg/m^2)			
(missing total = 8: AML = 5, MDS = 3)			
<25	75 (81.5%)	29 (82.9%)	46 (80.7%)
≥25	17 (18.5%)	6 (17.1%)	11 (19.3%)
5. Hemoglobin; (g/dl); Median (IQR)	8.4 (2.6)	7.6 (2.0)	8.8 (3.1)
6. WBC; (x10^9/L); Median (IQR)	4.9 (5.7)	12.7 (35.6)	4.3 (3.3)
7. Platelet; (x10^9/L); Median (IQR)	60.0 (136.8)	46.0 (70.0)	91.0 (174.1)
8. % blast in bone marrow; Median (IQR) (missing total = 1: AML = 1, MDS = 0)	3.0 (59.0)	70.0 (43.0)	1.0 (2.0)

N, number of patients
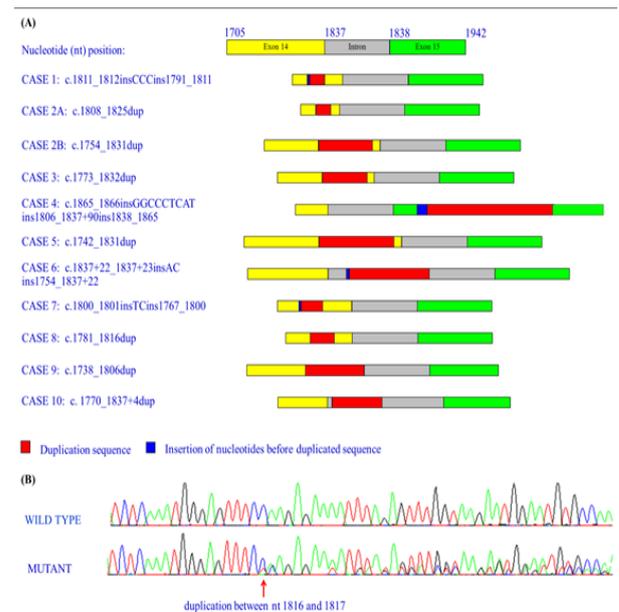
Figure 1. (A) Nucleotide Positions of Duplicated Sequence and Patterns of Mutations of *FLT3-ITD*, (B) Example of Pattern of Duplicated Sequences of Case 8

Table 2. Demographic and Clinical Data of 40 CN-AML Patients with Wild Type and Mutation of *FLT3-ITD, NPM1, DNMT3A*

Characteristic	FLT3-ITD			NPM1			DNMT3A		
	Wild type (n=30)	Mutant (n=10)	P-value	Wild type (n=33)	Mutant (n=7)	P-value	Wild type (n=36)	Mutant (n=4)	P-value
Gender [a]			0.231			0.652			1
Male; N (%)	11 (36.7)	1 1 (0.0)		11 (33.3)	1 (14.3)		11 (30.6)	1 (25.0)	
Female; N (%)	19 (63.3)	9 (90.0)		22 (66.7)	6 (85.7)		25 (69.4)	3 (75.0)	
Age [b]									
Median (Years)	48.5	48	0.463	47	58	0.024*	47.5	51.5	0.787
Hb [b]									
Median (g/dl)	7.5	7.6	0.595	7.6	6.9	0.094	7.6	7.7	0.821
WBC [b]									
Median (X10^9/L)	7.7	28.3	0.303	16.6	6.4	0.817	6.6	103	0.005*
Platelet [b]									
Median (X10^9/L)	50.4	30.5	0.235	49.7	27.8	0.455	39.4	52.7	0.857
BM Blast [b]									
Median (%)	70	84.5	0.134	74	70	0.773	72	75	0.75
FLT3-ITD [a]			-			1			0.256
Wild Type; N (%)	-	-		25 (75.8)	5 (71.4)		28 (77.8)	2 (50.0)	
Mutant; N (%)	-	-		8 (24.2)	2 (28.6)		8 (22.2)	2 (50.0)	
NPM1 [a]			1			-			0.552
Wild Type; N (%)	25 (83.3)	8 (80.0)		-	-		30 (83.3)	3 (75.0)	
Mutant; N (%)	5 (16.7)	2 (20.0)		-	-		6 (16.7)	1 (25.0)	
DNMT3A [a]			0.256			0.552			-
Wild Type; N (%)	28 (93.3)	8 (80.0)		30 (90.9)	6 (85.7)		-	-	
Mutant; N (%)	2 (6.7)	2 (20.0)		3 (9.1)	1 (14.3)		-	-	

*, P-value <0.05 was considered statistically significant; [a], Gender and correlation between genes were analyzed by Fisher's exact test; [b], Age, hemoglobin, white blood cell count, platelet count, and % of bone marrow blast, were analyzed by Wilcoxon-Mann-Whitney tests.

The most frequent mutation was c.863_864insTCTG (Type A), which was identified in 12.5% of CN-AML patients or in 50.0% of the patients with the *NPM1* mutation. The remaining two types included the insertion of four base pairs of CTGC insertions between positions 860 and 861 in one case (2.5% of CN-AML), already reported in the catalogue of somatic mutations in cancer (COSMIC) database. The insertion of four base pairs of CTCT insertions between positions 863 and 864 was also shown in one case (2.5% of CN-AML), and this type has not been reported previously in the COSMIC database.
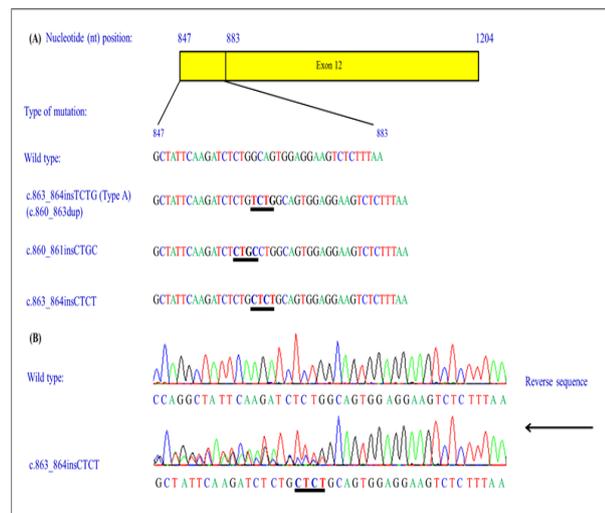
DNMT3A Mutation

DNMT3A^{mut} was detected in only four out of 40 CN-AML cases (10.0%). There were three types of missense mutations, which involved protein changes including R882P (2.5%), R882C (5.0%), and R882H (2.5%) among CN-AML patients (Figure 3).

Figure 2. (A) Sequence Analysis of Nucleotide Positions and Patterns of *NPM1* Mutations in Exon 12, (B) The Examples of Wild Type and C.863_864insCTCT Pattern of Reverse Sequences.
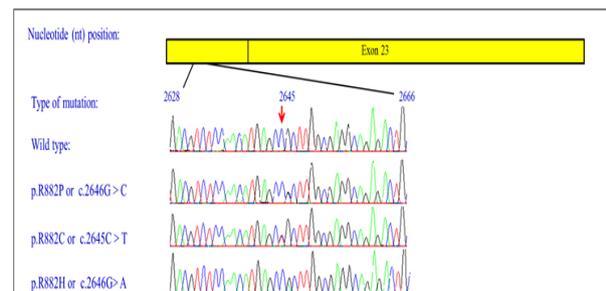

Figure 3. Sequence Analysis of Nucleotide Positions and Patterns of *DNMT3A* Mutations in Exon 23.

Table 3. Univariate and Multivariate Analyses of Risk Factors and 3 Genes Mutations in 100 Patients

Variable	n/N	OR (95% CI)	P-value
Univariate Analysis			
Sex			
Male	3/35 (8.6%)	1	
Female	13/65 (20.0%)	2.67 (0.71, 10.09)	0.148
Age			
≤60 Years	14/63 (22.2%)	5.00 (1.07, 23.41)	0.041*
>60 Years	2/37 (5.4%)	1	
Occupation			
Student/Retired Worker/ Housewife	1/13 (7.7%)	1	
Non-Agriculture	13/65 (20.0%)	3.00 (0.36, 25.21)	0.337
Agriculture	2/22 (9.1%)	1.20 (0.10, 14.70)	0.887
Residing in Upper Northern Thailand			
1-20 Years	2/13 (15.4%)	1	
21-40 Years	5/19 (26.3%)	1.96 (0.32, 12.12)	0.467
41-60 Years	7/40 (17.5%)	1.17 (0.21, 6.47)	0.86
> 60 Years	2/28 (7.1%)	0.42 (0.05, 3.40)	0.418
BMI, (Kg/M²)			
< 25	12/75 (16.0%)	1	
≥ 25	2/17 (11.8%)	0.70 (0.14, 3.47)	0.662
(Missing 8)			
History of Hypertension			
No	9/77 (11.7%)	1	
Yes	7/23 (30.4%)	3.31 (1.07, 10.21)	0.038*
History of Diabetes			
No	14/90 (15.6%)	1	
Yes	2/10 (20.0%)	1.36 (0.26, 7.07)	0.717
Family History of Cancer			
No	8/74 (10.8%)	1	
Yes	8/26 (30.8%)	3.67 (1.21, 11.13)	0.022*
Smoking			
Nonsmoker/Passive Smoker	14/68 (20.6%)	1	
Smoker	2/32 (6.2%)	0.26 (0.06, 1.21)	0.085
Alcohol Consumption			
Nondrinker	11/52 (21.2%)	1	
Drinker	5/48 (10.4%)	0.43 (0.14, 1.36)	0.151
Vegetable Consumption			
No	6/46 (13.0%)	1	
Yes	10/54 (18.5%)	1.52 (0.51, 4.55)	0.459
Fruit Consumption			
No	5/36 (13.9%)	1	
Yes	11/64 (17.2%)	1.29 (0.41, 4.05)	0.666
Coffee Consumption			
No	8/66 (12.1%)	1	
Yes	8/34 (23.5%)	2.23 (0.76, 6.60)	0.147
Exercise			
No	12/63 (19.0%)	1	
Yes	4/37 (10.8%)	0.52 (0.15, 1.73)	0.284

Table 3. Continued

Variable	n/N	OR (95% CI)	P-value
Good Sleep			
No	2/19 (10.5%)	1	
Yes	14/81 (17.3%)	1.78 (0.37, 8.57)	0.475
Benzene Exposure			
No	13/68 (19.1%)	1	
Yes	3/32 (9.4%)	0.44 (0.12, 1.66)	0.225
Insecticide Exposure			
No	14/68 (20.6%)	1	
Yes	2/32 (6.2%)	0.26 (0.06, 1.21)	0.085
Thinner Exposure			
No	13/79 (16.5%)	1	
Yes	3/21 (14.3%)	0.85 (0.22, 3.29)	0.81
Microwave User			
No	13/73 (17.8%)	1	
Yes	3/27 (11.1%)	0.58 (0.15, 2.21)	0.422
X-Ray Exposure			
No	6/31 (19.4%)	1	
Yes	10/69 (14.5%)	0.71 (0.23, 2.15)	0.541
Exhaust Gases Exposure			
No	9/51 (17.6%)	1	
Yes	7/49 (14.3%)	0.78 (0.27, 2.82)	0.647
Hair Dye Exposure			
No	12/59 (20.3%)	1	
Yes	4/41 (9.8%)	0.42 (0.13, 1.42)	0.164
House Near Road			
No	10/51 (19.6%)	1	
Yes	6/49 (12.2%)	0.57 (0.19, 1.72)	0.319
House Near Garden or Farm			
No	9/55 (16.4%)	1	
Yes	7/45 (15.6%)	0.94 (0.32, 2.77)	0.913
Residing in Smoke Haze of Burning Area			
No	2/9 (22.2%)	1	
Yes	14/91 (15.4%)	0.64 (0.12, 3.39)	0.596
Multivariate Analysis			
Variable	n/N	Adjusted OR (95% CI)	P-value
Age			
≤60 Years	14/63 (22.2%)	7.85 (1.49, 41.28)	0.015*
>60 Years	2/37 (5.4%)	1	
History of Hypertension			
No	9/77 (11.7%)	1	
Yes	7/23 (30.4%)	5.38 (1.51, 19.20)	0.010*
Family History of Cancer			
No	8/74 (10.8%)	1	
Yes	8/26 (30.8%)	2.71 (0.79, 9.39)	0.115
Smoking			
Nonsmoker/ Passive Smoker	14/68 (20.6%)	1	
Smoker	2/32 (6.2%)	0.55 (0.09, 3.27)	0.508
Insecticide Exposure			
No	14/68 (20.6%)	1	
Yes	2/32 (6.2%)	0.29 (0.06, 1.50)	0.139

*P-value<0.05 was considered statistically significant; n/N, number of patient with mutations/number of patients

FLT3-ITD, NPM1, DNMT3A Mutations with Demographic, Clinical Data and Gene Mutations

Sixteen of 40 CN-AML patients revealed the presence of gene mutations. They included two cases with mutations of both *FLT3-ITD* and *NPM1* (AML$^{FLT3-ITD/NPM1}$); two cases with *FLT3-ITD* and *DNMT3A* (AML$^{FLT3-ITD/DNMT3A}$); one case with *NPM1* and *DNMT3A* (AML$^{NPM1/DNMT3A}$); six cases with *FLT3-ITD* only (AML$^{FLT3-ITD}$); four cases with *NPM1* only (AML^{NPM1}); and one case with *DNMT3A* only (AML^{DNMT3A}). No patients revealed having all three gene mutations (AML$^{FLT3-ITD/NPM1/DNMT3A}$).

A comparison of demographic clinical data and these gene mutations was analyzed in only CN-AML cases (Table 2). *NPM1*^{mut} was associated with older age compared to wild type (58 vs 47 years). In addition, *DNMT3A*^{mut} patients had higher WBC count than those with wild type.

The Risk Factors and All Mutation of FLT3-ITD, NPM1, DNMT3A Genes in CN-AML and CN-MDS Patients (N = 100)

The variables of risk factors including demographic data, health information, the risk of smoking, alcohol consumption, lifestyle, occupational exposure, and environmental exposure were determined for their associations with mutant and wild type cases by univariate logistic regression (Table 3). Three factors correlated to three genes mutations with significant differences. The patients with the age ≤ 60 years had a five-fold risk of mutation (OR = 5.00, 95% CI = 1.07-23.41) compared to patients > 60 years. Patients with history of hypertension had a three-fold (OR = 3.31, 95% CI = 1.07-10.21) higher significant risk of mutation compared to non-history of hypertensive patients. Patients with a family history of cancer showed nearly a four-fold degree of significant risk (OR = 3.67, 95% CI = 1.21-11.13) compared to groups that did not have cancer in family.

The multivariate analysis was adjusted for age, history of hypertension, family history of cancer, smoking, and insecticide exposure (Table 3). After adjustment, the risk of three genes mutations increased significantly in patients who had an age of less than or equal to 60 years (OR =7.85, 95% CI = 1.49-41.28), and patients with a history of hypertension (OR =5.38, 95% CI = 1.51-19.20).

Discussion

Gene mutations were studied among many populations of AML but have been less reported among MDS, especially in the normal karyotype. The frequency of gene mutations was different among each population (Fernandez-Mercado et al., 2012; Park et al., 2012; Ahmad et al., 2014). In this study, the hotspot mutation exons of *FLT3*, *NPM1*, and *DNMT3A* genes sequencing were performed among all CN-AML and CN-MDS patients. This study showed these gene mutations were detected only in the CN-AML patients.

FLT3-ITD^{mut} was found in 25.0% of CN-AML. When comparing the frequency of mutations in only normal karyotype of AML patient from previous reports, the frequency of mutations in this study was lower than in Western countries (47.9% in UK (Fernandez-Mercado et al., 2012); 31.0% in Germany (Schlenk et al., 2008)). However, in Asian population, the frequency of mutations in CN-AML were 19.7% in China (Wang et al., 2010), 22.4% in Korea (Park et al., 2012), and 34.6% in Egypt (Shamaa et al., 2014). In Thailand, *FLT3-ITD* mutations were found in 24.6% and 19.2% from the studies in Central and Northeast Thailand, respectively (Auewarakul et al., 2005; Kumsaen et al., 2016). However, frequency of these mutations could not be compared with in this research because the previous papers did not clearly show the number of normal karyotype patients. *FLT3-ITD*^{mut} was not detected in CN-MDS patients in this study, similar to studies of Rocquain (2010) that did not find *FLT3-ITD*^{mut} in CN-MDS (Rocquain et al., 2010). Previous investigations also detected its presence in a low frequency (2.2%) in CN-MDS (Bains et al., 2011). Although MDS patients displayed a low frequency of these gene mutations, they are important in predicting AML progression (Bains et al., 2011). The sizes of the mutant ITD fragments varied from 18-159 bp in this study. The lengths of mutant fragments were found to be different in many reports and varied from 3 to 165 bp in India (Ahmad et al., 2010), 21 to 201 bp in Central Thailand (Auewarakul et al., 2005), 27 to 171 bp in Northeast Thailand (Kumsaen et al., 2016), and 26 to 57 bp in China (Zhong et al., 2012). Increasing ITD size leads to a decrease in the overall survival (OS) and relapse free survival (RFS) of patients (Stirewalt et al., 2006). Zhong et al., (2012) classified *FLT3-ITD*^{mut} into two types and identified them as Type I and Type II. Type I ITD had duplicated sequences without insertion nucleotides, but Type II ITD had an insertion before the duplicated sequences. Consequently, there were seven Type I (6 cases) and four Type II (4 cases) in this study and these results corresponded with the findings of previous studies that Type I was more prevalent than Type II (Auewarakul et al., 2005; Ahmad et al., 2010). AML patients who had *FLT3-ITD*^{mut} with Type I tended to have significantly worse OS than Type II or the wild type (Zhong et al., 2012). The single ITDs were found more than double ITDs in this study which was in accordance with previous studies, while some studies showed triple ITDs (Ahmad et al., 2010). *FLT3* gene is expressed in the normal hematopoietic stem cell/progenitor cells and important for lymphohematopoietic stem cell function. *FLT3-ITD* mutation activates signaling via RAS-MAP/AKT kinase and the STAT5 pathway. This mutation may increase reactive oxygen species (ROS) production, DNA damage and misrepair, leukemic transformation, and chemotherapy resistance (Sallmyr et al., 2008).

NPM1^{mut} was observed in 17.5% of CN-AML patients in the current study, a lower frequency than those of many previous studies from Western and Asia (Schlenk et al., 2008; Park et al., 2012). Moreover, frequency of *NPM1*^{mut} in this study also lower than those of CN-AML in a study of Central Thailand where the frequency was 38.1% (Boonthimat et al., 2008). The differences in these mutation frequencies may be due to many different factors including the genetic backgrounds of each population (Wangkumhang et al., 2013), geographic region and environmental factors. *NPM1*^{mut} are rare in MDS patients

(Rocquain et al., 2010; Bains et al., 2011) and this gene mutation in MDS patients was not found in this study, similar to a previous study (Rocquain et al., 2010). A previous study reported that approximately 80% of mutant *NPM1* were Type A mutation (Park et al., 2012), and we found that Type A *NPM1*mut was detected more than Non-Type A. The frequency of Type A *NPM1*mut in this study was 50.0% among *NPM1* mutant patients, while the mutation was 79.2% among the Korean population (Park et al., 2012). Several studies supported our data that *NPM1*mut was significantly associated with older age than those in wild type, but the mean or median age in the two age groups was ≤ 60 years (Boonthimat et al., 2008; Jeon et al., 2013). Moreover, *NPM1*mut was reported to be more common in older age than in younger age patients (Boonthimat et al., 2008). *NPM1* gene regulates the translocation of nucleophosmin (NPM) protein between the nucleus and cytoplasm (Borer et al., 1989). It plays an important role in the alternate-reading-frame protein (ARF)-p53 tumor-suppressor pathway and *NPM1*mut causes accumulation of NPM in the cytoplasm, which is a critical step in malignant transformation (Falini et al., 2005; Colombo et al., 2006).

*DNMT3A*mut was found in 10.0% of CN-AML but other studies reported the incidence range of 13.3%-34.2% in CN-AML (Marcucci et al., 2012; Ahmad et al., 2014). In addition, the frequency of *DNMT3A*mut in CN-AML from Central Thailand were four in 46 patients (8.7%) (Sirirat et al., 2017). No CN-MDS patients had *DNMT3A*mut in this study, whereas previous studies had 6.3% of the cases (El Ghannam et al., 2014). CN-AML patients in this study showed R882C in two cases, while R882P and R882H were each in one single case. However, R882H, R882C, R882S and R882P were also reported, and R882H was the most frequent pattern (Marcucci et al., 2012). Similar to previous studies Hou et al., (2012) and Marcucci et al., (2012), our patients carrying *DNMT3A*mut had a significantly higher WBC than the wild type patients. *DNMT3A*mut was associated with higher WBC and poor prognosis in de novo AML (Hou et al., 2012). WBC counts in *DNMT3A*mut were higher than those in wild type cases (Markova et al., 2012). The reason is that the proportion of co-mutation *AML*FLT3-ITD/DNMT3A is higher than those among the AMLDNMT3A patients. When lacking co-mutation with *FLT3-ITD*, WBC counts had no differences between *DNMT3A*mut and wild type patients (Markova et al., 2012). In this study, the incidence of AMLFLT3/DNMT3A mutations was also higher than AMLDNMT3A only. *DNMT3A* gene encodes the DNA methyltransferase (DNMT) 3A enzyme, which catalyzes the addition of the methyl group to the fifth position of the cytosine residue of the CpG dinucleotide of DNA to generate 5 methylcytosine (Brenner and Fuks, 2006). The down regulation of the target gene was mediated via the methylation of upstream CpG islands. Therefore, *DNMTA3*mut leads to an alteration of enzyme function and disrupts the tetramerization activity of *DNMT3A*, which may have contributed to initiate the oncogenesis (Holz-Schietinger et al., 2012).

The incidence of *FLT3-ITD*, *NPM1*, and *DNMT3A* mutations in CN-MDS was fewer than in CN-AML patients (Bains et al., 2011; Swelam et al., 2011; El

Ghannam et al., 2014). No mutation of these genes was observed in the current study, which corresponded with the report of an absence of *FLT3-ITD* or *NPM1* mutations among the French population (Rocquain et al., 2010). Although *FLT3-ITD*mut was rare in MDS, it was associated with a progression to AML (Bains et al., 2011).

The frequency of gene mutations in normal karyotype patients was lower compared to other studies. Moreover, coexisting mutations in this study were *AML*FLT3/NPM1, *AML*FLT3/DNMT3A, and *AML*NPM1/DNMT3A, while *AML*FLT3/NPM1/DNMT3A3 was not detected. Patients with *AML*NPM1/DNMT3A had a shorter overall survival (OS) and event free survival (EFS) than *AML*FLT3/NPM1 and *AML*FLT3/NPM1/DNMT3A presented the worst clinical outcomes (Loghavi et al., 2014). Factors of genetic background, lifestyle and environmental carcinogens might contribute to differences in mutation patterns. Moreover, laboratory methods and sample size of the study may lead to different frequency and mutation type.

In spite of the determination that effective treatment is hopeful for all AML and MDS patients, effective prevention of the disease is also an extremely important factor. The three gene mutations in this population were associated with the age ≤ 60 years and a history of hypertension. In this study, the median age of the mutant patients was lower than the wild type (50.5 vs 56.5 years). The risk of gene mutations increased among patients ≤ 60 years. From another report in Thailand, the age of Thai AML patients was typically younger than Western patients (Auewarakul et al., 2003). According to the importance of age-related susceptibility to environmental toxicants, it was reported that the epithelial tissues of adolescence or young adulthood were sensitive life stages for increasing cell proliferation (Perera, 1997). Nagasaki atomic bomb survivors who were exposed at a younger age had a high risk of cancer (Nakashima et al., 2008).

In this research, patients with a history of hypertension had an increased risk of mutation compared to those without. Previous reports on MDS in Thailand showed that 54.7% of MDS had co-morbid diseases involving hypertension (Maneechedtha et al., 2011). The association between hypertension and leukemia is still limited. However, there have been reports concerning hypertension associated with an increased risk of various cancers. Both men and women with hypertension tended to have an increased risk of kidney cancer and other types of cancers (Radisauskas et al., 2016). Likewise, meta-analysis researches indicated that hypertension was associated with an increased risk of prostate cancer (Liang et al., 2016) and endometrial cancer (Aune et al., 2017). The mechanism of hypertension and risk of cancer is still unclear, especially regarding gene mutations. It was observed that persons with high blood pressure had an increased chemical reactivity of lymphocytes to carcinogen exposure leading to an accumulation of DNA damage when compared with persons with normal blood pressure (Pero et al., 1976). Furthermore, individuals who have increased diastolic blood pressure tended to have chromosomal aberrations, particularly chromatid breaks (Pero et al., 1976).

In summary, the frequency of *FLT3-ITD*, *NPM1*, and *DNMT3A* mutations in CN-AML patients in upper

Northern Thailand was found to occur at lower rates than in the Western patients. It was different from some Asian countries and other parts of Thailand. No mutation of these genes was observed in CN-MDS. Some types of these gene mutation differed from previous studies. These may be attributed to the different geography, lifestyle, and genetic background. The age ≤60 years and history of hypertension were found to be significantly associated with an increased the mutations of these three genes. The other factors may not be involved with inducing myeloid leukemogenesis via the pathway of *FLT3-ITD*, *NPM1*, and *DNMT3A* mutations in patients living in upper Northern Thailand. The investigation of these three genes in the intermediate risk group with a normal karyotype is useful for a better understanding of the molecular leukemogenetic steps in CN-AML and CN-MDS patients. Furthermore, this study may be beneficial for planning treatment of the patients or cancer prevention in the population of upper Northern Thailand in the future.

Our research findings are worth further study on other gene mutations and the specific details of each risk factor with an increased number of test subjects that will confirm the importance of environmental factors influencing gene mutations.

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