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Research Article

Genetic Mutations and Their Clinical Implications in Older Patients with Acute Myeloid Leukemia

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ARTICLE INFO

Article history:
Received: 13 August, 2020
Accepted: 21 October, 2020
Published: 6 November, 2020

Keywords:
Acute myeloid leukemia (AML)
genetic mutation
cytogenetic

ABSTRACT

Objective: A number of leukemia-associated and patient-specific factors are related to the prognosis in older acute myeloid leukemia (AML) patients. In this study, we focus on the genetic mutations of older patients with AML and their impact on the clinical and prognosis.

Methods: We retrospectively analysed the clinical, cytogenetic and laboratory data of 427 *de novo* non-M3 AML patients treated in our hospital from January 2000 to March 2014. We compared relevant mutations in 21 genes between AML patients aged 60 years or older and those younger and exposed their prognostic implications. Then clinical curative effect survival rate of patients in two groups was observed after followed-up.

Results: Compared with the younger patients, the elderly had significantly higher incidences of *PTPN11*, *NPM1*, *RUNX1*, *ASXL1*, *TET2*, *DNMT3A* and *TP53* mutations but a lower frequency of *WT1* mutations. The older patients more frequently had one or more adverse genetic alterations. Multivariate analysis showed that *DNMT3A* and *TP53* mutations were independent poor prognostic factors among the elderly, while *NPM1* mutation in the absence of *FLT3/ITD* was an independent favorable prognostic factor. Furthermore, the status of mutations could well stratify older patients with intermediate-risk cytogenetic into three risk groups.

Conclusion: Older AML patients showed distinct genetic alterations from the younger group. Integration of cytogenetic and molecular mutations can better risk-stratify older AML patients. Development of novel therapies is needed to improve the outcome of older patients with poor prognosis under current treatment.

Introduction

Acute myelocytic leukemia (AML) is a hematopoietic stem cell originated malignant tumor which threatening the health of humankind. Even the prognosis of young patients has been improved gradually in the last four decades, the survival rate of elder patients remains quite low, and reports regarding the clinical significance about AML gene mutation in aged patients are still very rare [1, 2]. Today, it has been widely accepted that acquired gene mutation is closely related to the occurrence, development and prognosis of AML. Many leukemia related factors can lead to the unsatisfactory prognosis of AML besides the inherent systemic conditions such as accompanied diseases, poor body status, and intolerance of enhanced chemotherapy [3]. Cytogenetic study which has been the instructor for the risk classification and treatment design of AML for a very long time, is the key backbone for the treatment and

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http://dx.doi.org/10.31487/j.COR.2020.09.02

prognosis of AML [4, 5]. Appelbaum *et al*. found a higher risk of cytogenetic adverse in aged patients, especially the mutations in chromosome numbers 5, 7 and 17 [3].

Many acquired gene mutations, such as *NPM1*, *CEBPA*, *RUNX1*, *WT1*, *DNMT3A*, *ASXL1*, *IDH2* and *FLT3* which significantly affect the prognosis, has been found in the AML patients, especially among the one with medium cytogenetic risk [6-17]. But the relationship between gene mutation of aged AML patients and clinical significance still remained less explored. This study might pave the road to targeted therapy of the patients who showed compromised prognosis under current treatment.

Materials and Methods

I Clinical Documents

Clinical, cytogenetic and lab data of 427 patients (with 149 over 60 years old) diagnosed as non-M3 AML for the first time in our hospital from January 2000 till March 2014 were collected. All patients showed no congenital hematopoiesis diseases, history of leukopenia, family history of myelocyte tumor, nor treatment associated AML. Diagnosis and grading of AML patients were carried out under the standard of FAB. The whole experiment has passed the censorship of ethics committee, and informed consent was signed by every patient. 310 of the chosen patients were subjected to standard guided alleviation therapy (Demethyl Roxithromycin 12mg/d for day 1-3, cytarabine 200mg/d for day 1-7). The rest of patients were subjected to placebo or low dose chemical therapy (2/3 dose of standard guided alleviation therapy).

II Cytogenetics

Bone marrow stem cells were obtained through transient non-stimulative culture. Metaphase chromosomes were marked by Trypsin-Giemsa combining technology.

III Analysis of Gene Mutation

21 gene mutations were analysed including category I mutation of *FLT3/ITD, FLT3/TKD, NRAS, KRAS, JAK2, KIT, and PTPN11*; category II mutation of *CEBPA, RUNX1, NPM1, WT1, TP53*, and mucoprotein complex gene (including *STAG1/2, SMC1A, SMC3, and RAD21*), and the mutation of acquired modification gene (including *MLL/PTD, ASXL1, IDH1, IDH2, TET2, DNMT3A20*) [18-29].

Bone marrow cells were then anti-coagulated by EDTA before the extraction of total RNA by Trizol one step method. Then the RNA was reverse transcribed to cDNA by MMLV reverse transcriptase. Transcription factor enhancer combined factor gene (E2A) was selected as positive internal reference. Gene mutations were analysed by direct gene sequencing. One mutation site could be recognized when the following conditions are satisfied: 1) sequencing peak curve indicates heterozygosis with two single recognizable peaks, and low background; 2) sequencing of forward and backward should both be heterozygosis and in consistency with each other, one negative mutation occurred in two sequencings will be considered non-mutation; positive mutant patients must be tested by PCR amplification of gDNA, and a positive result will be accepted as positive mutation; 3) peak of base group showed a downward tendency in heterozygote when compared the sequencing maps of both heterozygote and homozygote. Abnormal results will be repeated twice to be confirmed.

IV Statistical Analysis

Discrete variables are accurately tested by Fisher. In occasion of abnormal distribution, Mann-Whitney U test will be applied for the comparison of continuous variables and the median of distribution. The affection of clinical prognosis of age, molecular mutation, and other variables is evaluated. Only the patients under routine standard chemical therapy are accepted. Complete remission (CR) is treated as therapeutic effective. Overall survival (OS) is defined as death caused by any reason during the period between the day of the first diagnosis and the day of the last follow up. Disease free survival (DFS) is defined as the time span between CR and detectable relapse or death. Independent prognostic factor of OS and DFS is discussed by multifactor Cox proportional risk regression analysis. The proportional risk hypothesis is based on the multivariate Cox proportional risk regression through pre-regression test of time dependent covariant Cox. Variables include age, leukocyte count as diagnosis, karyotype, mutations of *NPM1/ FLT3-ITD, WT1, CEBPA, RUNX1, MLL/ PTD, ASXL1, TET2, IDH2, DNMT3A, and TP53.* $P<0.05$ is considered as statistically significant. All statistical analysis is carried out by SPSS 18.0.

Results

I Comparison of Clinical and Pathological Characteristic Between Older and Younger Patients

217 of the total 427 AML patients are male, 210 are female (Table 1). 156 patients are over 60 years old (older group), with average age of 69.3 (60-90 years old). No significant difference in sex, blood type, and lactic dehydrogenase level exist between older group and younger group (Tables 1 & 2).

Table 1: Clinical features of AML patients stratified by age.

		Total（n=427）	Older patient（n=156）	Younger patient（n=271）	*P* value
Sex	Male	217	81	136	0.1923
	Female	210	75	135	
Age		53.2±8.7	69.3±5.7	41.8±7.4	0.2164
FAB	M0	9	5	4	0.4683
	M1	110	32	78	0.3272
	M2	151	56	95	0.1231
	M4	98	37	61	0.1456

	M5	30	16	14	0.2783
	M6	18	3	15	0.3492
	Undetermined	11	5	6	0.4125
Induction response	CR	232	31	201	<0.001
	PR/refractory	58	22	36	0.3472
	Induction death	27	15	12	0.1875
	Relapse	123	21	102	0.3412

Table 2: Lab data of AML patients stratified by age.

		Mean（n=427）	Older patient（n=156）	Younger patient（n=271）	*P* value
Lab data	WBC（/μl）	20 889 (100–734900)	21345 (700–738900)	21 236 (110–535 000)	0.6751
	Hb（g/dl）	7.9 (3.0–17.0)	8.2 (3.0–15.0)	8.0 (3.0–16.0)	0.4585
	Platelet（×1000 /μl）	42.0 (3.0–950.0)	43.5 (3.0–625.0)	44.5 (3.0–756.0)	0.3456
	Blast (/μl)	8674 (0–512345)	7568 (0–564135)	8692 (0–456080)	0.3934
	LDH (U/l)	837 (240–16 000)	843 (254.0–16 000)	905 (250–14500)	0.5465

II Comparison of Cytogenetic Abnormality Between Older Group and Younger Group

Among all, 386 patients' karyotype data are available, including 143 older patients and 243 younger patients (Table 3). Higher cytogenetic mal-mutation rate was noticed in older group (19.6% *vs.* 8.6%, $P = 0.01$), with t (8; 21) (2.8% *vs.* 15.6%, $P<0.001$). And higher rates of abnormal of complex chromosomes (13.3% *vs.* 5.8%, $P = 0.002$), monosomy 7/7Q deficiency (6.3% *vs.* 3.3%, $P = 0.001$), and monosomy 5/5Q deficiency (7.7% *vs.* 2.1%, $P = 0.001$) were found in older group, but with a low occurrence of t (7; 11) (0 *vs.* 2.9%, $P = 0.006$). No significant difference in simple chromosome abnormality with two or less mutations related to chromosome number 8, 11, 13 and 21 were found between two groups.

Table 3: Association of age with cytogenetic abnormalities.

		Total（n=386）	Older patient（n=143）	Younger patient（n=243）	*P* value
Karyotype	Favorable	56	6	50	<0.001
	Intermediate	265	112	153	0.6721
	Unfavorable	61	28	21	0.01
	Normal	191	80	111	0.1250
	Simple	150	43	107	0.0031
	Complex	33	19	14	0.0021
	t(8;21)	42	4	38	<0.001
	inv (16)	15	4	11	0.0489
	t(11q23)	19	6	13	0.0324
	t(7;11)	7	0	7	0.006
	− 5/5q −	14	11	3	0.001
	− 7/7q −	17	9	8	0.001
	+8	25	13	12	0.3147
	+11	5	3	2	0.4568
	+13	3	2	1	0.2314
	+21	8	2	6	0.1245

III Comparison of Molecular Gene Mutations Between Older Group and Younger Group

Complete mutation screening of 21 genes were carried out for the study of difference of gene mutation in the etiopathogenesis of older and younger AML patients. The most common molecular mutations among the population are *FLT3/ITD* (23.7%), secondly *NPM1* (21.8%), *DNMT3A* (15.0%), *TET2* (13.3%), and *CEBPA* (12.6%). Among older patients, the most common molecular mutations are *NPM1* (30.7%), secondly *TET2* (26.4%), *FLT3 / ITD* (23.8%), *DNMT3A* (21.5%), and *RUNX1* (17.2%) (Table 4). Median of diagnosed molecular mutations of older patients is significantly higher than younger patients (2.0 *vs.* 1.0, $P<0.001$).

Significant higher mutations rates of *PTPN11*, *NPM1*, *RUNX1*, *ASXL1*, *TET2*, *DNMT3A, and TP53* were found in older patients compared with younger patients. (7.5% *vs.* 3.3%, $P=0.003$; 30.8% *vs.* 16.2%, $P=0.001$; 17.0%*vs.* 11.4%, $P=0.008$; 19.5% *vs.* 7.8%, $P<0.001$; 26.4% *vs.* 5.5%, $P<0.001$; 21.4% *vs.* 11.1%; $P=0.002$; 15.1% *vs.* 4.1%, $P=0.006$). While *WT1* mutation was rarely found in older patients over 60 years old (3.1 *vs.* 8.7%, $P=0.005$). No significant difference of other genetic alterations was noticed between two groups. The frequency of one or more adverse genetic mutations (including *FLT3/ITD*, *WT1*, *RUNX1*, *ASXL1*, *DNMT3A, and TP53*) is higher in older patients (76.3% *vs.* 45.8%, $P<0.001$).

Table 4: Distribution of molecular genetic alterations by age.

		Patients with alteration（%）			
		Whole cohort	Older patients	Younger patients	*P* value
FLT3/ITD	427	23.7（101）	23.9（38）	23.2（63）	0.865
FLT3/TKD	427	7.0（30）	6.9（11）	7.0（19）	0.645
NRAS	427	13.3（57）	13.8（22）	12.9（35）	0.582
KRAS	427	3.0（13）	1.9（3）	3.7（10）	0.341
PTPN11	427	4.9（21）	7.5（12）	3.3（9）	0.003
KIT	427	4.7（20）	4.4（7）	4.8（13）	0.423
JAK2	427	0.7（3）	0.6（1）	0.7（2）	0.947
WTI	427	8.0（34）	3.1（5）	10.7（29）	0.0136
NPM1	427	21.8（93）	30.8（49）	16.2（44）	0.001
CEBPA	427	12.6（54）	5.7（9）	16.6（45）	0.0248
RUNX1	427	13.6（58）	17.0（27）	11.4（31）	0.008
MLL/PTD	427	6.3（27）	5.7（9）	6.6（18）	0.458
ASXL1	427	12.2（52）	19.5（31）	7.8（21）	P<0.001
IDH1	427	5.6（24）	6.9（11）	4.8（13）	0.782
IDH2	427	13.1（56）	16.4（26）	11.1（30）	0.241
TET2	427	13.3（57）	26.4（42）	5.5（15）	<0.001
DNMT3A	427	15.0（64）	21.4（34）	11.1（30）	0.002
TP53	427	8.2（35）	15.1（24）	4.1（11）	0.006
Cohesin	335	9.1（39）	8.8（14）	9.2（25）	>0.999

IV The Analysis of the Affection of Clinical Prognosis of Gene Mutation in Older Patients by Cox Proportional Risk Model

Cases of standard chemical therapy among older patients were less than younger patients, (61/156, 39.1% *vs.* 249/271, 91.9%, *P*<0.001). Within the group, the OS of the patients in standard chemical therapy group was longer than placebo group (median, 11.0 *vs.* 4.0 months, *P*<0.001, Figure 1). Among all patients, 310 AML patients were subjected to standard guided remission program, with 232 CR. The CR rate of older group was significantly lower than younger group (19.5% *vs.* 74.2%, *P*<0.001, Table 1). In the average 73 months follow up (0.1-180), the OS and DFS of the older group were significantly shorter than younger group (median 11.0 *vs.* 57.0 months, *P*<0.001, Figure 2A; median 4.0 *vs.* 10.0 months, *P*=0.002, Figure 2B).

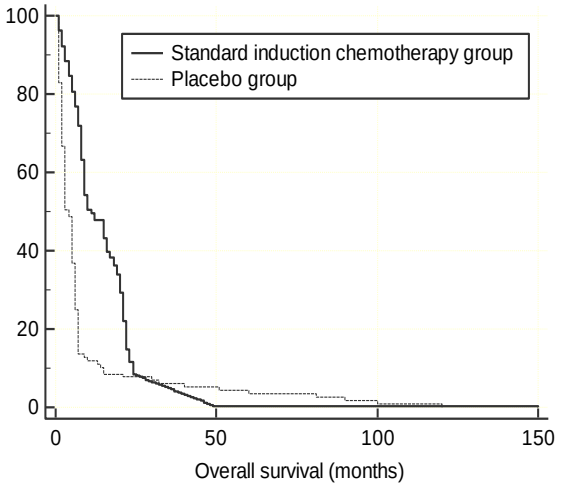

Figure 1: The Kaplan-Meier survival curve for OS in AML patients stratified by having standard induction chemotherapy or not.

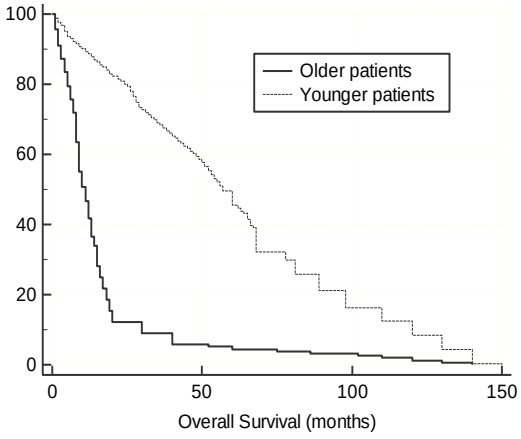

Figure 2A: The Kaplan-Meier survival curve for OS in AML patients stratified by age.

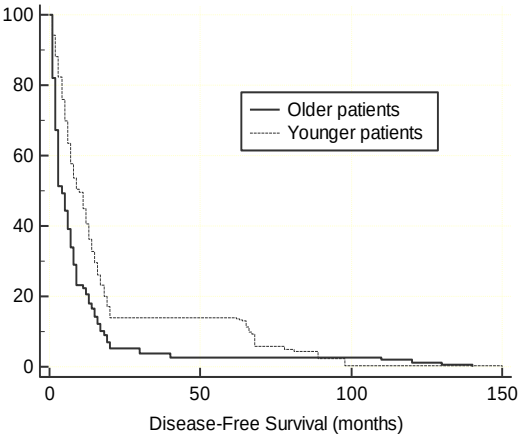

Figure 2B: The Kaplan-Meier survival curve for DFS in AML patients stratified by age.

Age, higher leukocyte count (>450000/μl), and *WT1*, *DNMT3A* and *TP53* mutations were the adverse prognosis factors revealed by multifactor Cox proportional risk regression analysis among population (Table 5). On the other hand, *NPM1*$^{+}$*/ FLT3-ITD*$^{-}$ and *CEBPA* double mutation is the independent favorable prognosis factor. *DNMT3A and TP53* mutations were independent adverse prognosis factors in the multifactor Cox proportional risk regression analysis of older patients, while *NPM1*$^{+}$*/ FLT3-ITD*$^{-}$ is still a favorable prognosis factor.

Table 5: Multivariable Cox analysis about DFS and OS.

		DFS				OS			
		PR	95%CI		*P*	PR	95%CI		*P*
			Lower	Upper			Lower	Upper	
Whole cohort	Age	1.486	1.023	2.314	0.002	2.586	1.680	3.892	0.001
	WBC	1.359	1.024	2.035	0.035	1.864	1.025	2.684	0.002
	Karyotype	1.567	1.068	2.984	0.028	1.756	0.766	3.236	0.123
	NPM1/FLT3-ITD	0.246	0.168	0.650	0.006	0.423	0.196	0.875	0.004
	CEBPA$^{double\text{-}mutation}$	0.586	0.368	0.964	0.027	0.489	0.203	0.965	0.013
	RUNX1	1.698	1.123	2.645	0.034	1.796	0.864	3.125	0.059
	WT1	1.975	1.238	2.941	0.003	1.925	1.029	3.258	0.042
	ASXL1	0.998	0.621	1.789	0.758	1.368	0.843	2.465	0.458
	TET2	0.996	0.635	1.645	0.861	1.241	0.639	1.963	0.923
	IDH2	0.842	0.523	1.321	0.346	0.654	0.351	0.944	0.013
	MLL/PTD	1.456	0.764	2.415	0.402	1.325	0.582	3.359	0.421
	DNMT3A	2.014	1.314	3.014	0.001	2.011	1.214	3.684	<0.001
	TP53	2.358	1.216	5.240	0.019	4.982	2.017	12.092	0.001
Older patients									
	WBC	1.568	0.741	3.125	0.359	1.945	0.782	4.285	0.123
	Karyotype	0.895	0.362	3.147	0.978	1.456	0.634	5.369	0.345
	NPM1/FLT3-ITD	0.457	0.158	1.235	0.056	0.365	0.064	0.865	0.012
	CEBPA$^{double\text{-}mutation}$	0.968	0.403	2.645	0.752	0.571	0.150	2.654	0.356
	RUNX1	0.965	0.456	3.687	0.552	1.842	0.531	4.026	0.341
	ASXL1	1.532	0.642	3.452	0.769	1.365	0.511	3.684	0.765
	TET2	1.245	0.513	2.065	0.652	1.055	0.523	2.658	0.954
	IDH2	0.965	0.180	1.245	0.069	0.358	0.160	1.482	0.116
	MLL/PTD	1.647	0.597	4.639	0.486	2.349	0.698	9.621	0.095
	DNMT3A	1.769	0.860	3.564	0.235	3.158	1.356	8.032	0.002
	TP53	2.325	0.652	10.451	0.368	4.024	1.023	18.456	0.032

V Kaplan-Meier Survival Analysis

During the long term follow up post treatment, life quality of the patients with any genetic mutation was worse than those of no genetic mutation (including *FLT3/ITD, DNMT3A or TP53*). OS and PFS were longer in non-gene mutation group than adverse gene mutation group (median 68.0 *vs.* 14.0 months, *P*=0.003, Figure 3A, median 16.0 *vs.* 6.0 months, *P*=0.004, Figure 3B). survival rate in non-gene mutation group was significantly higher than adverse gene mutation group after the *Log-rank test through* Kaplan-Meier survival analysis (χ^2=49.7.1, *P*<0.001; χ^2=39.1, *P*< 0.0001) (Figure 3).

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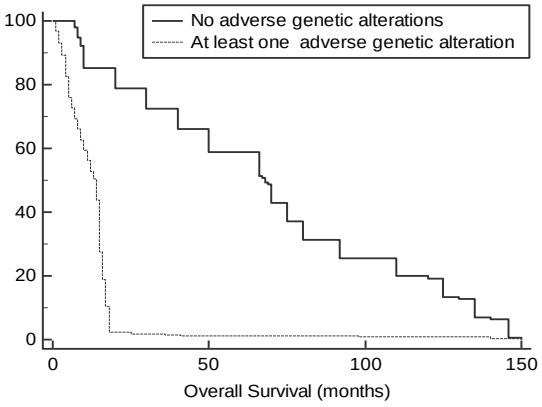

Figure 3A: The Kaplan-Meier survival curve for OS in AML patients stratified by having adverse genetic alterations or not.

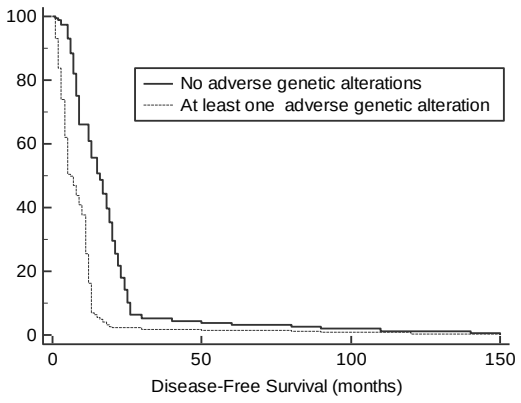

Figure 3B: The Kaplan-Meier survival curve for DFS in AML patients stratified by having adverse genetic alterations or not.

Discussion

Most patients in the studies regarding the prognosis factors of AML were of younger age with less complications and good clinical status, which would be inappropriately to represent all AML patients. In this study, we didn't set age limitation for the analysis of first diagnosed AML patients, thus compared the difference in gene mutations between older and younger patients and discussed the clinical significance. We found obvious characteristics in clinical biology and genetic mutation of aged patients which predict the prognosis of these patients.

Karyotype is the most powerful prognosis factor of AML. Aged patients accompanied by a higher frequency of cytogenetic adverse risk, such as complex chromosome abnormal or chromosome number 5 and 7 mutation, but abnormal of core-binding factor was rarely seen. For a better risk classification of aged AML patients, ELN professors in Europe first provided the standard classification procedure based on cytogenetics and three gene molecule mutations (*FLT3/ITD*, *NPM1* and *CEBPA*) [4]. And other related genes were also brought into consideration in the risk classification of AML patients. But the studies about molecular changes and clinical significance of aged AML patients are very limited till now. Adverse prognosis factors *RUNX1* and *SXL1* gene mutations are more common in aged AML patients with normal cytogenetics. OS is shorter in aged patients with *WT1* or *TP53* mutations, while CR and OS are better in patients with *NPM149* mutation. Ostronlff *et al*. found that *NPM1* mutation contributed to the survival of patients between 55-65 years old who without *FLT3/ITD* mutation but showed no effect on patients over 65 [30].

This report thoroughly studied 21 gene molecular genetic mutations of aged non-M3 AML patients. We firstly provided the distribution of genetic changes and different results of gene mutations in different age groups of patients. Secondly, the mutation rate of *PTPN11*, *NPM1*, *RUNX1*, *ASXL1*, *TET2*, *DNMT3A* and *TP53* was higher in aged patients, while the mutation rate of *WT1* was lower. Most gene mutations were more common in aged patients followed by adverse prognosis with the exception of *NPM1* mutation. Besides, one or more adverse genetic changes (including *FLT3/ITD*, *WT1*, *RUNX1*, *ASXL1*, *DNMT3A and TP53*) were of higher frequency in older patients than younger patients. Not only the higher adverse cytogenetic frequency, aged patients also faced a higher gene mutation rate and adverse prognosis related molecular genetic mutations which unsatisfactory clinical results. Another reason is that they are more prone to the toxic effect of the chemical therapy agent and suffer a higher mortality rate. Taken together, aged AML patients have more obvious clinical biology characteristics, and higher frequency of high risk cytogenetic and gene mutation, with worse prognosis. Integration of cytogenetics and molecular mutation may contribute to the classification of aged patients by different results and risks. This study is of great value for the development of new therapy and the improvement of clinical results of aged patients with adverse prognosis.

Acknowledgments

This work was supported by the National Natural Sciences Foundation of China (No.81670196) and Nantong Science and Technology Project (HS2019003).

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