Variation of nicotinic subtype α7 and muscarinic subtype M3 acetylcholine receptor expression in three main types of leukemia

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Abstract. Cholinergic receptors, such as α7-nicotinic acetylcholine receptor (α7-nAChR) and M3-muscarinic acetylcholine receptor (M3-mAChR), have been demonstrated to serve a significant role in the proliferation, differentiation and apoptosis of leukemic cells. However, the expression of these receptors in samples from patients with leukemia remains unclear. The present study aimed to determine the expression of M3-mAChR and α7-nAChR in the bone marrow or peripheral blood of 51 patients with leukemia, including acute myeloid leukemia (AML; n=33), acute lymphoblastic leukemia (ALL; n=13), and chronic myeloid leukemia (CML; n=5). Peripheral blood mononuclear cells (PBMCs) were also isolated from healthy subjects (n=5) for comparison. Western blot analysis was performed to determine the protein expression profiles, and a pattern of decreased α7-nAChR levels in patients with leukemia was observed. Among the leukemia types, the lowest expression of α7-nAChR and M3-mAChR were identified in patients with T-cell ALL/lymphoma (T-ALL). CML exhibited the highest level of M3-mAChR, which was significantly different from APL and AML-M4, yet not from healthy subjects ($P<0.05$). Therefore, different expression profiles of α7-nACR and M3-mAChR were detected amongst the leukemia types. Collectively, the present study supports the potential role of cholinergic signaling in mediating leukemogenesis. However, further studies in larger cohorts are required to validate these findings.

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Introduction

Leukemia includes a group of heterogeneous neoplastic malignancies that develop in the bone marrow and affect normal hematopoiesis. Conventionally, leukemia has been classified according to its cellular origin, whether of myeloid or lymphoid lineage, and by the course of the illness, whether acute or chronic. The four major types are acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL). They differ significantly in terms of the morphological, cytogenetic, immunophenotypic, and molecular features of the malignant cells (1). The French-American-British (FAB) classification divides AML into nine subtypes, identified as M0 through M7. These nine are based on the level of maturity of the myeloid cells. The category of undifferentiated AML, or M0, reflects the difficulty in delineating whether a cancer cell is truly a myeloid-type or a lymphoid-type malignancy when it presents neither myeloid nor lymphoid markers (2). These specific characteristics of leukemia suggest the heterogeneity of the underlying biological alterations involved in cancer cell transformation and the variations in the levels of hematopoietic progenitor cell hierarchy.

Acetylcholine (ACh) is a major central and peripheral neurotransmitter and is also involved in the control of several non-neuronal functions, including immune function (3). Immune cells, especially lymphocytes, express essential components of the non-neuronal cholinergic system. They include the chemical messenger, ACh; the ACh-synthesizing enzyme, choline acetyltransferase (ChAT); an ACh-degrading enzyme, acetylcholinesterase (AChE); and both muscarinic (m) and nicotinic (n) ACh receptors (AChRs) (4). There is accumulating evidence for the involvement of the non-neuronal cholinergic pathway in regulating and modulating the immune system by means of its effects on the differentiation and proliferation of lymphocytes, cytokine production, antigen presentation, and inflammation (5).

Several studies on the involvement of the non-neuronal cholinergic pathway in tumorigenesis have been published,

including lung, colon, cervix, prostate, breast, and bile duct cancers, wherein more information regarding cholinergic autocrine and paracrine signaling is available (6-9). Molecular analyses of the expression of essential cholinergic components in malignant tumors of different histogenesis have indicated that those tumors largely exhibit different cholinergic component expression from normal tissue (10). In addition, enhancement of ACh production has often been observed in tumors as well as some differences in the expression patterns of ACh receptors. Among the various types of cholinergic AChRs, subtype α7 nAChR (α7-nAChR) and subtype M3 mAChR receptor (M3-mAChR) have been identified as the cholinergic AChRs most capable of promoting cancer progression, for example, by the induction of cancer cell growth and metastasis (6,9,11).

Previous studies have demonstrated the downregulation of certain types of cholinergic ACh receptors during the thymocyte maturation process, denoting their possible role in T-cell development (12). Furthermore, the plasticity of cholinergic AChRs during T-cell differentiation has also been demonstrated in murine splenic T-cell models (13). Extensive data obtained from human mononuclear leukocytes (MNLs), isolated T- and B-cells, and various leukemia cell lines, have revealed the diversity of the non-neuronal cholinergic system, particularly of cholinergic AChRs in human immune cells (14,15). Our previous *in vitro* study of NB-4 acute promyeolocytic leukemic cells also demonstrated that the expression of α7-nAChR and M3-mAChR changes following all-trans retinoic acid-induced differentiation treatment (16). In addition, induction of AChE activity was evident in whole blood and lymphocyte samples obtained from newly diagnosed pediatric patients with T- or B-ALL. However, AChE activity decreased during the remission period (17). It is important to note the recent hypothesis that non-neuronal cholinergic machinery may be involved in leukemogenesis, especially in T-cell leukemia, and that its components, such as cholinergic AChRs, may represent relevant therapeutic targets for leukemia (18). To date, there has been no new information regarding the involvement of the non-neuronal cholinergic system amongst different types of leukemia. We propose that the expression patterns of cholinergic systems may differ among the types and subtypes of leukemia. In the present study, we compared the expression levels of the major cholinergic AChRs, including α7-nAChR and M3-mAChR, in healthy subjects and in patients with the three main types of leukemia, to reveal the potentiality of the cholinergic pathway as a pharmacological target in hematopoietic derived neoplasia.

Materials and methods

Patients. Inclusion criteria for the subjects were as follows: i) Diagnosis of leukemia at Siriraj Hospital (Bangkok, Thailand); ii) patients with leukemia were in the novel diagnostic-phase and not undergoing treatment that might influence the expression of cholinergic AChRs in lymphocytes; iii) written informed consent was obtained. The exclusion criteria were as follows: i) Presence of multiple tumors; ii) being pregnant or too young (age <15 years); iii) presence of acute or chronic diseases such as diabetes, parasitosis or any immune dysfunction. The exclusions were intended to minimize potential complications due to the impact of these complex diseases on immune cells. This study was conducted in accordance with the provisions of the International Conference on Harmonization of Good Clinical Practice guidelines and the Helsinki Declaration. The study was approved by the Committee on Human Rights Related to Research Involving Human Subjects of the Chulabhorn Research Institute (CRI project number: 33/2554, approval date: 29/02/2012) and the Ethics Committee of Siriraj Hospital (Project number: 255/2555, approval date: 01/08/2012). Written informed consent was obtained from each patient prior to sample collection. A total of 51 peripheral blood (PB) or bone marrow (BM) samples were obtained from patients at the time of diagnosis between 01/2013 and 12/2016. PB samples were also taken from healthy subjects (n=5) to serve as a control group. The peripheral blood mononuclear cells (PBMCs) of patients with leukemia and healthy subjects were isolated from 6 ml of heparinized venous blood by density gradient centrifugation using Ficoll-Paque (Isoprep; Robbins Scientific, Sunnyvale, CA, USA) at 500 x g for 30 min. The mononuclear cells were washed three times in Hank's balanced salt solution (Gibco-Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Western immunoblotting assay. PBMCs and BM samples were lysed in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 20 mM NaF, 1 mM PMSF, 1% Triton X-100, and 1X protease inhibitor cocktail set I (Calbiochem; Merck KGaA, Darmstadt, Germany). Sample lysates were sonicated and then incubated at 4˚C for 30 min. Samples were centrifuged at 16,000 x g for 15 min at 4˚C. Sample supernatants were collected and stored at -80˚C for further analysis. Concentrations of proteins in the supernatants were determined by using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sample (50 μg total protein) was mixed with Laemmli loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, and 5% 2-mercaptoethanol) and then boiled at 95˚C for 5 min. Proteins were separated via 7.5% SDS-PAGE in a Mini-PROTEAN II system (Bio-Rad Laboratories, Inc.). The separated proteins were then transferred onto a nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK), and the membrane was incubated in blocking buffer containing 5% non-fat dry milk in TBS-T buffer (10 mM Tris-HCl, pH 8.0, 0.05% Tween-20, and 150 mM NaCl) for 1 h at room temperature, followed by overnight incubation at 4˚C with the primary antibodies. Antibodies against α7-nAChR (sc-5544; 1:1,000) and M3-mAChR (sc-9108; 1:500) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The antibody against GAPDH (2118; 1:2,000) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). After washing with TBS-T buffer 3 times (10 min each), the membrane was incubated with a horseradish-peroxidase conjugated secondary antibody (GE Healthcare Life Sciences) for 2 h at room temperature. The protein bands stained by the targeted antibodies were visualized using an enhanced chemiluminescence assay kit (GE Healthcare Life Sciences) followed by exposure to x-ray film (Pierce, Perbio, Brazil). To avoid variation between gels, the exposure time of α7-nAChR, M3-mAChR, and GAPDH, was fixed at 2, 20, and 2 min, respectively. Relative protein expression levels of α7-nAChR

Table I. Clinical characteristics of patients.

Disease diagnosis	No. of case	No. of males/females	Age (years)	Hemoglobin (g/dl)	Hematocrit (%)	WBC count ($x10^9$/l)	Platelet count ($x10^9$/l)	Blast cells (%)
AML-M0	6	3/3	16-76 (42)	6.2-8.5 (8)	18.2-25.5 (24.6)	2.4-273.4 (62.1)	8-133 (28)	80.5-88 (85.8)
AML-M1	5	4/1	15-81 (54)	6.9-12.2 (8.8)	21.3-37.0 (26.3)	4.8-190.2 (16)	24-507 (152)	24-92.2 (39.8)
AML-M2	8	4/4	16-66 (47.5)	4.2-11.7 (8.1)	13.5-33.8 (23.4)	0.5-131 (20.6)	8-85 (33)	65.7-80.4 (76.6)
AML-M3	6	4/2	33-85 (56.5)	7.3-10 (7.95)	21-29.8 (23)	0.6-133.8 (13.9)	9-113 (41)	64.5-92.5 (82.3)
AML-M4	8	5/3	21-71 (58.5)	4.9-9.9 (8.4)	15.7-30.5 (25.7)	9.2-205.1 (31.4)	20-189 (38.5)	66.3-85 (78.8)
CML	5	3/2	26-57 (49)	8.8-12.3 (9.8)	26.4-37.3 (32.1)	2.4-312.4 (5.7)	100-601 (209)	ND
T-ALL	9	8/1	19-50 (26)	5-16.6 (8.9)	16-50.8 (26.9)	3.1-280 (9.4)	17-406 (131)	44.3-90.4 (74.4)
B-ALL	4	2/2	15-56 (29)	5.6-12.5 (8.3)	18.8-27.9 (25.8)	6.65-153.1 (56.2)	21-63 (31)	65.1-77.7 (65.2)
Normal[a]	5	2/3	22-34 (28)	12.1-14.5 (13.3)	38.1-44.1 (39.7)	4.55-7.84 (5.7)	170-341 (231)	ND

[a]Normal is healthy subjects. Data in the parentheses are the median. AML, acute myeloid leukemia; M0, AML with minimal differentiation; M1, AML without maturation; M2, AML with maturation; M3, acute promyelocytic leukemia; M4, acute myelomonocytic leukemia; CML, chronic myeloid leukemia; T-ALL, T lymphoblastic leukemia/lymphoma; B-ALL, B lymphoblastic leukemia/lymphoma; ND, not determine.

Figure 1. Expression levels of α7-nAChR in patients with leukemia. (A) Representative immunoblots of α7-nAChR in the three main types of leukemia, including five subtypes of AML, two subtypes of ALL, and CML. GAPDH was used as the loading control. (B) The ratio of α7-nAChR to GAPDH was determined by densitometric analysis. Data are presented as the means ± standard deviation. The number of cases in each group is presented in parentheses on the x axis. P<0.05 was considered to indicate a statistically significant difference. α7-nAChR, nicotinic subtype α7 acetylcholine receptors; AML-M0, AML with minimal differentiation; AML-M1, AML without maturation; AML-M2, AML with maturation; APL, acute promyelocytic leukemia; AML-M4, acute myelomonocytic leukemia; CML, chronic myeloid leukemia; T-ALL, T lymphoblastic leukemia/lymphoma; B-ALL, B lymphoblastic leukemia/lymphoma.

and M3-mAChR were calculated from the band intensities using computerized densitometry with ImageQuantTL software (GE Healthcare Life Sciences) . Notably, the M3-mAChR immunoblot had two bands, which were considered and quantified as M3-mAChR in accordance with a previous study (19).

Statistical analysis. All data are expressed as the means ± standard deviation (SD). To determine whether the data set was normally distributed, a Shapiro-Wilk normality test was performed. As some groups contained only small sample numbers, statistically significant differences were assessed using the non-parametric Kruskal-Wallis one-way analysis of variance for rank with the post hoc Dunn's test. A P-value <0.05 is considered to indicate a statistically significant difference.

Results

Patient characteristics. The study included 5 healthy subjects and 51 patients with leukemia [AML (n=33), CML (n=5), and

Table II. Relative expression levels of α7-nAChR and M3-mAChR in patients with leukemia compare to healthy subjects.

	Relative expression levels (% of healthy subjects)			
	α7-nAChR		M3-mAChR	
Leukemia types	Mean	Median	Mean	Median
Normal[a] (5)	100	100	100	100
AML-M0 (6)	51	43	70	80
AML-M1 (5)	79	75	129	147
AML-M2 (8)	70	66	127	145
APL (6)	57	54	47	57
AML-M4 (8)	47	42	57	58
CML (5)	49	48	164	187
T-ALL (9)	26	19	40	49
B-ALL (4)	62	60	135	147

[a]Normal is healthy subjects. Data in the parentheses are number of cases. Data are relative expression levels of α7-nAChR or M3-mAChR and presented as % of mean or median of leukemic groups to mean or median of healthy subject group. AML, acute myeloid leukemia; M0, AML with minimal differentiation; M1, AML without maturation; M2, AML with maturation; M3, acute promyelocytic leukemia; M4, acute myelomonocytic leukemia; CML, chronic myeloid leukemia; T-ALL, T lymphoblastic leukemia/lymphoma; B-ALL, B lymphoblastic leukemia/lymphoma.

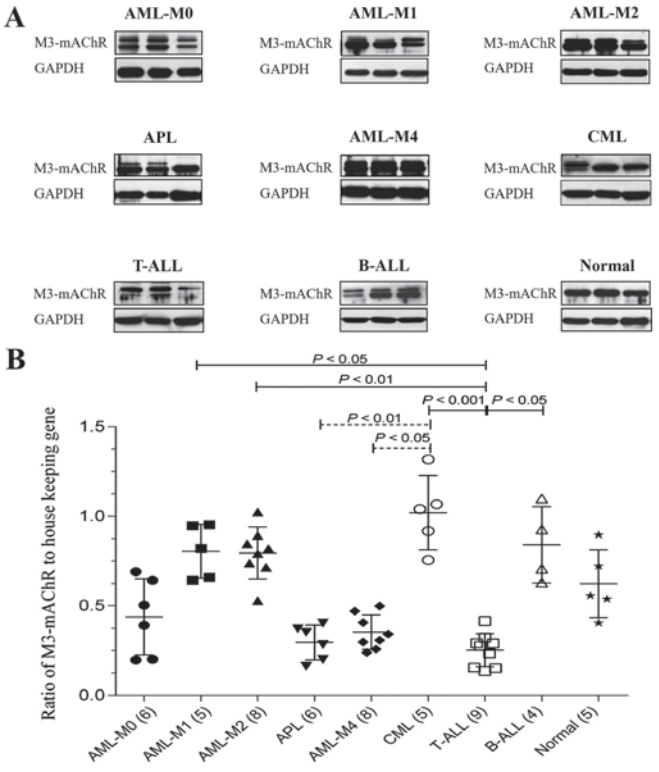

Figure 2. Expression levels of M3-mAChR in patients with leukemia. (A) Representative immunoblots of M3-mAChR in the three main types of leukemia, including five subtypes of AML, two subtypes of ALL, and CML. (B) Ratio of M3-mAChR to GAPDH as determined by densitometric analysis. Data are presented as the means ± standard deviation. The number of cases in each group is presented in parentheses at the x axis. P<0.05 was considered to indicate a statistically significant difference. M3-mAChR, muscarinic subtype M3 acetylcholine receptors; AML-M0, AML with minimal differentiation; AML-M1, AML without maturation; AML-M2, AML with maturation; APL, acute promyelocytic leukemia; AML-M4, acute myelomonocytic leukemia; CML, chronic myeloid leukemia; T-ALL, T lymphoblastic leukemia/lymphoma; B-ALL, B lymphoblastic leukemia/lymphoma.

ALL (n=13)]. According to the WHO classification (2), AML cases were further classified into subtypes: AML with minimal differentiation (AML-M0; n=6), AML without maturation (AML-M1; n=5), AML with maturation (AML-M2; n=8), acute promyelocytic leukemia (AML-M3; n=6), or acute myelomonocytic leukemia (AML-M4; n=8). ALL was further classified as T lymphoblastic leukemia/lymphoma (T-ALL; n=9), and B lymphoblastic leukemia/lymphoma (B-ALL; n=4). Clinical characteristics are listed in Table I. AML and ALL occur in children as well as in adults (subject age range 15-85) but CML has only been found in adults (subject age range 26-57). Hemoglobin levels and percentages of hematocrit in all groups of leukemia seem to be relatively lower than in healthy subjects. As predicted, the white blood cell count (WBC) was higher; meanwhile the platelet count was much lower in the patients with leukemia than in the healthy subject group.

Expression levels of α7-nAChR and M3-mAChR in patients with leukemia. Expression levels of α7-nAChR in PBMCs or BM in different types of patients with leukemia were compared with healthy subjects. It might be expected that all types of patients with leukemia would have lower expression levels of α7-nAChR than healthy subjects, but significant expression levels were observed in T-ALL, CML, AML-M40 and AML-M4 (Fig. 1 and Table II). The expression level of α7-nAChR was lowest in T-ALL, significantly different from in AML-M1, AML-M2 and AML-M4.

Regarding M3-mAChR expression, the results showed a large variation in M3-mAChR expression among leukemia types (Fig. 2 and Table II). The expression level of M3-mAChR was lowest in T-ALL, significantly different from in B-ALL, CML, AML-M1 and AML-M2. In addition, CML showed the highest level of M3-mAChR, significantly different from in APL and AML-M4. Notably, there was no significant difference between expression in the control group any in type of leukemia types.

Discussion

The putative involvement of the non-neuronal cholinergic system in the etiology of hematopoietic derived neoplasia is of particular importance in view of various recent reports (16-18). Several studies have provided evidence demonstrating that blood cells, especially lymphocytes, possess non-neuronal cholinergic components including cholinergic AChRs (14,20). Our results showed a variation of cholinergic AChRs, including α7-nAChR and M3-AChR, in the BM or PBMC samples from patients with the three main types of leukemia: AML, ALL, and CML. In detail, α7-nAChR in T-ALL, CML, but not B-ALL, were lower in patients with leukemia than in healthy

subjects. It has been reported that cholinergic signals regulate thymic differentiation and selection (21). Furthermore, previous studies have shown that α7-nAChR is involved in both T and B lymphocyte development in the bone marrow and spleen (22). In AML subtypes based on levels of myeloid maturation, variation in α7-nAChR expression was observed. The α7-nAChR expression was lowest in AML-M0 (AML with minimal differentiation); its level of expression increased to a maximum in AML-M1, then continuously declined in AML-M2, APL, and AML-M4. This pattern of variation was also observed in the expression of α4-nAChR subunits during B lymphocyte development in wild-type mice (22).

Among AML subtypes, we found that M3-mAChR expression was lowest in APL and AML-M4. Our previous *in vitro* study on the NB-4 acute promyeolocytic leukemic cell line demonstrated that M3-mAChR markedly increases after all-*trans*-retinoic acid-induced differentiation treatment (16). Hence, administration of a specific M3-mAChR agonist along with differentiation-inducing drugs may be a potential treatment for the APL subtype. Amongst the leukemia types, the highest expression of M3-mAChR was detected in CML, where most myeloid cells are mature. Collectively, these observations support the association of M3-mAChR in myeloid maturation. It is notable that in previous studies of the CML K562 cell line, muscarinic receptor activation stimulated intracellular cAMP, decreased c-Fos and cyclin D1 expression, and inhibited cell proliferation (19,23). Altogether, mAChR activation by its agonist may also be a potential approach for treatment of CML. However, this hypothesis needs further study.

Previous studies have demonstrated that ACh production in various leukemic T-cell lines, including CEM, Jurkat, HSB-2, MOLT-3, and MOLT-4, was considerably higher compared to fresh PBMCs obtained from healthy subjects (24). Moreover, it has been suggested that ACh may not be rapidly hydrolyzed in T-ALL as AChE is decreased in these types of leukemic cells, compared with in mature normal T-cell lymphocytes (25). Previous studies have shown that Jurkat, T-cell-derived leukemic cells, increased M3-mAChR expression (26). In addition, it has been proposed that high levels of ACh in T-ALL may act as an autocrine growth factor and play a significant role in leukemia T-cell clonal expansion via shaping of intracellular calcium signaling pathways (18). However, our study showed the lowest expression of both α7-nAChR and M3-mAChR was in T-ALL patients. It could be hypothesized that the reduction of cholinergic receptors may represent an adaptation mechanism of cholinergic over-activation.

In conclusion, our observations highlight the differential expression profiles of α7-nAChR and M3-mAChR among the three main types of leukemia, and that these expression patterns may mediate leukemogenesis. Moreover, these findings may support the utilization of cholinergic AChRs as potential prognostic markers and alternative therapeutic treatments. However, further studies with larger cohorts, and functional studies, are necessary to elucidate this proposal.

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Availability of data and materials

All data generated and analyzed during this study are included in this article.

Authors' contributions

TS performed the experiment, analyzed the data, and drafted the manuscript. SC and KC performed the experiments. CUA participated in the design of the study and supervised subject selection and sample collection. OP performed the sample collection. JS was responsible for initiation, conception, experimental design, execution of the entire project and for critical revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Committee on Human Rights Related to Research Involving Human Subjects of the Chulabhorn Research Institute (CRI project number: 33/2554, approval date: 29/02/2012) and the Ethics Committee of Siriraj Hospital (Project number: 255/2555, approval date: 01/08/2012). All patients provided written informed consent for the publication of data in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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