Expression of the Chemokine MCP-1 and Chemokine Receptors CXCR4 and CCCR2 in Egyptian Acute Myeloid Leukemia Patients

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Abstract: Background: AML blasts of different FAB subsets express specific chemokines and chemokine receptors depending on their degree of maturation which might account for some aspects in their pattern of extramedullary invasion (EMI) and accumulation of leukemic cells. **Objectives:** We aimed to define the pattern of chemokine MCP-1 and chemokine receptors CXCR4 and CCR2 expression by AML blasts in two AML FAB subgroups, myeloid M0/M1/ M2 and monocytic M4/M5 groups to determine their impact on tumor load and EMI. **Patients and Methods:** The study was performed on 50 de novo AML patients. Expression of CXCR4 and CCR2 was measured by flow cytometry while MCP-1 expression was detected by reverse transcriptase polymerase chain reaction (RT-PCR). **Results:** Median TLC was 65.6 x 10^9/L in MCP1 positive patients versus 37.2 x 10^9/L in MCP1 negative cases (p = 0.07). MCP1 was positive in 14/20 (70%) patients with EMI versus 6/20 (30%) patients only without EMI (p = 0.05). CXCR4 was positive in most AML patients (38/50, 76%) with no significant difference between AML FAB subgroups (p =0.9). However, median CXCR4 percent positivity by flow cytometry was 79% (0.3-98) in the M4/M5 group versus 57.5% (1.9-89) in the M0/M1/ M2 (p = 0.08). CXCR4 was positive in 35/43 patients (81%) with hypercellular bone marrow (BM) at diagnosis (p =0.02). CCR2 positivity was higher in M4/M5 group (8/21, 38%) than M0/M1/M2 group (2/29, 7%) (p =0.006). Meanwhile, MCP-1 expression was positive in 20/48 (41.7%) of our AML cohort and was insignificantly higher in M4/M5 group (10/21, 48%) than M0/M1/M2 group (10/29, 34%) (p =0.2). Regarding EMI, lymphadenopathy was found in 90% of patients in M4/M5 group versus 30% in M0/M1/M2 group (p =0.001). **Conclusion:** Data suggest that MCP-1 and CXCR4 have major impact on tumor load in AML at time of diagnosis. In addition, MCP1 have a striking role in EMI irrespective of the FAB subtype. Its ligand CCR2 seems to be restricted to monocytic group (M4/M5) which showed significant lymphadenopathy when compared to M0/M1/M2 group.

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1. Introduction

AML is an aggressive disease characterized by accumulation of immature malignant cells in the bone marrow (BM) which may later invade the blood stream and localize in extramedullary sites [1]. The 5 years overall survival (OS) for patients less than 60 years of age receiving the most intensive conventional chemotherapy is less than 50% [2]. AML is considered a very heterogenous disorder, and the recently published WHO classification was therefore based on a combination of clinical history, morphology, cytogenetic and molecular abnormalities [3].

The mechanisms regulating the trafficking of leukemic myeloid blasts are still poorly understood. Chemokines are a family of soluble proteins that are involved in wide range of biological processes with relevance for hematological malignancies including cell trafficking, regulation of cell proliferation and apoptosis, immunoregulation, normal hematopoiesis and angiogenesis [4]. A substantial evidence of AML blasts migration requires the sequential engagement of specific cytokines and their receptors [1]. Monocyte chemoattractant protein (MCP1) and its ligand CCR2 are chemokines that could be involved in this process [1, 5]. The local chemokine network in human AML is probably further modulated by the hypoxic BM microenvironment and the local release of chemokines by non leukemic BM stromal cells [6]. The accumulation of malignant cells can be indeed favored by the production of chemokines by tumor cells themselves and/or surrounding non-tumoral cells [7]. Furthermore, CXCR4 expression was widely described and proved to have a prognostic impact in AML [8].

In this study, we aimed to define the pattern of chemokine (MCP-1) and chemokine receptors (CXCR4 and CCR2) expression by AML blasts in two AML FAB subgroups, myeloid M0/M1/ M2 group and monocytic M4/M5 group. Chemokines expression was correlated to tumor load manifested

by high total leucocytic count (TLC), degree of bone marrow cellularity and infiltration by blasts and extramedullary invasion (EMI) as represented by organomegally and lymphadenopathy.

2. Patients and Methods

The present study was carried in the Clinical Pathology Department, National Cancer Institute (NCI), Cairo University during the period between July 2007 and December 2009.

Patients: Fifty de novo AML patients (28 males and 22 females) presented to the Hemato-Oncology department prior to any treatment. Patients were evaluated for expression of CXCR4 and CCR2 by flow cytometry and for expression of MCP-1 by RT-PCR. Written informed consent was obtained from every patient. Study was carried after approval of the institutional review board according to declaration of Helsinki.

All patients were subjected to complete history and physical examination with particular attention to age, gender, presenting symptoms, performance status, presence of fever, signs of infections, bleeding manifestations, hepatomegaly, splenomegaly, lymphadenopathy and symptoms of central nervous system (CNS) infiltration (headache, vomiting, blurring of vision). Tumor load was defined as high TLC at diagnosis, degree of bone marrow (BM) infiltration (cellularity and bone marrow blast % at diagnosis) and EMI in spleen, lymph nodes (LN), gum, CSF and skin [9]. Hepatomegaly as a parameter of EMI was excluded due to the high incidence of bilharziasis and viral hepatitis in Egypt.

Methods: Laboratory investigations done included complete blood count (CBC) with TLC, blasts percentage, hemoglobin level and platelet count. Biochemical analysis included serum urea, creatinine, uric acid, liver profile [serum bilirubin, alkaline phosphatase, AST, ALT, serum albumin], fasting blood sugar and 2 hrs postprandial and serum LDH.

Bone marrow aspirate smears were examined for assessment of cellularity and blasts percentage. **Cytochemistry (**myeloperoxidase [or Suddan Black] supplemented by Periodic acid shiff, acid phosphatase, specific esterase and non-specific esterase) were performed whenever necessary. All patients were classified according to the French-American-British [FAB] classification.

CSF examination was performed to patients with symptoms of CNS involvement, AML M4 and M5 and patients with TLC >100x 10^9/L.

Immunophenorypic analysis on peripheral blood (PB) or BM blasts was performed at diagnosis using multicolor flow cytometry (Coulter Epics, XL, Hialeh) [10]. A wide panel of FITC (flourescein) or PE (phycoerythrin) conjugated monoclonal antibodies (MoAb) was used including pan leucocytic marker (CD45), myeloid markers (MPO, CD33, CD13, and CD14), B-lymphoid markers (CD 19, CD 10, and CD22), T-lymphoid markers (CD3, CD4, CD5, CD7, and CD8), stem cell marker CD34 and HLA-DR in addition to anti ***CXCR4*** Mo Ab, FITC *(DAKO)* **and** anti ***CCR2*** PE *(R & D systems).*

Detection of surface markers: The whole blood staining method was performed. In short, 10 ul labeled MoAb was added to 100 ul blood, incubated in the dark for 20 minutes then processed by Q system (coulter Corp, Hialeh, Fl) where immunoprep reagent A for lysing, B as stabilizer and C as fixative were consecutively added.

Detection of intracellular markers: Hundred ul of whole blood was lysed using lysis solution (Becton and Dickenson) for 10 minutes. Cells were washed once and resuspended in 1 ml PBS. A mixture of 500 ul 4% paraformaldehyde as fixative, 500 ul PBS and 5 ul tween 20 as detergent was added to the cells and incubated or 10 min. The cells were washed and 10 ul Mo Ab was added and incubated for 3 min at 4ºC. Cells were washed, suspended in 500ul PBS and analyzed.

Interpretation: Any antigen was considered positive when ≥ 20% of blast cells were stained above the negative control except for CD34, MPO and CD10 where ≥ 10% was considered positive [8]. Cell surface expression of CXCR4 and CCR2 was considered positive above 20%.

MCP-1 detection by RT-PCR:

RNA Extraction: Total RNA was extracted from 300 ul PB or BM samples using a salting out procedure *(Purescript, Gentra, Minneapolis, MN, USA)* according to manufacturer's instructions. Samples were stored at -70°c till used**.** RNA quality was assayed by gel electrophoreses on ethidium bromide stained 1% agarose containing 2.2 mol/L formaldehyde.

Reverse Transcription*:* cDNA synthesis was performed by reverse transcriptase using Gene Amp Gold RNA PCR Reagent Kit *(Applied Biosystems, USA),* 1ug RNA was used in 20 uL volume including 1x RT-PCR buffer, 2.5 mM Mg CL2, 1 mM dNTPS blend, 10 U/20 ul RNase inhibitor, 10 mM DTT, 1.25 um Random Hexamer and 15 U/ 20 ul Multiscribe Reverse Transcriptase enzyme. Cyclic conditions consisted of 25°C for 10 min and 42°C for 1 hour.

PCR reaction for MCP-1 detection consisted of 5x RT PCR buffer, 1.75 mM MgCL2, 0.8 uL 200uM of each dNTPs, 2-5 U of Ampli Taq Gold DNA polymerase enzyme (5 units/ ul), 1 uL of 7.5 pmol of each primer and 2-3 ul cDNA. DEPC water was added to a total reaction volume of 50 ul. MCP-1 primer sequence was sense: 5-CTC ATA GCA GCC ACC TTC AT-3 and antisense: 5- GCT TTT CCT

CTT GAA CCA CA-3 *(R & D systems, US Biological, USA).*

Cycling conditions: Initial denaturation at 95°C for 5 min was performed in a thermocycler *(biometra, Germany)* followed by 35 cycles of amplification consisting of denaturation at 95°C for 45 sec, annealing at 65°C for 45 sec and extension at 72°C for 45 sec. A final extension step of 10 min at 72°C was added. A housekeeping gene, B-actin was run with every PCR reaction to check cDNA integrity and exclude any PCR failure. Each run included positive control cell line supplied from R & D, negative control cell line, HL 60 and non template control (NTC). All PCR products obtained through individualized RT-PCR reactions were separated on 2% agarose gel electrophoresis. Positive sample reaction appeared at 198 bp. Positive control appeared at 320 bp.

Statistical Methods:

Data was analyzed using SPSS win statistical package version 15 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation (SD) or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. Comparison between two groups regarding quantitative variables was done using non-parametric t-test. Survival analysis was done using Kaplan-Meier method and Cox-regression method. All tests used were two-tailed. *P*-value ≤ 0.05 was considered significant [11].

3. Results

This work was carried out on 50 de novo AML patients: 28 males (56%) and 22 females (44%) with a median age of 37.5 years (1 month - 82 years). According to FAB classification, one patient (2%) was classified as M0, 14 (28%) as M1, 14 (28%) as M2, 18 (36%) as M4 and 3 patients (6%) as M5. M0/ M1/ M2 patients were classified together as one group and constituted 58% (29/50) of all cases while M4/ M5 group constituted 42% (21/50). Median follow up period of patients was 18 months (3- 28.6) Table 1 represents major laboratory parameters and table 2 shows clinical data of 50 AML patients.

Table (1): Laboratory data of 50 AML patients

Parameters	Median (range)
TLC (10^9/L)	55.7 (1-351)
Hb (g/dL)	7 (3.9-13)
Platelets (10^9/L)	41.5 (4-187)
BM blast % at diagnosis	66 (12-98)
BM blast % at complete remission (CR)	1 (0-5)

Table (2): Clinical data of 50 AML patients

Parameter	Number	Percent
Hepatomegaly	25	50%
Splenomegaly	25	50%
Hepatosplenoegaly	17	34%
Lymphadenopathy	27	54%
Spleen + Lymphadenopathy	12	24%
Gum involvement	2	4%
CSF infiltration	0	0%
Skin involvement	0	0%
Extramedullary infiltration (involvement of spleen, LN, gum, CSF and/or skin)	28	56%
BM cellularity at time of diagnosis		
Hypercellular	43	86%
Normocellular	7	14%

Median age of M0/ M1/ M2 group was 45 years (18-70) while median age was 24 years (1 month -82 years) in the M4/ M5 group (*P* = 0.01). Median Bone marrow blast percent (%) at time of diagnosis in M0/ M1/ M2 group was 75% (12-98%) versus 61% (15-87%) in M4/ M5 group (*P* = 0.08). None of other clinical or laboratory parameters as gender, TLC, Hb, platelet count, splenomegaly, lymph node (LN) involvement or BM cellularity carried a statistically significant difference between both studied FAB groups.

Expression of CXCR4 by flow cytometry: CXCR4 was positive in 38/50 patient (76%) and was negative in 12 (24%) patients. Median percentage expression was 68% (0.3-98%). Twenty two /28 patients (78%) were positive for CXCR4 in the M0/M1/M2 group and 16/21 (76%) patients in the M4/ M5 group (*P* = 0.9).

Median CXCR4 % positivity was 57.5% (1.9-89) in the M0/ M1/ M2 group versus 79% (0.3-98) in the M4/ M5 group ($P = 0.08$).

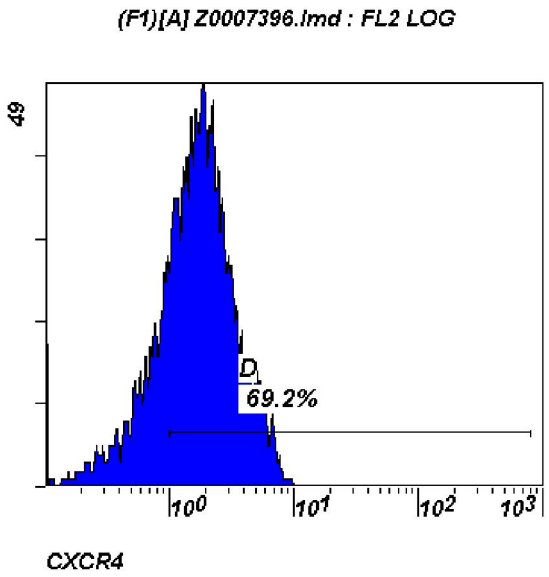

Figure (1): Flow cytometric histogram of an M5 case positive for CXCR4.

Expression of CCR2 by flow cytometry: CCR2 was positive in 10/50 (20%) and was negative in 40/50 patients (80%) in the whole AML cohort. Two patients were positive for CCR2 (2/29, 7%) in the M0/M1/ M2 group while 8/21 (38%) patients in the M4/M5 group were positive ($P = 0.006$, Figure 2).

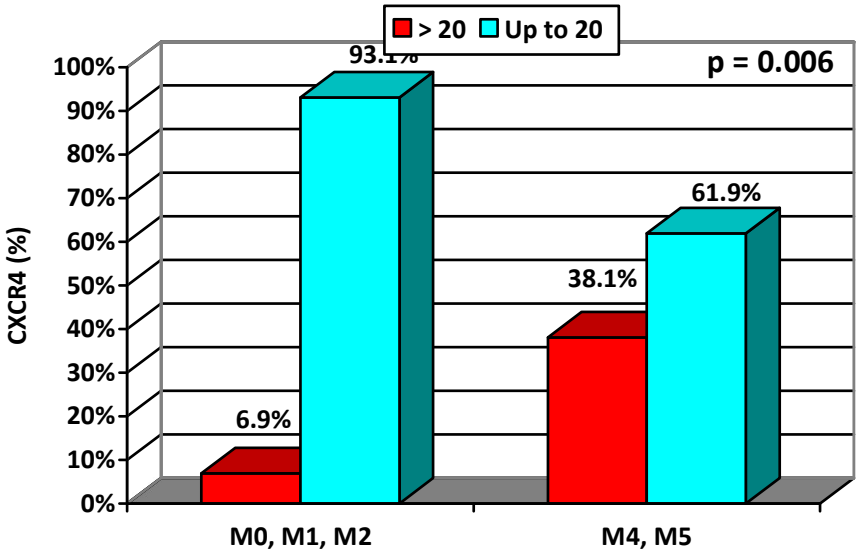

Figure (2): CCR2 positive patients in different phenotypic groups of AML patients

Median CCR2 MFI ratio expression was 1.5 (0.5-11) in the whole AML group. Median was 1.1 (0.49-11) in M0/M1/M2 group while in the M4/M5 group, median was 3.5 (1.5-9.4) ($P = 0.01$).

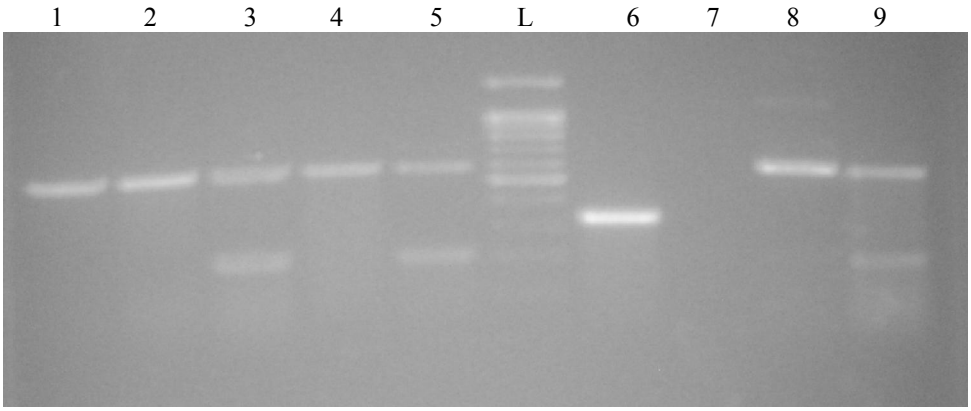

Fig. (3): MCP-1 expression by RT-PCR: L= Molecular weight marker (Ladder) Lanes 1, 2, 4 and 8= Negative MCP-1 samples. Lanes 3, 5 and 9= Positive MCP-1 samples (198bp). Lane 7= Non Template Control. Lane 6= Positive Control (320bp).

MCP-1 EXPRESSION BY RT-PCR: Forty-eight cases were tested: 20 patients (41.7%) were positive while 28 patients (58.3%) were negative (Figure 3). Ten out of 29 patients (34%) in M0/M1/M2 group were positive versus 10/21 (48%) in M4/M5 group (*P* = 0.2).

Relation between the level of expressions of CXCR4, CCR2 and MCP-1 and tumor load:

EMI was present in 15/28 (54%) of patients in the M0/M1/M2 group versus 13/28 (46%) of patients in the M4/ M5 group (*P* = 0.4).

Lymphadenopathy was found in 19/21 (90%) of patients in the M4/M5 group versus 8/29 (30%) in the M0/ M1/ M2 group (*P* =0.001).

Regarding CXCR4 and CCR2 expression levels, no statistic significant relation to tumor load was detected. However in MCP-1 positive patients, median TLC was 65.6 x 10^9/L (1-351.0 x 10^9/L) versus 37.2 x 10^9/L (3-267 x 10^9/L) in MCP-1 negative cases (*P* = 0.07). In addition, 14/20 (70%) patients with EMI had positive MCP1 expression versus 6/20 (30%) patients only with no EMI (*P* = 0.05).

BM cellularity: 35/43 of patients (81%) with hypercellular BM were positive for CXCR4 versus 3/7 patients (43%) with normocellular marrow (p=0.02). However, BM hypercellularity did not show any statistically significant relation with CCR2 or MCP1 positivity (*P* =0.6).

Relation of chemokines expression to BM blast % at time of diagnosis: No statistically significant association was encountered between BM blast % at diagnosis and CXCR4, CCR2 or MPC1 positivity (*P* =0.7, *P* =0.2 and *P* =0.5 respectively).

Overall Survival was higher in M0/M1/ M2 group than the M4/M5 group, however the relation did not reach statistical significance (*P* = 0.3). CXCR4, CCR2 and MCP1 did not show any impact on survival (*P* =0.2, *P* =0.3 and *P* =0.6, respectively).

4. Discussion

AML is a very heterogeneous disorder. Besides cytogenetic and molecular abnormalities, interactions of stromal cells and extracellular matrix with leukemic blasts can also generate antiapoptotic signals that contribute to neoplastic progression and persistence of treatment related minimal residual disease [12]. Cytokine induced, receptor-mediated phosphorylation patterns of intracellular mediators can identify subsets of patients with different prognosis [8].

In this study, median age range was 37.5 years and male to female ratio was 1.2:1. In similar studies, male to female ratio was comparable however median age was higher (64yrs) [8, 13]. The different FAB subsets in our patients were also comparable to western value [14]. Fifty six % (28/50) of our patients had EMI in spleen, LN, gum, CSF and/or skin. Another study detected EMI in 40% of patients and nearly all fell in the M4/ M5 group. This group also excluded liver involvement from their study because of endemic hepatitis [1]. In our study, 90% of cases associated with LN enlargement fell in the M4/M5 versus 30% only in M0/M1/M2 group (p=0.001). However, no significant difference between the two groups regarding EMI as a whole was observed. One group found that 40% of AML patients with EMI were mostly M4/M5 [15] while EMI was reported in 30% of patients in another series [16].

CXCR4 was positive in most of our AML patients (38/50, 76%) regardless the FAB subtype confirming previous published results [1, 17]. Mean CXCR4 expression in patients with no EMI was 62.7±30.7% which was significantly higher than that

of patients with EMI (49.4±31.9%) (P =0.04). An explanation stated is that CXCR4 allows tumor cells to access cellular niches in the BM that favor tumor cell survival and growth [17]. In support of this evidence was the significant relation between CXCR4 expression and BM cellularity at time of diagnosis. The majority of our patients with hypercellular BM (81%) were CXCR4 positive while 57% of normocellular marrows were CXCR4 negative (P =0.02). However no significant difference in CXCR4 expression between the 2 FAB subgroups studied was found. This goes hand in hand with another report that found CXCR4 widely expressed in 84% of AML patients [1].

MCP-1 expression was positive in 34% of M0/M1/M2 group versus 48% in the M4/M5 group in our patient cohort. Although higher in monocytic group, no statistically significant difference was detected between the 2 groups (p=0.2). In contrast, others found MCP-1 positive in 37.5% of M0/M1 group, 60% in M2 group and 100% in M4/M5 group, with a significant difference between the 3 groups [1]. One group explained this variability of results by measuring MCP-1 during AML blasts culture and showing that the time at which maximal amounts of MCP-1 was produced differed between AML samples [19].

Regarding CCR2 expression, 7% of patients in the M0/M1/M2 group were positive versus 38% of patients in the M4/M5 group (p=0.006). It was stated that CCR2 is uniquely expressed by all M4/M5 subsets and not other AML FAB subsets [1]. From 20 positive patients for MCP-1 expression, five were positive for its ligand CCR2 while from 28 MCP1 negative patients; only four were positive for CCR2 giving a concordance of 60% (29/48) and disconcordance of 40% (19/48) between both markers. Our data agree with others who stated that production of chemokines and their specific receptors such as MCP-1/CCR2 allows functional cross talks within the malignant clone that helps its accumulation. This was significant in finding that 90% of patients with lymphadenopathy fell in the M4/M5 group as both markers were higher than in the M0/M1/M2 group. In addition, median TLC was higher in the MCP-1 positive than negative group and CXCR4 was positive in nearly all patients with hypercellular BM underscoring the effect of these 2 cytokines on tumor load at time of diagnosis. However, our results are not in complete agreement with studies that reported MCP-1 expression to be responsible for EMI as a whole in monocytic leukemia as EMI was found higher in our M0/M1/M2 group. It is the chemokine itself that seems to be responsible for EMI whenever expressed by AML blasts. As a proof of different biological behavior in our patient cohort, the frequency of MCP-1 positivity was comparable in both AML subgroups but positive MCP1 was significantly associated with patients showing signs of EMI.

In conclusion, MCP-1 is associated with higher TLC at diagnosis and seems to play a significant role in EMI. It has a higher expression in monocytic leukemias, although insignificant however, its ligand CCR2 is nearly restricted to M4/M5 FAB AML subtypes suggesting that chemokine/receptor interactions orchestrate EMI in AML. CXCR4 showed unrestricted positivity in most AML FAB subtypes and was significantly associated with tumor load at diagnosis especially hypercellular BM and was inversely associated with EMI. In AML, chemokine expression is one of many variable factors that modulate this very heterogeneous disease. Other exogenous growth factors which are cytokine dependent in proliferation like GM-CSF, SCF and Flt3L should be investigated concomitantly. A larger scale of patients needs to be included for accurate characterization of the function played by these specific cytokines in different AML FAB subtypes.

Competing interest: The authors declare no competing financial interests.

Author Contribution: MA designed and performed research and analyzed data. YN and NE performed research, carried out molecular genetic studies and flow cytomery and analyzed laboratory data. FE performed research, analyzed molecular genetic data and wrote the paper. HR and YM designed and facilitated the research. All authors read and approved the final manuscript.

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