# Expression of (NM23-H1) gene in acute lymphocytic leukemia and its clinical significance

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## Abstract

**Introduction:** The aim of this article is to determine the expression of the NM23-H1 gene in acute lymphocytic leukaemia, and to evaluate the relationship between NM23-H1 expression, clinical features and laboratory features.

**Material and methods:** The study included 25 newly diagnosed ALL patients and a control group of 10 age- and sex-matched healthy individuals with no family history of leukaemia. All patients and controls were subjected to careful history taking, complete blood picture, bone marrow aspirate examination, cytochemistry (peroxidase and Sudan black) and immunophenotyping (using the ALL panel to detect B cell markers and T cell markers). Venous blood samples from patients and controls were analysed for NM23-H1 mRNA expression level by real time PCR.

**Results:** NM23-H1 mRNA was overexpressed in ALL cases compared to the control group, with a mean level of 0.99 ±0.74, 0.22 ±0.16 respectively. The resistant cases expressed a significantly higher NM23-H1 level (mean 0.975) than those who achieved remission (mean 0.805), and passed out cases expressed a highly significant level of the studied gene (mean 1.780).

**Conclusions:** There is a highly significant positive correlation between the level of expression of the NM23-H1 gene and the outcome of patients with ALL.

**Key words:** *NM23-H1* gene, ALL, real time PCR.

## Introduction

Leukaemias are diseases which develop as a consequence of abnormal, uncontrolled proliferation of a single mutant haematopoietic progenitor cell that has the capability to expand by indefinite self-renewal, giving rise to malignant, poorly differentiated haematopoietic cells that progressively infiltrate the bone marrow [1].

They have in common a high fatality rate due to complications of bone marrow failure or infiltration of tissues because of uncontrolled generalized irreversible proliferation of one line of leukocytes; thus it is often associated with abnormal white blood counts and eventually leads to anaemia, thrombocytopenia and severe infections [2].

Acute leukaemia is broadly classified into myeloid and lymphoid cell types and subdivided according to stages of differentiation. The diagnostic methods for identification of AML and ALL and their subtypes are based

on morphological, cytochemical and immunophenotyping patterns, besides genetic and molecular analysis [3].

Acute lymphoblastic leukaemia (ALL) results from the clonal proliferation and accumulation of progenitors that exhibit cell markers associated with the earliest stage of lymphoid maturation. This leukaemic clone may exhibit features of either B-cell or T-cell commitment or sometimes mixed-lineage leukaemia [4].

Acute lymphoblastic leukaemia is one of the most important diseases in oncology due to the progress that has occurred in its understanding and treatment over the past 50 years. Enhanced understanding of ALL came with the demonstration that the blast cells could be defined by specific markers, then studies were subsequently amplified to allow definition of lymphoid lineage and degree of maturation which have clinical significance [5].

A non-differentiating mouse myeloid leukaemia cell line produced differentiation inhibiting factors (I-factors). Suppression of these I-factors resulted in non-differentiating leukaemic cells becoming sensitive to differentiation inducers. One of these I factors was purified as a homologue of NM23 [6]. The *NM23* gene was originally identified by the differential hybridization of a cDNA library with total RNA extracted from mildly and highly metastatic melanoma cell lines [7]. The differentiation inhibitory factor *NM23* can inhibit the differentiation of murine and human myeloid leukaemia cells, and *NM23* expression is greatly increased during blast formation in normal lymphocytes [8]. These findings suggest that NM23 genes play a role in the growth and differentiation of normal and malignant haematopoietic cells [9].

Two types of human *NM23* gene have been identified: *NM23-H1* and *NM23-H2*. They show 88% amino acid sequence homology and the genes are located on the same region of chromosome 17q21 [10]. Based on an analysis of the promoter regions of the *NM23-H1* and *NM23-H2* genes, they are independently and differentially regulated. However, a statistically significant correlation between the expression of NM23-H1 and NM23-H2 was observed in ALL, whereas a poor prognosis and a low percentage of complete remission were associated only with the NM23-H1 expression level [11].

It was reported that *NM23* genes were overexpressed in acute myelogenous leukaemia (AML) and that NM23-H1 expression was significantly correlated with a poor prognosis in AML, especially in AML-M5, in ALL, MDS, and CML-BC [12].

It has been reported that high-grade non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma exhibited significantly higher levels of NM23-H1 expression than low-grade NHL [13]. These studies suggest that NM23-H1 expression in human haematopoietic malignancies is associated with the aggressiveness of the disease [14].

Drug resistance, either inherent or acquired, is an important cause of treatment failure in haematological neoplasms. Ferguson *et al*. reported a functional link between NM-23 expression and cancer cell sensitivity to the alkylating agent [15]. Other mechanisms may be involved in drug resistance. *NM23* genes can modulate differentiation, proliferation (cell cycle), and drug resistance in AML, and these three events are closely linked. Inducing differentiation in leukaemic cells is associated with the concomitant block of the cell cycle at the G0/G1 phase [15].

Transforming growth factor-β (TGF-β) is a negative regulator of proliferation, inducing growth arrest in the G1 phase of the cell cycle in many cell types, and the loss of the cellular response to this ligand that occurs during oncogenesis in some systems. Transfection of melanoma and breast carcinoma cells with the *NM23* gene decreases the response to TGF-β [16, 17]. The role of *NM23* expression in the malignant growth of leukaemia cells remains to be clarified, but *NM23* may play key roles in various aspects of haematopoietic cell biology, including differentiation, proliferation (cell cycle), and drug resistance.

## Material and methods

### Material

**Patient samples:** peripheral blood was obtained from 25 newly diagnosed ALL patients. They were attending the National Cancer Institute haematology/oncology unit (El manial – Fom el khaleeg – Cairo University). Their age ranged from 2 to 56 years with a mean of 25.96 ±16.48. There were 8 females (32%) and 17 males (68%). Patients were primarily diagnosed as having ALL on the basis of history taking, clinical examination laying stress on the presence of lymphadenopathy, hepatomegaly, splenomegaly and laboratory investigations. Informed consent was taken from the patients and the parents of the children according to guidelines of the local ethical committee of the National Research Centre.

**Control samples:** 10 age- and sex-matched healthy individuals with no family history of leukaemias and normal CBC were included. Their age ranged between 3 and 52 years with a mean of 24.3 ±16.18. There were 4 females (40%) and 6 males (60).

Patients were subjected to the following:

a) routine laboratory investigations:
- complete blood picture,
- bone marrow examination,

- cytochemistry: peroxidase, Sudan black tests,
- immunophenotyping: using ALL panel to detect B cell markers;

b) special investigations:

- analysis of *NM23-H1* gene expression using real time PCR (polymerase chain reaction) for cases and controls.

## Methods

Study of NM23-H1 m-RNA expression by real-time PCR in peripheral blood in patients with ALL.

### Sample collection

From every patient and control subject 3 ml of venous blood was withdrawn under complete aseptic conditions into EDTA Vacutainers for assay of expression of NM23-H1 m-RNA.

### RNA purification using RNA isolation kit for total RNA isolation from whole human blood (QIAamp RNA Blood mini kit, catalogue number 52304), Qiagen, Germany (www.qiagen.com)

#### *Principle*

QIAamp spin columns represent a technology for total RNA preparation that combines the selective binding properties of a silica-gel-based membrane with the speed and convenience of microspin technology.

#### *Reagents*

- QIAamp Spin columns (clear) (50),
- QIAampshredderTM Spin columns (lilac) (50),
- collection tubes (1.5 ml) (50),
- buffer EL (5OX120),
- buffer RLT (45 ml): 10 µl of β-ME (β-mercaptoethanol) per 1 ml of RLT buffer,
- were added to this buffer before use,
- buffer RW1 (45 ml),
- buffer RPE (11 ml): it is supplied as concentrate – before use 4 volumes of ethanol (96%) were added to make a working solution,
- RNase-free water (10 ml),
- ethanol (96-100%),
- 70% ethanol in water,
- β-mercaptoethanol (14.5 ml),
- tube for erythrocytes lysis (1.5 ml – 15 ml depending on sample size),
- sterile RNase-free pipette tips,
- microcentrifuge with rotor for 2 ml tubes.

#### *Real time PCR for NM23-H1 quantification*

– Primers and probes for NM23-H1

– Sequence specific primers for NM23-H1: (gene bank accession No. X68193)

Forward primer: 5'-ATG GCC AAC TGT GAG CGT ACC-3'

Reverse primer: 5'-CAT GTA TTT CAC CAG GCC GGC-3'

LightCycler hybridization probe:

Nm23-H1 FL: 5'-GAA ATT CATGCA AGC TTC CGA AGA TCT X -3'

Nm23-H1 LC: 5'-CTC AAG GAA CAC TAC GTT GAC CTG AAG G P-3'

– GAPDH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping gene is treated as internal control (gene bank accession No. M33197)

• Sequence specific primers for GAPDH:

Forward primer: 5'-ACATCGCTCAGACACCATGG-3'

Reverse primer: 5'-GTAGTTGAGGTCAATGAAGGG-3'

LightCycler hybridization probe

GAPDH FL: 5' -TGTCCCCACTGCCAACGTGTCAG X-3'

GAPDH LC: 5'- GGTGGACCTGACCTGCCGTCTAGA P -3'

4–5 µl extracted RNA and GAPDH standards used to prepare the standard curve.

The RNA level of the NM23-H1 gene is normalized by the level of the GAPDH RNA present in the sample. For relative quantitation the values obtained are compared to those from the standard RNA dilutions which are amplified by the RT-PCR in parallel.

#### *Procedure*

For every patient, two PCR reaction mixtures were prepared, for assay of NM23-H1 mRNA and GAPDH mRNA expression levels in the RNA isolated from peripheral blood collected from patients and controls. The results are given as the NM23-H1/GAPDH ratio [18].

### Calibration curve

- Quantification: is to know the input copy number of the RNA in the sample.
- To determine the copy number of target transcripts (NM23-H1 mRNA) the GAPDH standard was used to generate a calibration curve. Total RNA of healthy donors was serially diluted in log step from $10^7$ copies to $10^3$ copies in µl volume.
- A calibration curve was created by plotting the threshold (Ct) vs. a known copy number, for each template in the dilution. The copy numbers for all known samples were determined by real time software according to the calibration curve. The calibrators were defined to contain arbitrary units of GAPDH RNA and all calculated concentrations were relative to these concentrations.

### Interpretation of results

Instead of using end point fluorescence to calculate quantity it was calculated in real time based on the reaction exponential PCR phase (cp-crossing point).

### Quantification

The LightCycler apparatus measured the fluorescence of each sample in every cycle at the end of the annealing step. The second derivative maximum method was used to determine the crossing point (cp) automatically for individual samples.

This was achieved by a software algorithm (Ver.305) that identified the first turning point of the fluorescence curve, corresponding to the first maximum of the second derivative curve, which serves as the cp.

LightCycler software constructed the calibration curve by plotting the cp versus the logarithm of the number of copies for the calibrator. The number of copies in unknown samples was calculated by comparing their cps with the calibration curve. To correct the differences in both RNA quality and quantity between samples, data were normalized using the ratio of target cDNA concentration to that of GAPDH [19].

**Table I.** Descriptive statistics of the result of the real time PCR analysis for NM23-H1 gene

| | NM23-H1 expression | | | *P* value |
|---|---|---|---|---|
| | Mean ± SD | Maximum | Minimum | |
| ALL patients (*n* = 25) | 0.01 | 2.8 | 0.99 ±0.74 | 0.001 (HS) |
| Control (*n* = 10) | 0.02 | 0.57 | 0.22 ±0.16 | |

*P < 0.05 is considered significant*
*HS – highly significant*

**Table II.** Comparison of the level of expression of NM23-H1 gene among different ALL subtypes in ALL cases (*n* = 25)

| Subtype | Count | Minimum gene expression | Maximum gene expression | Mean NM23-H1 expression | SD | *P* value |
|---|---|---|---|---|---|---|
| C-ALL | 7 | 0.01 | 1.9 | 1.233 | 0.806 | 0.64 (NS) |
| Pre-B | 12 | 0.3 | 2.2 | 0.848 | 0.632 | |
| Pro-B | 6 | 0.4 | 2.8 | 0.992 | 0.936 | |

*P < 0.05 is considered significant*
*NS – non significant*

**Table III.** Comparison of the outcome with NM23-H1 expression in ALL (*n* = 25)

| Out-come | Count | Minimum gene expression | Maximum gene expression | Mean NM23-H1 expression | SD | *P* value |
|---|---|---|---|---|---|---|
| Remission | 12 | 0.1 | 1.8 | 0.805 | 0.554 | 0.001 (HS) |
| Resistance | 10 | 0.01 | 2.8 | 0.975 | 0.920 | |
| Death | 3 | 1.6 | 1.9 | 1.780 | 0.15 | |

*P < 0.05 is considered significant*
*HS – highly significant*

Using baseline data gathered from the samples or input from the user, the system software calculated a fixed threshold at the middle of the linear algorithm phase of amplification. At the point where the fluorescence of the sample passed the threshold, the software determined the threshold cycle (ct).

At the threshold point the software then interpolated the sample quantity from a standard curve that ran concurrently with the sample.

### Expression

Target concentration is expressed in relation to the concentration of the housekeeping gene. Relative NM23-H1 expression = copy no. of NM23-H1/copy no. of GAPDH = conc. of NM23-H1/conc. of GAPDH [20].

### Statistical analysis

Data were statistically described in terms of range, mean, standard deviation (± SD), frequencies (number of cases) and relative frequencies (percentages) where appropriate comparison of quantitative variables between different groups in the present study was done using Mann-Whitney U test for independent samples in comparing 2 groups, Kruskal-Wallis analysis of variance (ANOVA) test in more than 2 groups. For comparing categorical data, chi-square ($\chi^2$) test was performed. Exact test was used instead when the expected frequency was less than 5. Correlations between various variables were done using Spearman rank correlation equation. A probability value (*p* value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel version 7 (Microsoft corporation, NY, USA) and SPSS (Statistical Package for the Social Science, SPSS Inc., Chicago, IL, USA) statistical program.

## Results

Comparing the *NM23-H1* gene expression in ALL patients and the control group, the NM23-H1 expression in ALL patients ranged from 0.01 to 2.8, with a mean value of 0.99 ±0.74, while the NM23-H1 expression in the control group ranged from 0.02 to 0.57, with a mean value of 0.22 ±0.16. The study showed a statistically highly significant increase in the relative NM23-H1 expression in the ALL patients compared to the control group with a *p* value of 0.001 (Table I, Figure 1).

Comparing the expression of *NM23-H1* mRNA between males and females, its mean expression was 1.1 in males and 0.7 in females, with a *p* value of 0.086, which is statistically non-significant.

Comparing the expression of the *NM23-H1* gene between different ALL subtypes, its mean expression in C-ALL was 0.80, in PreB 0.63, in ProB 0.93, with a *p* value of 0.64, which is statistically non-significant (Table II).

A statistically highly significant correlation was found between *NM23-H1* gene expression and the outcome of patients with *p* value 0.001 (Table III, Figure 2).

### Correlative study

A merely statistically significant positive correlation was found between the expression level of the *NM23-H1* gene and the LDH level of ALL patients with a *p* value of 0.05 (Figure 3). A non-significant negative correlation was found between the expression level of NM23-H1 and the platelet level of the ALL patients with a *p* value of 0.064 (Figure 4). A statistically significant negative correlation was found between the expression level of NM23-H1 and the haemoglobin level of the ALL patients with a *p* value of 0.03 (Figure 5).

## Discussion

The degree of differentiation is an important prognostic factor in leukaemia. For example, patients with leukaemia of the undifferentiated phenotype have a lower response rate to treatment and poor survival. Induction of differentiation is closely linked to loss of leukemogenicity and blocks expression of the malignant phenotypes. Conversely, a disorder of the cellular differentiation of malignant cells reflects the clinical behaviour and therapeutic responses [21].

Normal haematopoiesis can be controlled by various positive and negative regulatory molecules. In acute leukaemia these signals continue to operate but in an unbalanced fashion, allowing emergence and eventual dominance of a malignant clone. Leukaemic cells are arrested in less differentiated stages of development. These results suggest that negative regulators are also important to regulate differentiation of leukaemic cells in addition to positive regulators [6].

In our study there was a statistically significant positive correlation between real time PCR relative NM23-H1 expression and LDH ($p = 0.05$). This is in agreement with Yokoyama *et al.*, who found a statistically significant positive correlation between LDH and NM23-H1 expression ($p = 0.006$).

In our study a statistically significant negative correlation was found between Hb level and the relative expression of the *NM23-H1* gene ($p = 0.03$), which was not found in other studies. Leukaemic cells by immunophenotyping showed 7 cases of common ALL (28%), 12 cases of pre-B

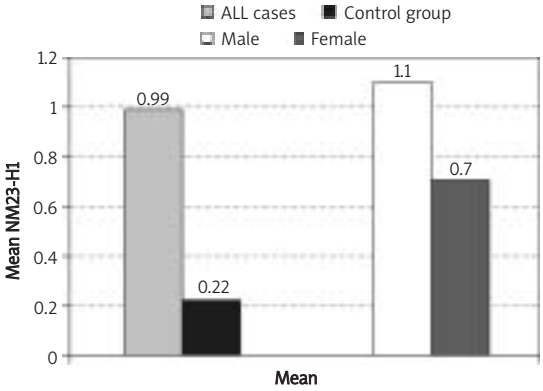


**Figure 1.** Comparison between NM23-H1 expression in ALL cases and control group

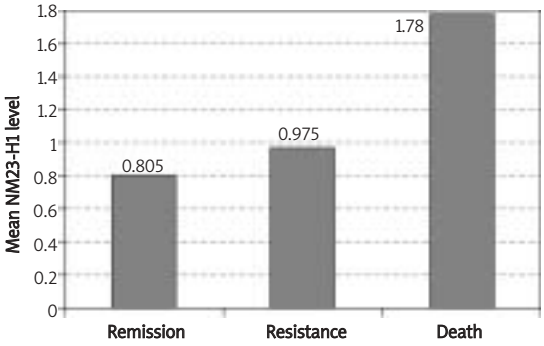


**Figure 2.** Comparison of the outcome with level of NM23-H1 expression

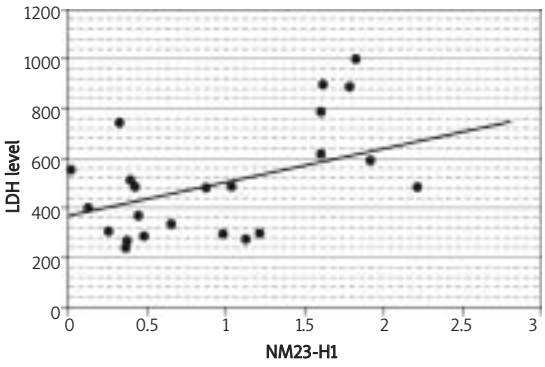


**Figure 3.** Correlation between NM23-H1 expression and LDH level among ALL cases ($r = 0.38$, $p = 0.045$)

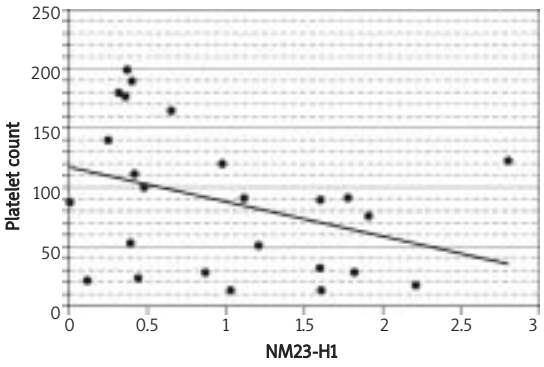


**Figure 4.** Correlation between NM23-H1 expression and Platelet count among ALL cases ($r = –0.37$, $p = 0.064$)

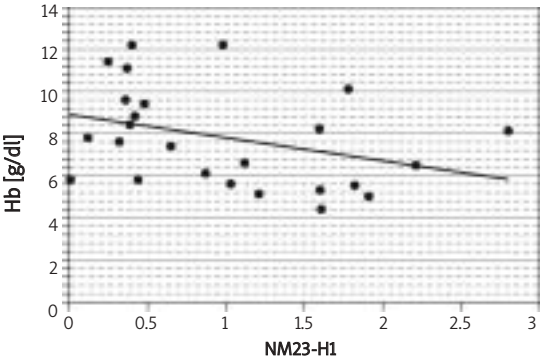


**Figure 5.** Correlation between NM23-H1 expression and Hb level among ALL cases ($r = –0.42$, $p = 0.03$)

(48%) and 6 cases of pro-B (24%). There was no significant correlation between the ALL subtype and NM23-H1 expression ($p = 0.64$).

A highly statistically significant correlation was found between the expression level of NM23-H1 in ALL cases and the control group. In ALL cases NM23-H1 expression level showed a mean of 0.99 ±0.74 in comparison to the control group, which showed a mean of 0.22 ±0.16 ($p = 0.001$). These findings are approved by most of the studies done on the differentiation inhibitory factor NM23-H1. In accordance with this Yokoyama *et al*., found a statistically significant correlation between the expression level of NM23-H1 in ALL cases and the control group, in a study on 9 newly diagnosed ALL cases ($p = 0.007$) [11].

In our study the *NM23-H1* gene showed normal expression level in 14 cases (56%) and overexpression in 11 cases (44%). This is confirmed by Wu and Zhao, who found a high expression level in 76% of cases. In our study a good response to chemotherapy was inversely correlated with NM23-H1. Twenty cases (48%) achieved remission, 10 cases showed resistance to chemotherapy (40%) and 3 cases died (12%). The resistant cases expressed a significantly higher NM23-H1 level (mean = 0.975) than those who achieved remission (mean = 0.805), and dead cases expressed a highly significant level of the studied gene (mean = 1.780) [14].

All the studies done on the investigated gene have confirmed the relationship between the prognosis of the ALL cases and the degree of gene expression. Wu and Zhao's study on 82 ALL cases showed that patients who achieved complete remission expressed a mean level of NM23-H1 of 0.135, and those who were resistant to chemotherapy had a mean gene level of 1.35 with a highly statistically significant difference ($p < 0.001$) [14].

In our study there was a highly significant negative correlation between the level of expression of the *NM23-H1* gene and the outcome of the patients ($p$ value 0.001). This confirms that the high expression level of the studied gene is associated with poor prognosis and outcome of the ALL patients.

In conclusion, NM23-H1 mRNA levels could significantly predict a poor prognosis in ALL. It can be considered as an important prognostic factor for planning the treatment strategy. In combination with other prognostic factors, it might give us a more useful method for selecting a high-risk population at diagnosis to plan the best therapeutic strategy. NM23-H1 mRNA may also be a useful prognostic factor for many other neoplasms that over-express NM23-H1 mRNA as well as in ALL.

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