



The role of tumor necrosis factor receptor superfamily member 4 (TNFRSF4) gene expression in diagnosis and prognosis of acute myeloid leukemia

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Abstract

Objectives Acute myeloid leukemia (AML) is still challenging in predicting the prognosis due to its high heterogeneity. Molecular aberrations and abnormalities play a significant prognostic role in AML patients. Our aim of the study was to investigate the prognostic role of TNFRSF4 gene expression in AML patients and its potential effect on treatment protocols.

Methods Bone marrow mononuclear cells were analyzed for TNFRSF4 expression by real-time quantitative PCR as well as of FLT3/ITD and NPM1 mutations in 80 newly diagnosed AML patients and 80 control subjects.

Results TNFRSF4 was significantly overexpressed in the AML patients ($p < 0.001$). TNFRSF4 expression was associated with unfavorable clinical outcomes including treatment response, relapse free survival, and overall survival. On multivariate testing, TNFRSF4 high expression proved to be an independent prognostic marker for clinical remission and relapse free survival but not overall survival.

Conclusion TNFRSF4 expression was revealed as an unfavorable prognostic marker and might be a target for immunotherapy in the future.

Keywords AML · Gene expression · TNFRSF4 · Prognosis

Introduction

Acute myeloid leukemia (AML) is a group of heterogeneous malignancy of the hematopoietic stem cells in the bone marrow characterized by uncontrolled proliferation of clonal myeloid blast cells [1]. These blasts replace the normal hematopoietic cells leading to cytopenias [2, 3].

Although recognition of the molecular pattern is of utmost importance for the prognosis and treatment of AML, it is still extremely difficult and challenging to classify AML due to the high heterogeneity of the disease [1]. Depending only on the cytogenetic analysis is not satisfactory, because there is almost 50% of AML patients who have normal cytogenetic testing with variable outcomes [1, 4, 5]. Therefore, studying the molecular background for the occurrence and progression of AML has become essential for early

diagnosis to avoid treatment delay and to improve the overall prognosis [3, 6, 7].

Mutations in nucleophosmin (NPM1), TP53 and FMS-like tyrosine kinase 3/internal tandem duplication (FLT3/ITD) genes are the most common molecular abnormalities in AML [1, 8, 9]. These mutations may lead to disturbance of the transcription factors and faulty gene expressions in AML pathogenesis [1, 10].

NPM1 is mainly present in the nucleolus where it interacts with proteins involved in ribosomal synthesis and transport of ribosomal proteins through the membrane of the nucleus [11–13]. NPM1 binds to R-motifs which are proteins that contain multiple copies of the amino acid arginine [14]. It has been proved that NPM1 mutations occur in high frequencies in AML with normal karyotypes disrupting the cytoplasmic NPM1 functions as an interacting partner and transporter protein leading to the assumption that NPM1 mutation might be an early risk for leukemogenesis [13]. An important role for NPM1 in leukemias and lymphomas has been investigated in previous studies [13].

FLT3 belongs to the receptor tyrosine kinase class III (RTK) family [15, 16]. In normal hematopoiesis, FLT3 gene

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expression is confined to myeloid and lymphoid progenitors [15, 17]. In hematologic malignancies, FLT3 mutations are highly expressed in 70% to 100% of the AML blasts [15, 18]. There are two major types of FLT3 mutations in AML: internal-tandem duplication mutation in the juxtamembrane domain (ITDs) and point mutations in the tyrosine kinase domain (TKD) [15]. Mutations of FLT3 gene occur in up to 35% of AML patients, and specifically, the internal tandem duplication (ITD) which is the most common type that accounts for about 25% of all AML cases [19]. FLT3-ITD is considered a negative risk factor in patients with AML [19].

The tumor necrosis factor ligand superfamily number 4 (TNFSF4) is present on cell surfaces of dendritic cells, endothelial cells, and B lymphocytes, with its receptor TNFRSF4, also known as OX40 or CD134, being expressed on activated T lymphocytes [20]. TNFSF4/TNFRSF4 pathway is one of the main positive costimulatory signal pathways for immune cell stimulation [20, 21]. It induces T helper two differentiation, stimulates CD8+ T cells and promotes cytokine synthesis [20, 22]. It can also activate NF-kappa B pathway via TRAF2 and TRAF5 [3, 23]. Studies on TNFRSF4 gene expression suggest its role in T cell-APC interaction leading to immunologic activation producing proinflammatory cytokines resulting in various active diseases as SLE and atherosclerosis [20, 24, 25]. Some recent research has studied TNFRSF4's role in the immunotherapy of some tumors [3, 26, 27].

Consequently, and in the context of TNFRSF4 gene expression on CD8+ T cells and T regulatory cells, various studies proved TNFRSF4 gene expression to be significantly increased in relapsed AML [3, 28]. Therefore, we decided to investigate the expression profile of TNFRSF4 in AML, to explore its potential prognostic value among AML patients and its potential benefit in future therapies.

Materials and methods

Our research was carried out on 80 newly diagnosed non-M3 AML patients with normal cytogenetic analysis at Alexandria University Hospital between August 2018 and August 2020. A total of 80 age- and sex-matched subjects with normal bone marrow, and without malignancy who were examined at the hospital at the same time were enrolled as the control group. The sample size was calculated using the G power version 3.1 statistical software program with a 0.05 level of significance and 80% power of the study. Written informed consent was signed by each participant in the study. This study was approved by the Ethics Committee of Alexandria Faculty of Medicine.

All our AML patients were subjected to laboratory investigations that included complete blood count (CBC), BM aspirate and/or biopsy, blood film morphology, flow

cytometry, and cytogenetic analysis at the time of diagnosis. AML was diagnosed by the French-American-British classification (FAB) classification and the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues [2]. 20% was the cut-off of the blast count for the diagnosis of AML. The exclusion criteria included M3-AML, abnormal cytogenetic analysis, or previously diagnosed AML on treatment. Details of the study groups are listed in Table 1.

All AML patients received induction chemotherapy with 3 and 7 regimen including daunorubicin 45–90 mg/m² intravenously for three days and infusion of cytosine arabinoside 100 mg/m² over 24 h daily for one week [29]. Response to induction therapy was assessed using BM examination on day 28 after the induction therapy. All patients were then re-evaluated quarterly for relapse by BM aspirate examination. By the end of the induction phase, patients were said to be in complete remission (CR) if they had < 5% blast cells in the BM aspirate with no Auer rods, neutrophil count of $\geq 1.5 \times 10^9/L$, platelet count of $> 100 \times 10^9/L$, as well as bone marrow cellularity of at least 20%. Partial response (PR) was diagnosed if they had from 5 to 25% blasts in BM aspirate examination. Resistance to therapy was defined as persistence of over 25% blasts in the bone marrow, persistence of blast cells in the peripheral blood, and/ or blasts found at an extramedullary site at the end of the induction therapy [30].

DNA and RNA extraction

Diagnostic bone marrow (BM) specimens were aspirated at the Department of Clinical Pathology, University of Alexandria, Egypt at the initial diagnosis. DNA and RNA were isolated from BM mononuclear cells (BMNCs) according to the manufacturer's instructions using the QIAamp DNA and RNA blood mini kits (Qiagen, USA), respectively. The quantity and quality of the extracted DNA and RNA were determined using NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA). A ratio value of 1.8 was considered to indicate DNA purity. Single-stranded cDNA was synthesized from purified RNA sample using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) for RNA reverse transcription and the PCR amplification was carried out on the SimpliAmp Thermal Cycler (Applied Biosystems, USA).

FLT3/ITD and NPM1 mutations detection

Exons 14 and 15 of the FLT3 gene were amplified using MyTaq Red Mix (Bioline, London, United Kingdom), 14F, and 15R primers. The master mix was composed of dNTPs (dATP, dCTP, dGTP, dTTP) 0.4 mM each and 4 mM magnesium chloride and 0.05 units/ml of Taq polymerase in a

Table 1 Comparison between the two studied groups according to different parameters

| | AML (n = 80) | Control (n = 80) | Test of Sig | p |
|--------------------------------|------------------|------------------|---------------------|----------|
| Sex | | | | |
| Male | 48 (60%) | 61 (76.3%) | $\chi^2 = 4.864^*$ | 0.027* |
| Female | 32 (40%) | 19 (23.8%) | | |
| Age (years) | 44 ± 11.9 | 59.4 ± 6.4 | t = 10.226* | < 0.001* |
| ≤ 60 | 71 (88.8%) | 48 (60%) | $\chi^2 = 17.348^*$ | < 0.001* |
| > 60 | 9 (11.3%) | 32 (40%) | | |
| CBC | | | | |
| Platelets × 10 ⁹ /L | 55 (9 – 578) | 241 (126 – 492) | U = 424.0* | < 0.001* |
| WBCs × 10 ⁹ /L | 13.5 (0.6 – 302) | 6.5 (4 – 10.2) | U = 2844.0* | < 0.001* |
| Hb (g/dl) | 8.6 ± 2.4 | 14 ± 1.1 | t = 18.530* | < 0.001* |
| Initial Pb blasts | 11.5 (0 – 94) | – | – | – |
| Initial BM blasts | 52.5 (22 – 98) | – | – | – |
| FAB subtype | | | | |
| M0 | 11 (13.8%) | – | – | – |
| M1 | 14 (17.5%) | – | – | – |
| M2 | 18 (22.5%) | – | – | – |
| M3 | 0 (0%) | – | – | – |
| M4 | 19 (23.8%) | – | – | – |
| M5 | 14 (17.5%) | – | – | – |
| M6 | 1 (1.3%) | – | – | – |
| M7 | 3 (3.8%) | – | – | – |
| NPM1 | | | | |
| Un mutated | 53 (66.3%) | – | – | – |
| Mutated | 27 (33.8%) | – | – | – |
| FLT3 ITD | | | | |
| Un mutated | 42 (52.5%) | – | – | – |
| Mutated | 38 (47.5%) | – | – | – |
| Clinical remission | | | | |
| CR | 51 (63.8%) | – | – | – |
| NCR | 29 (36.3%) | – | – | – |
| TNFRSF4 | 1 (0.2 – 30.9) | 0.6 (0.3 – 1.6) | U = 1928.5* | < 0.001* |

Data was expressed by Mean ± SD

For normally distributed quantitative variables, Data was expressed by Median (Min. – Max.) For abnormally distributed quantitative variables

χ^2 Chi square test, *t* Student t-test, *U* Mann Whitney test

p: p value for comparing between the two groups

*Statistically significant at $p \leq 0.05$

reaction buffer. The following primers were used to amplify exons 14 and 15 of the FLT3 gene. The 14 Forward primer sequence was 5' GCAATTTAG-GTATGAAAGCCAGC-3'. The 15 Reverse primer sequence was 5' CTTTCAGCATTTTGACGGCAACC-3'. PCR conditions were as follows: initial denaturation step at 95 °C for 7 min followed by 35 cycles that included 1 min 94 °C for denaturation, 30 s 60 °C for annealing and 90 s 72 °C for extension, followed by a final extension of 7 min at 72 °C. FLT3/ITD mutations were detected using 2% agarose gel electrophoresis, stained with ethidium bromide. Then visualization of the DNA bands was done using a 302 nm ultraviolet transilluminator. The normal

FLT3 gene showed a fragment length of 328 bp produced from the wild-type allele. While the FLT3/ITD mutation produced a larger fragment than the wild type.

For NPM1 mutation detection, the allele-specific oligonucleotide reverse transcriptase PCR (ASO-RT-PCR) strategy was used to analyze the NPM1 exon 12 mutation. 5'CCA AGA GGC TAT TCA AGA TCT CTC TC-3' was used as a forward primer and 5'ACC ATT TCC ATG TCT GAG CAC C-3' was used as a reverse primer to amplify the cDNA. 50 ng cDNA was amplified using 20 pmol of each primer in a 25 µl reaction mix. The PCR master mix was composed of buffer containing dNTPs (dATP, dCTP, dGTP, dTTP) 0.4 mM each, 1.5 mM

MgCl₂ and 2.5 units/ml of Taq polymerase. PCR conditions were as follows: initial denaturation at 95 °C for 15 min followed by 42 cycles of PCR (each cycle consisted of 95 °C for 30 s, 67 °C for 30 s, 60 °C for 20 s), and final extension step of 5 min at 72 °C. Amplification of a 258 bp fragment of the Abelson (ABL) gene was used as an internal quality control. The Amplified products were then electrophoresed using 2% agarose gels and examined under UV light with ethidium bromide stain. A 320-bp fragment was detected in NPM1 mutation status, while the unmutated samples did not show any bands.

Reverse transcription-quantitative PCR for gene expression

Quantitative analysis of TNFRSF4 gene expression level in bone marrow cells was carried out on Stratagene Mx3000P Real-time PCR system (Agilent, Germany). qPCR was conducted using the presynthesized cDNA, Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA), and sequence-specific primers. The primers sequences were shown as follows: forward, 5'-ACA ACG ACG TGG TCA GCT CCAA-3' and reverse, 5'-CAG CGG CAG ACT GTG TCC TGT- 3'. The PCR conditions were composed of pre-denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 34 s. GAPDH and 18sRNA housekeeping genes were used as endogenous controls; their expression was constant in all samples. The 2^{-ΔΔCT} CT method was used to analyse the relative mRNA expression levels [31].

Statistical analysis

SPSS statistical software v20.0 was used for statistical calculations. Statistical analysis was performed using the Chi-square test and Fisher's Exact test for categorical data. Quantitative data were presented as mean and standard deviation (SD). Student's t-test was used to compare significant differences between two groups for normally distributed quantitative variables. For abnormally distributed data, the Mann-Whitney test was used. Multivariate logistic regression model was adjusted for multiple comparisons. Kaplan–Meier curve was made to assess the relationship between genetic mutations or target gene expression levels and CR, RFS, or OS. Survival studies were tested using Cox proportional hazards models. Statistical significance was made when the *p* value < 0.05.

Results

Characteristics of healthy controls and AML patients

In the control group, the mean age was 59.4 years, with 61 (76.3%) males and 19 (23.8%) females. The median WBC count was 6.5 with a range of 4 to 10.2 X 10⁹ cells/l. In the group of patients with AML, the mean age was 44 years, with 48 (60%) males and 32 (40%) females. The median WBC count was 13.5 with a range of 0.6 to 302 X10⁹/l in the patients group. Regarding the FAB classification, 11 (13.8%) of the AML patients were classified as M0, 14 (17.5%) as M1, 18 (22.5%) as M2, 19 (23.8%) as M4, 14 (17.5%) as M5, 1 (1.3%) as M6 and 3 (3.8%) as M7. In terms of molecular analysis, 38 (47.5%) AML patients showed internal tandem duplications in FMS-like tyrosine kinase 3 (FLT3-ITD) gene, and 27 (33.8%) AML patients exhibited mutations in nucleophosmin 1 (NPM1) gene (Table 1).

Association of TNFRSF4 expression with AML risk

The relative expression of TNFRSF4 was significantly increased in AML patients compared with the control group (*p* < 0.001; Fig. 1), with a mean of 1 in the patients group compared to a mean of 0.6 in the control group (Table 1). ROC curve analysis showed that TNFRSF4 was able to differentiate AML patients from healthy controls (area under the curve (AUC) = 0.699, 95% CI = 0.614–0.783), with a sensitivity of 71.25% and a specificity of 61.25% at the best cut-off point (Table 2).

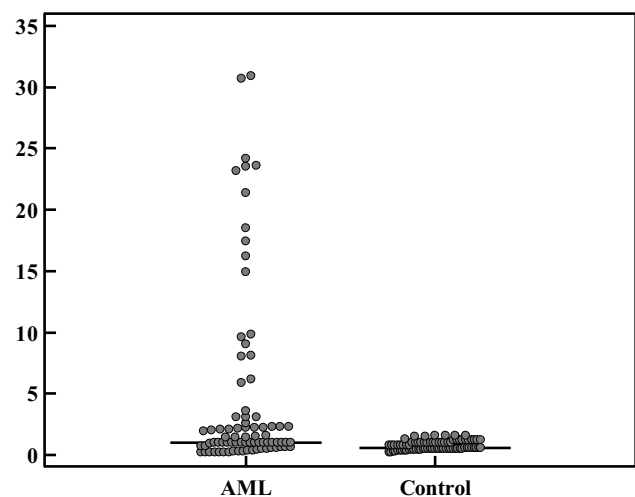


Fig. 1 Comparison between the two studied groups according to TNFRSF4 (*p* < 0.001)

Table 2 Agreement (sensitivity, specificity) for TNFRSF4 to diagnose AML patients (n = 80) from control (n = 80)

| | AUC | p | 95% C.I | Cut off [#] | Sensitivity | Specificity | PPV | NPV |
|---------|-------|---------|---------------|----------------------|-------------|-------------|------|------|
| TNFRSF4 | 0.699 | <0.001* | 0.614 – 0.783 | >0.96 | 71.25 | 61.25 | 64.8 | 68.1 |

AUC Area Under a Curve, *p* value Probability value, CI Confidence intervals, NPV Negative predictive value, PPV Positive predictive value

*Statistically significant at $p \leq 0.05$

Association of TNFRSF4 expression with FAB classification and molecular genetics

TNFRSF4 expression was associated with both elevated NPM1 mutation ($p = 0.022$) and elevated FLT3-ITD gene mutation ($p = 0.045$). In addition, there was a significant statistical relationship with FAB classification ($p = 0.023$) (Table 3).

Predictive value of TNFRSF4 for treatment response

In the TNFRSF4-low expression group, 85.4% of cases achieved CR, while 14.6% did not. In the TNFRSF4-high expression group, 41% of cases achieved CR, while 59% did not (Table 3). Further analysis suggested that the CR rate was significantly lower in the TNFRSF4-high expression group than in the low-expression group ($p < 0.001$) (Table 3). Furthermore, the intensity of consolidation showed statistical significance with TNFRSF4 expression, with higher intensity of consolidation in the TNFRSF4-high expression group ($p = 0.014$) (Table 3).

Predictive value of TNFRSF4 for AML relapse rate (RFS) and OS

The number of patients who showed RFS was 46.2% in the TNFRSF4-high expression group compared with 87.8% in the TNFRSF4-low expression group. Similarly, high TNFRSF4 expression levels were associated with higher AML relapse rates compared with low TNFRSF4 expression levels (53.8% vs 12.2% respectively, $p < 0.001$). Regarding the OS, the number of patients who showed OS was lower in the TNFRSF4-high expression group (46.2%) compared with that in the TNFRSF4-low expression group (78%) ($p = 0.003$; Table 3).

Furthermore, survival analysis was tested using the Kaplan–Meier analysis. Firstly, regarding gene mutations, NPM1 mutations were associated with favorable OS and RFS ($p < 0.001$ for OS and $p = 0.040$ for RFS), while FLT3/ITD mutations were associated with poor OS and RFS ($p < 0.001$ for OS and $p < 0.001$ for RFS). Secondly, regarding target gene expressions, high TNFRSF4 levels

were associated with poor OS and RFS ($p = 0.022$ for OS and $p < 0.001$ for RFS) (Fig. 2a–f).

Associations with genetic mutations

The CR rate showed statistical significance with both NPM1 mutations and FLT3-ITD gene mutations ($p = 0.038$ for NPM1 and $p = 0.004$ for FLT3-ITD; Table 4). On the other hand, the intensity of consolidation showed statistical significance only with FLT3-ITD mutations ($p = 0.028$). Furthermore, the relapse rate was statistically significant in FLT3/ITD mutation (52.6% in the mutated state vs 14.3% in the non-mutated state, $p < 0.001$), while the group with NPM1 mutation showed significantly decreased relapse rates in comparison with the unmutated group (48.1% vs 24.5% respectively, $p = 0.033$) (Table 4). Regarding the OS, it was statistically significant with both NPM1 and FLT3-ITD genetic mutations. The percentage of patients who survived in the NPM1 mutated group was 81.5% compared with 52.8% in the non-mutated NPM1 mutated group, and 42.1% survived in the FLT3-ITD mutated group compared with 81% in the non-mutated FLT3-ITD group ($p = 0.012$ for NPM1 mutation and $p < 0.001$ for FLT3-ITD mutation) (Table 4).

Prognostic factors for CR

Univariate Cox regression analysis demonstrated that TNFRSF4 high expression ($p < 0.001$, HR = 0.041–0.350, 95% CI = 0.119), high intensity of consolidation ($p = 0.003$, HR = 0.088–0.610, 95% CI = 0.231), NPM1 mutation ($p = 0.041$, HR = 0.140–0.960, 95% CI = 0.367) and FLT3-ITD mutation ($p = 0.005$, HR = 0.093–0.650, 95% CI = 0.245) were predictors of lower CR in AML patients (Table 5). Subsequent multivariate analysis adjusted for these variables demonstrated that high expression of TNFRSF4 ($p = 0.003$, HR = 0.054–0.550, 95% CI = 0.172) and FLT3-ITD mutation ($p = 0.027$, HR = 0.081–0.860, 95% CI = 0.264) were considered as independent risk factors for poor CR in AML patients (Table 5).

Table 3 Relation between TNFRSF4 and different parameters in AML group (n = 80)

| | TNFSF4 | | Test of Sig. | p |
|---------------------------|---------------------------|-------------------------|---------------------|--------------------------|
| | Low (≤ 1) (n = 41) | High (> 1) (n = 39) | | |
| Sex | | | | |
| Male | 26 (63.4%) | 22 (56.4%) | $\chi^2 = 0.409$ | 0.523 |
| Female | 15 (36.6%) | 17 (43.6%) | | |
| Age (years) | 41.2 \pm 11.7 | 46.9 \pm 11.4 | t = 2.223* | 0.029* |
| ≤ 60 | 40 (97.6%) | 31 (79.5%) | $\chi^2 = 6.539^*$ | ^{FE} p = 0.013* |
| > 60 | 1 (2.4%) | 8 (20.5%) | | |
| FAB subtype | | | | |
| M0 | 8 (19.5%) | 3 (7.7%) | $\chi^2 = 13.368^*$ | ^{MC} p = 0.023* |
| M1 | 7 (17.1%) | 7 (17.9%) | | |
| M2 | 9 (22%) | 9 (23.1%) | | |
| M3 | 0 (0%) | 0 (0%) | | |
| M4 | 13 (31.7%) | 6 (15.4%) | | |
| M5 | 2 (4.9%) | 12 (30.8%) | | |
| M6 | 1 (2.4%) | 0 (0%) | | |
| M7 | 1 (2.4%) | 2 (5.1%) | | |
| Platelets $\times 10^9/L$ | 55 (10 – 302) | 55 (9 – 578) | U = 770.5 | 0.780 |
| WBCs $\times 10^9/L$ | 8 (1 – 302) | 18 (0.6 – 228) | U = 787.0 | 0.904 |
| Hb (g/dl) | 8.9 \pm 2.4 | 8.2 \pm 2.3 | t = 1.407 | 0.163 |
| NPM1 | | | | |
| Un mutated | 32 (78%) | 21 (53.8%) | $\chi^2 = 5.236^*$ | 0.022* |
| Mutated | 9 (22%) | 18 (46.2%) | | |
| FLT3 ITD | | | | |
| Un mutated | 26 (63.4%) | 16 (41%) | $\chi^2 = 4.018^*$ | 0.045* |
| Mutated | 15 (36.6%) | 23 (59%) | | |
| Initial Pb blasts | 12 (0 – 94) | 11 (0 – 90) | U = 760.0 | 0.703 |
| Initial BM blasts | 48 (22 – 98) | 58 (22 – 98) | U = 726.0 | 0.479 |
| Clinical remission | | | | |
| CR | 35 (85.4%) | 16 (41%) | $\chi^2 = 17.01^*$ | <0.001* |
| NCR | 6 (14.6%) | 23 (59%) | | |
| Intense of consolidation | | | | |
| Low | 30 (73.2%) | 18 (46.2%) | $\chi^2 = 6.08^*$ | 0.014* |
| High | 11 (26.8%) | 21 (53.8%) | | |
| Relapse | | | | |
| No | 36 (87.8%) | 18 (46.2%) | $\chi^2 = 15.81^*$ | <0.001* |
| Relapse | 5 (12.2%) | 21 (53.8%) | | |
| Survival | | | | |
| Survival | 32 (78%) | 18 (46.2%) | $\chi^2 = 8.675^*$ | 0.003* |
| Died | 9 (22%) | 21 (53.8%) | | |

Data was expressed by Mean \pm SD

For normally distributed quantitative variables, Data was expressed by Median (Min. – Max.) For abnormally distributed quantitative variables

χ^2 Chi square test, *FE* Fisher Exact, *MC* Monte Carlo, *t* Student t-test, *U* Mann Whitney test

p: p value for comparing between TNFSF4 and different parameters

*Statistically significant at $p \leq 0.05$

Prognostic factors for RFS

Univariate Cox regression analysis showed that high TNFRSF4 expression ($p = 0.001$, HR = 2.347–26.65; 95%

CI = 7.908), FLT3/ITD ($p = 0.001$, HR = 1.937–12.28; 95% CI = 4.878), and WBC counts ($p = 0.020$, HR = 1.001–1.013, 95% CI = 1.007) were predictors of

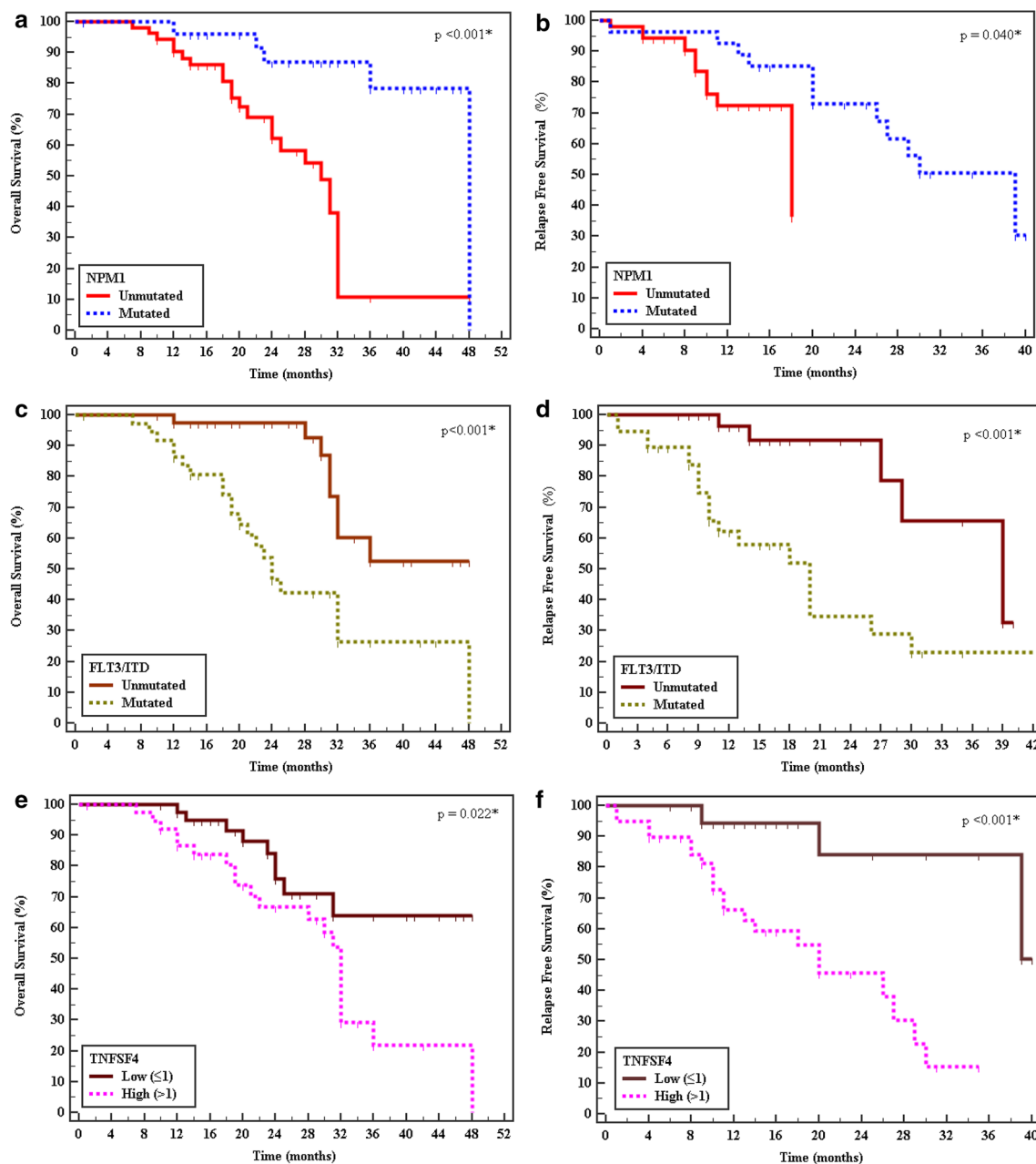


Fig. 2 **a** Kaplan–Meier survival curve for Overall Survival in AML group according to NPM1. **b** Kaplan–Meier survival curve for Relapse Free Survival in AML group according to NPM1. **c** Kaplan–Meier survival curve for Overall Survival in AML group according to FLT3/ITD. **d** Kaplan–Meier survival curve for Relapse Free Sur-

vival in AML group according to FLT3/ITD. **e** Kaplan–Meier survival curve for Overall Survival in AML group according to TNFSF4. **f** Kaplan–Meier survival curve for Relapse Free Survival in AML group according to TNFSF4

unfavourable RFS in AML patients (Table 5). Subsequently, multivariate analysis was performed for those variables and revealed that both high TNFRSF4 expression ($p = 0.003$, HR = 1.926–22.03, 95% CI = 6.513) and FLT3-ITD gene mutation ($p = 0.009$, HR = 1.388–9.660, 95% CI = 3.661) were independent risk factors for poor RFS in AML patients (Table 5).

Prognostic factors for OS

Univariate Cox regression analysis revealed that TNFRSF4 high expression ($p = 0.030$, HR = 1.087–5.230, 95% CI = 2.385), high intensity of consolidation ($p = 0.046$, HR = 0.015–4.327, 95% CI = 2.095), NPM1 mutation ($p = 0.001$, HR = 0.071–0.507, 95% CI = 0.190) and FLT3-ITD mutation ($p = 0.001$, HR = 1.687–8.554, 95%

Table 4 Relation between NPM1 and FLT3 ITD with different parameters in AML group (n = 80)

| | NPM1 | | FLT3 ITD | |
|--------------------------------|---------------------|------------------|---------------------|------------------|
| | Un mutated (n = 53) | Mutated (n = 27) | Un mutated (n = 42) | Mutated (n = 38) |
| Sex | | | | |
| Male | 30 (56.6%) | 18 (66.7%) | 28 (66.7%) | 20 (52.6%) |
| Female | 23 (43.4%) | 9 (33.3%) | 14 (33.3%) | 18 (47.4%) |
| χ^2 (p) | 0.755 (0.385) | | 1.637 (0.201) | |
| Age (years) | | | | |
| ≤ 60 | 48 (90.6%) | 23 (85.2%) | 38 (90.5%) | 33 (86.8%) |
| > 60 | 5 (9.4%) | 4 (14.8%) | 4 (9.5%) | 5 (13.2%) |
| χ^2 (^{FE} p) | 0.519 (0.477) | | 0.264 (p = 0.729) | |
| Mean ± SD | 43.6 ± 12 | 44.7 ± 11.8 | 44.6 ± 11 | 43.2 ± 12.8 |
| t (p) | 0.371 (0.712) | | 0.527 (0.599) | |
| FAB subtype | | | | |
| M0 | 9 (17%) | 2 (7.4%) | 7 (16.7%) | 4 (10.5%) |
| M1 | 12 (22.6%) | 2 (7.4%) | 7 (16.7%) | 7 (18.4%) |
| M2 | 7 (13.2%) | 11 (40.7%) | 8 (19%) | 10 (26.3%) |
| M3 | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| M4 | 15 (28.3%) | 4 (14.8%) | 9 (21.4%) | 10 (26.3%) |
| M5 | 9 (17%) | 5 (18.5%) | 8 (19%) | 6 (15.8%) |
| M6 | 0 (0%) | 1 (3.7%) | 0 (0%) | 1 (2.6%) |
| M7 | 1 (1.9%) | 2 (7.4%) | 3 (7.1%) | 0 (0%) |
| χ^2 (^{MC} >p) | 13.603* (0.020*) | | 4.809 (0.595) | |
| Platelets × 10 ⁹ /L | 55 (10 – 401) | 49 (9 – 578) | 64.5 (10 – 578) | 43 (9 – 569) |
| U (p) | 685.50 (0.760) | | 615.50 (0.079) | |
| WBCs × 10 ⁹ /L | 18 (0.9 – 302) | 8 (0.6 – 92) | 4.3 (0.6 – 302) | 25 (0.6 – 228) |
| U (p) | 594.0 (0.216) | | 607.50 (0.066) | |
| Hb (g/dl) | 8.8 ± 2.4 | 8.1 ± 2.2 | 8.6 ± 2.4 | 8.5 ± 2.4 |
| t (p) | 1.143 (0.256) | | 0.361 (0.719) | |
| Initial Pb blasts | 11 (0 – 94) | 21 (0 – 90) | 18 (0 – 94) | 8 (0 – 90) |
| U (p) | 684.0 (0.748) | | 683.0 (0.267) | |
| Initial BM blasts | 48 (22 – 98) | 54 (22 – 94) | 54 (22 – 98) | 45.5 (22 – 98) |
| U (p) | 701.50 (0.887) | | 666.0 (0.203) | |
| Clinical remission | | | | |
| CR | 38 (71.7%) | 13 (48.1%) | 33 (78.6%) | 18 (47.4%) |
| NCR | 15 (28.3%) | 14 (51.9%) | 9 (21.4%) | 20 (52.6%) |
| χ^2 (p) | 4.293* (0.038*) | | 8.405* (0.004*) | |
| Intense of consolidation | | | | |
| Low | 34 (64.2%) | 14 (51.9%) | 30 (71.4%) | 18 (47.4%) |
| High | 19 (35.8%) | 13 (48.1%) | 12 (28.6%) | 20 (52.6%) |
| χ^2 (p) | 1.127 (0.288) | | 4.812* (0.028*) | |
| Relapse | | | | |
| No | 40 (75.5%) | 14 (51.9%) | 36 (85.7%) | 18 (47.4%) |
| Relapse | 13 (24.5%) | 13 (48.1%) | 6 (14.3%) | 20 (52.6%) |
| χ^2 (p) | 4.549* (0.033*) | | 13.372* (< 0.001*) | |
| Survival | | | | |
| Survival | 28 (52.8%) | 22 (81.5%) | 34 (81%) | 16 (42.1%) |
| Died | 25 (47.2%) | 5 (18.5%) | 8 (19%) | 22 (57.9%) |
| χ^2 (p) | 6.265* (0.012*) | | 12.845* (< 0.001*) | |

Data was expressed by mean ± SD. For normally distributed quantitative variables,

Data was expressed by Median (Min. – Max.) For abnormally distributed quantitative variables

χ^2 Chi square test, *FE* Fisher Exact, *MC* Monte Carlo, *t* Student t-test, *U* Mann Whitney test, *p* p value for comparing between different categories

Table 5 Univariate and multivariate Logistic and COX regression analysis for the parameters affecting RFS (n = 26 vs. 54) and OS (n = 30 vs. 50) in AML patients

| | CR | | | | | | RFS | | | | | | OS | | | | | |
|--------------------------------|------------|---------------------|--------|---------------------|--------|---------------------|------------|---------------------|--------|---------------------|---|--------------|------------|--------------|---|---------------|--|--|
| | Univariate | | | #Multivariate | | | Univariate | | | #Multivariate | | | Univariate | | | #Multivariate | | |
| | P | OR (95% C.I) | P | OR (95% C.I) | P | HR (95% C.I) | P | HR (95% C.I) | P | HR (95% C.I) | P | HR (95% C.I) | P | HR (95% C.I) | P | HR (95% C.I) | | |
| Age | 0.654 | 0.991 (0.953–1.030) | | | 0.385 | 1.016 (0.980–1.053) | | | 0.167 | 1.024 (0.990–1.059) | | | | | | | | |
| WBC count | 0.769 | 0.999 (0.991–1.007) | | | 0.020* | 1.007 (1.001–1.013) | 0.312 | 1.004 (0.997–1.010) | 0.125 | 1.004 (0.999–1.01) | | | | | | | | |
| Initial Pb blasts | 0.675 | 0.997 (0.981–1.013) | | | 0.763 | 0.998 (0.982–1.013) | | | 0.407 | 0.993 (0.976–1.010) | | | | | | | | |
| Initial BM blasts | 0.618 | 0.995 (0.977–1.014) | | | 0.402 | 1.007 (0.990–1.024) | | | 0.649 | 0.996 (0.981–1.012) | | | | | | | | |
| Intensity consolidation (high) | 0.003* | 0.231 (0.088–0.610) | 0.065 | 2.847 (0.937–8.653) | 0.256 | 1.571 (0.721–3.421) | | | 0.046* | 2.095 (1.015–4.327) | | | | | | | | |
| NPM1 | 0.041* | 0.367 (0.140–0.960) | 0.123 | 0.391 (0.118–1.290) | 0.051 | 0.301 (0.090–1.007) | | | 0.001* | 0.190 (0.071–0.507) | | | | | | | | |
| FLT3/ITD | 0.005* | 0.245 (0.093–0.650) | 0.027* | 0.264 (0.081–0.860) | 0.001* | 4.878 (1.937–12.28) | 0.009* | 3.661 (1.388–9.660) | 0.001* | 3.798 (1.687–8.554) | | | | | | | | |
| TNFRSF4 | <0.001* | 0.119 (0.041–0.350) | 0.003* | 0.172 (0.054–0.550) | 0.001* | 7.908 (2.347–26.65) | 0.003* | 6.513 (1.926–22.03) | 0.030* | 2.385 (1.087–5.230) | | | | | | | | |

OR Odd's ratio, HR Hazard ratio, C.I Confidence interval, LL Lower limit, UL Upper Limit

All variables with p < 0.05 was included in the multivariate

* Statistically significant at p ≤ 0.05

CI=3.798) were predictors of poor OS in AML patients. Subsequent multivariate analysis adjusted for the previous variables demonstrated that high consolidation intensity ($p=0.007$, HR=1.365–6.912, 95% CI=3.071), NPM1 mutation ($p < 0.001$, HR=0.035–0.338, 95% CI=0.109) and FLT3-ITD mutation ($p=0.001$, HR=1.856–10.33, 95% CI=4.380) were independent prognostic factors for shorter OS in AML patients (Table 5).

Discussion

Acute myeloid leukemia (AML) is a highly heterogeneous stem cell disorder that is caused by the uncontrolled proliferation of hemopoietic cells in the bone marrow [32]. Numerous genetic alterations and mutated genes expression of oncogenes and tumor suppressor genes have been detected in the pathogenesis of AML affecting the prognosis [1].

With the advanced technologies, many new molecular markers have been discovered, which in turn, has led to more precise and sophisticated AML classification affecting the prognosis and treatment modalities [1]. In our study, we chose a new molecular marker, TNFRSF4 gene expression, which might have a role in the carcinogenesis and tumorigenesis environment of different cancers including AML, and could be a target for immunotherapy in the future.

Previous studies have shown that TNFRSF4 is implicated in diverse malignant tumors. For example, Puntigam et al. detected significantly increased TNFRSF4 levels on circulating T regulatory cells of HPV + patients suffering from head and neck squamous cell carcinoma, presuming that antibody agonists of TNFRSF4 could provide a therapeutic tool through inhibition of T regulatory cells and enhancing anti-tumor activity [33]. Similarly, Kumar et al. previously reported that TNFRSF4 expression was high in cancers including melanoma, colon carcinoma, breast cancer, and B-cell lymphoma [26, 34]. In that study, they revealed that TNFRSF4 agonistic antibodies could be used for treatment protocols in melanoma, fibrosarcoma, colon cancer, and glioma, through inducing anti-tumor response in preclinical tumour models, mentioning that anti-TNFRSF4 antibodies are still in clinical trials, together with radiation and chemotherapy for many cancer subtypes [34, 35]. Moreover, TNFRSF4 signaling has been studied and proved in many autoimmune diseases. For instance, in systemic lupus erythematosus (SLE), TNFRSF4 has been located in multiple genetic loci, indicating that it might have a role in disease occurrence [34, 36]. In addition, TNFRSF4 interaction with its receptor has been reported in patients with collagen induced arthritis at the inflamed joints, with improvement of the inflammatory process after disrupting the TNFRSF4 mechanism [34, 37]. In another study, Zhou et al. studied TNFRSF4 and miR-744 in cardiac transplant patients and

demonstrated that miR-744 negatively regulated TNFRSF4 expression by binding of the 3'UTR of TNFRSF4 mRNA and that this TNFRSF4 inhibition reduced the rate of rejection of heart transplantation [38]. Daniel He et al., on the other hand, studied gene profiling of 730 immune-related genes in asthmatic patients and found out that there was an up-regulation of the TNFRSF4 protein expression and was considered as a potential therapeutic target in patients suffering from asthma [39]. Lastly, Gao et al. investigated various TNF genes associated with scleritis in Chinese people and reported that the TNFRSF4 GT haplotype was a risk factor for scleritis in his study population together with other TNF genes [40]. With that being said, we can say that many studies have been carried out to investigate TNFRSF4 gene expression in various cancers and autoimmune diseases, but studies addressing the direct role of TNFRSF4 in AML are still very few. Therefore, further research is required to demonstrate the potential effect of TNFRSF4 on AML leukemogenesis.

In our present study, we analyzed the clinicopathological implications of TNFRSF4 expression in AML and its possible relationship with various characteristics of the disease to improve the efficacy of the evaluation of AML. Our study revealed that TNFRSF4 was elevated in AML compared to healthy controls. We showed that higher TNFRSF4 gene expression was significantly related to and can predict poorer overall survival of AML patients. High TNFRSF4 was associated with mutated FLT3-ITD, mutated NPM1 genes, lower CR rate after induction therapy, high intensity of consolidation, and higher relapse rate. Considering our results, we hypothesized that TNFRSF4 high expression may be a part of an aggressive course of AML. Although very few studies investigated TNFRSF4 expression in AML, one study carried out by Gu et al. in 2020 has highlighted the prognostic role of TNFRSF4 expression in AML [3]. Our results coincide with their results that demonstrated clearly that higher TNFRSF4 expression was associated with NPM1 and FLT3 mutations and was positively correlated with bone marrow blast percentage, besides, they proved that TNFRSF4 high expression contributed to the poor clinical outcome of patients regarding the CR, OS, and relapse free survival (RFS). Another research conducted by Neubling et al. showed that TNFRSF4 is detected on AML blasts and that the TNFRSF4 can, after using agonistic TNFRSF4 antibodies, induce proliferation and secretion of cytokines that mediate growth and survival of leukemic cells. They also demonstrated that disruption of TNFRSF4/TNFRSF4 mechanism of action impaired Natural Killer cells reactivity against primary AML cells, strongly suggesting that TNFRSF4 is implicated in disease pathophysiology of AML and that developing TNFRSF4-targeted approaches can be successful in cancer immunotherapy. Moreover, another research found that TNFRSF4 co-stimulation *in vitro* could

reverse the CD8 T cell dysfunction partly in patients with AML [41], presuming that TNFRSF4 could play a role in the treatment of AML. In addition, one more study that was carried out on mice has revealed that direct ligation of TNFRSF4 on CD8 T cells increased tumor specific cytotoxicity and that concomitant treatment with agonistic antibodies to TNFRSF4 raises the chances of disease control after surgical or radiation therapy [42]. Our results proved that TNFRSF4 high expression negatively impacts AML prognosis. Thus, our results together with the previous studies results suggested clearly that it is very likely that TNFRSF4 expression plays an important role in AML and has a crucial role in determining the clinical outcome and treatment response of the disease, and that immunotherapy targeting TNFRSF4 receptors could be beneficial in treating patients with AML.

Furthermore, the current study showed that high TNFRSF4 expression was strongly related to worse AML disease progression, lower CR, higher intensity of consolidation, higher relapse rate, and shorter OS, which helped in clear differentiation between AML cases according to their TNFRSF4 gene expression level. High TNFRSF4 expression was an independent unfavorable prognostic marker for CR and RFS in AML patients. On contrary, OS was not affected by TNFRSF4 in the multivariate analysis. However, TNFRSF4 expression was significantly related to lower CR, shorter RFS, and OS by univariate analysis, suggesting that TNFRSF4 is at least playing a part in the prognostic evaluation of AML that could be used to predict clinical outcome and survival in AML patients more precisely.

A limitation of our study to be noted was that the TNFRSF4 expression was measured only at baseline, while changes in the expression levels after receiving treatment were not measured, therefore, further investigations would be recommended in AML patients to explore whether there will be changes in the expression later in the course of the disease. In addition, our study was observational, it could not differentiate the possible mechanisms behind our findings. Thus, functional studies are recommended to understand the research topic in depth.

In conclusion, our findings from this study indicate that TNFRSF4 high expression worsens the prognosis in AML patients with worse clinical outcome than in patients with low expression. Therefore, TNFRSF4 expression might be used to assess the prognosis and might have a role in treatment guidance in AML patients. Because agonistic antibodies to TNFRSF4 are proved effective in improving the prognosis of various cancers and autoimmune diseases, they are considered promising alternative treatment options for patients with AML. Based on our findings, we suggest that clinical trials evaluating the use of agonistic antibodies to TNFRSF4 should be considered for patients with AML showing high TNFRSF4 gene expression.

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Declarations

Conflict of interest The authors declare no conflict of interest.

Consent to participate Written informed consent was signed by each participant in the study. This study was approved by the Ethics Committee of Alexandria Faculty of Medicine.

Consent for publication Consent was obtained from participants to publish the results with keeping the confidentiality of their personal information.

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