Prognostic Value of Absolute Lymphocyte Count, Lymphocyte Percentage, Serum Albumin, Aberrant Expression of CD7, CD19 and the Tumor Suppressors (PTEN and p53) in Patients with Acute Myeloid Leukemia

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Abstract

**Background:** Acute myeloid leukemia (AML) is a hematopoietic neoplasm. Tumor suppressors have a magnificent role in preventing the AML process. The absolute lymphocyte count is a simple yet statistically powerful estimate in patients with acute leukemia besides the lymphocyte’s percentage. **Aim:** Investigating the prognostic value of absolute lymphocyte count, lymphocyte percentage, serum albumin, the aberrant expression of CD7 and CD19 and the tumor suppressor genes (PTEN and p53) in patients with AML. **Methods:** 35 de novo AML patients were included. They received the standard induction chemotherapy (3+7 protocol) and were followed up for one year after treatment. 15 normal healthy individuals, age and sex matched constituted the controls. **Results:** The mean overall survival of patients with lymphocyte percentage ≤25 was low compared to those with high lymphocyte percentage (>25%) (χ² =5.808, P=0.016). AML patients with low levels of ALC showed significantly shorter overall survival than patients with high levels (χ² =4.587, P=0.032). AML patients with low serum albumin were of low overall survival compared to those with normal level (χ² =8.698, P=0.003). Patients with aberrant CD7 expression showed short survival and unresponsiveness to treatment than CD7 negative patients. PTEN gene expression and p53 protein level were significantly lower in AML patients compared to the control group. **Conclusion:** The decrease in ALC, lymphocyte percentage, albumin concentration and the increase in monocyte percentage indicates bad prognosis in AML patients. The Aberrant CD7 expression, very low expression of PTEN and low level of p53 could estimate the unresponsiveness to standard chemotherapy.

**Keywords:** AML- ALC- albumin- CD7- CD19- PTEN- p53

Introduction

Acute myeloid leukemia is an aggressive clonal hematopoietic neoplasm [1] characterized by proliferation of immature myeloid cells, decreased apoptosis and genomic instability. These genetic alterations may include inappropriate expression of oncogenes or loss of function of tumor suppressor genes [2].

In Egypt, a series of 83,500 newly diagnosed cancer cases was studied by the National Cancer Institute (NCI), during the period between 2002 and 2010. In 75,036 cases of adults, acute leukemic cases constituted
7.7% of newly diagnosed cancer cases in both sexes. Sex distribution of AML cases shows slight male predominance with male: female ratio 1.15:1. The mean age of adult leukemia is 42.5 years [3].

Despite the progress of AML classification, there are simple prognostic markers with different clinical outcomes.

PTEN (Phosphatase and tensin homolog on chromosome 10) is a tumor suppressor gene regulating apoptosis and cell survival by inhibiting the PI3K/AKT signaling pathway [4] originating from epidermal growth factor receptor (EGFR) activation or activation of other tyrosine kinase receptors [5], which are involved in angiogenesis, growth, proliferation and survival [4]. The dysregulation of PTEN may give rise to many aspects of cancer development such as cell proliferation and apoptosis resistance [6]. PTEN gene mutation or deletion has been related to many types of cancers [7] and hematological malignancies [8].

PI3K/AKT pathway is also regulated by p53, a tumor suppressor protein encoded by the Tp53 gene that has an incredible job in DNA damaged incited cell death [9]. A reciprocal regulation may exist between the PI3K-Akt pathway and the tumor suppressor protein p53 [10] as PTEN is a transcriptional target of p53 [11]. Mutation or deletion of Tp53 gene disrupts p53 pathway fundamentally in choosing between established or hematological malignancies [8].

Accurate assessment of prognosis is central to the management of AML. By stratifying patients according to their risk of treatment resistance or treatment-related mortality (TRM), prognostic factors help guide the physician in deciding between standard or increased treatment intensity, consolidation chemotherapy or more investigational therapies [13]. Therefore, we aimed at investigating the prognostic value of lymphocyte count, lymphocyte percentage serum albumin, the aberrant expression of CD7 besides the tumor suppressor genes (PTEN and p53) in patients with AML.

Materials and Methods

The research incorporated fifty subjects divided into:
Group1: 15 healthy controls (age and sex matched)
Group2: 35 patients with de novo AML. Patients with secondary AML, therapy related AML, patients with promyelocytic anemia (M3), patients with comorbid disease (hepatic, renal and cardiac) and patients >60 years old or <18 years old were excluded.

These patients were recruited from patients admitted to the Hematology Department of the Medical Research Institute, Alexandria University, Egypt during the period from June/2016 to December/2016. A written consent was taken from patients and control after explaining the nature, steps and the research’s aim.

For remission induction, all patients received the 3+7 standard protocol (daunorubicin 60-90 mg/m²/day for 3 days + cytarabine 100-200 mg/m² for 7 days), then all patients were reevaluated (peripheral blood (PB) and bone marrow (BM) aspirate samples were repeated) for complete remission achievement (BM blasts <5%) on day 28 [14]. After that, the patients were monitored for one year.

Patients were thoroughly examined and were subjected to full history taking including (Age, Gender, Symptoms, Medical history, Family history of leukemia or other malignancies), Complete blood picture (CBC) [15], Bone marrow aspiration for Flow cytométric immunophenotyping [16], Colorimetric estimation of serum albumin [17], ELISA test for serum p53 [18] and real time PCR to determine PTEN gene expression [19].

Complete Blood Count (CBC)

CBC was done on an automated cell counter (Sysmex XT-1800I, Japan) to the patients before and after treatment.

Bone Marrow Aspiration and Immunophenotyping (For Patients Only)

Direct immune fluorescence staining was done on the bone marrow aspirates using specific directly conjugated fluorochrome-labeled monoclonal mouse anti-human antibodies as per the manufacturer instructions. Immunofluorescence was analyzed using Becton Dickinson FACS Caliber flow cytometer equipped with BD Cell Quest pro software (BD biosciences, San Jose, CA, USA) to diagnose acute leukemia. This panel includes:
- A primary panel was used to distinguish AML from ALL: CD13, CD33, CD117, anti-MPO, CD19, CD79a, CD7, and cCD3.
- CD34 and CD45 were also done where, CD34 is a non-lineage specific marker expressed in hematopoietic progenitor cells and CD45 is a pan myeloid marker.
- CD64 and CD11c were done if monocytic AML was expected.

The percentage of CDs expression from gated blast cells was recorded and the expression was considered positive when ≥20% of the gated cells expressed it at the diagnosis time.

Colorimetric Estimation of serum albumin

It was done to determine the concentration of serum albumin of the control group and the patients before and after treatment.

The method is based on the specific binding of bromocresol green (BCG), an anionic dye and the protein at acidic PH with the resulting shift in absorption wave length of the complex. The intensity of the colour formed is proportional to the concentration of albumin in the sample.

\[ \text{BCG} + \text{Albumin} \rightarrow \text{BCG-albumin complex} \]

ELISA Test

The ELISA test was done on the serum of the 2 groups to determine the serum concentration of p53 protein.

The microtiter plate wells was provided coated by purified human P53 antibody to make solid-phase
antibody, samples containing P53 antigen was added to wells, also combined P53 antibody which with horse redox peroxidase (HRP) labeled became antibody-antigen-enzyme antibody complex, after washing completely, the 3,3’,5,5’ tetra methyl benzidine (TMB) substrate solution was added where the TMB substrate became blue color at HRP enzyme- catalyzed. The reaction was terminated by the addition of sulphuric acid and the color change was measured spectrophotometrically at a wave length of 450 nm. The concentration of P53 in the samples was determined by comparing the optical density (O.D) of the samples to the standard curve.

Real Time PCR

It was done for the patients before treatment and the control group to determine the expression of the PTEN gene using comparative CT method, where ACTB was used as a house keeping gene. The relative amount of PTEN normalized to ACTB was calculated with the equation 2\(^{-\Delta \Delta CT}\) Where:

\[
\Delta \Delta CT = (C_{T \text{target gene}} - C_{T \text{ACTB}}) \text{patient} - (C_{T \text{target gene}} - C_{T \text{ACTB}}) \text{control}
\]

Fold change = 2\(^{-\Delta CT}\)

Thus, the expression produced by the control group will be equal to one, any positive value will represent an upregulation while a negative value will represent a downregulation in gene expression, as compared to the control group.

Statistical analysis

Statistical analysis was formed using IBM SPSS statistics “version 21” [20]. Quantitative data were described by mean as measure of central tendency & standard- error and minimum, maximum as measure of dispersion. Mixed design ANOVA done to detect statistical significant difference in the mean of quantitative variables before and after therapy.

Independent sample t test and Mann Whitney test were used to study statistical significance in the mean of quantitative variables between patients groups and healthy controls. Wilcoxon signed rank test was done to prove statistical significance in the mean of quantitative variables before and after treatment in patients group. All the statistical tests were two tailed and done at 0.05 level of significance.

Results

The studied patients (35) included 17 males (48.6%) and 18 females (51.4%); their ages ranged from 19 to 60 years with a mean± SE (44.85±2.17).

1. Flow Cytometric Immunophenotyping (FCI) Analysis in AML Patients.

Almost all the cases were positive for CD13, CD33, CD34, CD45 and anti MPO but only 9 cases (25.7 %) were CD117 positive, 4 cases (11.4 %) were CD64 and CD11c positive which represents AML with monocytic component. Aberrant expression of B-cell markers was found in only 2 cases (5.7%) showing expression of CD19 and aberrant expression of the T-cell marker (CD7) was seen in 9 cases (25.7%). On the other hand, all the cases were negative for CD79a and cCD3 indicating the absence of ALL.

2. Hematological Analysis:

Table (1): Hematological results of all the studied group

3. Tumor Suppressor Genes

3.1. PTEN Gene Expression

Figure 1. Kaplan-Meier Overall-survival Curve According to CD7 Positivity in AML Patients. The Kaplan-Meier overall-survival showed that there was statistical significant difference (P=0.000) in survival between CD7 positive patients and CD7 negative patients where the CD7 positive had 21.22 times more probability of death.

CD11c positive which represents AML with monocytic component. Aberrant expression of B-cell markers was found in only 2 cases (5.7%) showing expression of CD19 and aberrant expression of the T-cell marker (CD7) was seen in 9 cases (25.7%). On the other hand, all the cases were negative for CD79a and CD3 indicating the absence of ALL.

2. Hematological Analysis:

Table (1): Hematological results of all the studied group

Figure 2. Kaplan-Meier Overall-survival Curve According to Serum Albumin Level in AML Patients. Kaplan-Meier test was used to estimate the overall survival of AML patients according to the concentration of albumin in serum (g/dl) which revealed that AML patients with low serum level of albumin than its corresponding cut-off point (≤3.5) was of lower survival than those with high levels.
The expression of PTEN in the control group equals 1 and any positive value indicates over expression but the negative value indicates down expression. The patients before treatment showed down expression of PTEN gene compared to the control group (P=0.000) and among the patients, 60% of them showed low expression (>0.5) and 40% of them showed very low expression (≤0.5).

### 3.2. p53

The p53 concentration was significantly higher in the patients after treatment when compared to before treatment (P1=0.003).

### 4. Clinical Outcome:

After the patients were treated with 3+7 induction protocol, bone marrow examination was done on day 28 to determine the patients’ response to therapy and the patients were monitored for 1 year (Table 2).

Table (2): Patients’ response after chemotherapy.

### 5. Overall Survival Analysis

To study the prognostic values of all the parameters, Kaplan-Meier survival curve was constructed (Table 3, Figures 1-7).

Table (3): The overall survival of all the studied parameters

Figure (1): Kaplan-Meier overall-survival curve
Table 2. Patients’ Response after Chemotherapy

| Outcome                                      | No  | %     |
|----------------------------------------------|-----|-------|
| Complete remission from first time survived  | 21  | 60    |
| Complete remission from second time died     | 4   | 11.43 |
| Died                                         | 2   | 5.71  |
| Total                                        | 35  | 100   |

No, number of patients

according to CD7 Positivity in AML patients

Figure (2): Kaplan-Meier overall-survival curve according to serum albumin level in AML patients

Figure (3): Kaplan-Meier overall-survival curve according to lymphocyte % in AML patients.

Figure (4): Kaplan-Meier overall-survival curve according to ALC in AML patients.

Figure (5): Kaplan-Meier overall-survival curve according to ALC/AMC Ratio in AML patients.

Figure (6): Kaplan-Meier overall-survival curve according to PTEN gene expression in AML patients.

Table 3. The Overall Survival of all the Studied Parameters

| Parameter                                      | Mean overall survival (months) | Log rank | χ²     | P      |
|------------------------------------------------|-------------------------------|----------|--------|--------|
| CD7 positivity                                 |                               |          | 21.22  | 0.000* |
| Positive CD7                                   | 3.5                           |          |        |        |
| CD7 Negative                                   | 10.86                         |          |        |        |
| Albumin (g/dl)                                 |                               |          | 8.698  | 0.003* |
| Low albumin (≤ 3.5g/dl)                        | 6.61                          |          |        |        |
| High albumin (> 3.5g/dl)                       | 11.47                         |          |        |        |
| Lymphocyte (%)                                 |                               |          | 5.808  | 0.016* |
| Low lymphocyte% (≤25 %)                        | 6.7                           |          |        |        |
| High lymphocyte% (>25%)                        | 10.45                         |          |        |        |
| ALC (x10⁹/l)                                   |                               |          | 4.587  | 0.032* |
| ALC (≤ 5 x10⁹/l)                               | 6.81                          |          |        |        |
| ALC (> 5 x10⁹/l)                               | 10.37                         |          |        |        |
| ALC/AMC (≤ 10)                                 |                               |          | 0.722  | 0.395  |
| ALC/AMC (> 10)                                 | 10                            |          |        |        |
| Blast cells in BM (%)                          |                               |          | 6.679  | 0.010* |
| Low blast cells (≤50%)                         | 10.25                         |          |        |        |
| High blast cells (>50%)                        | 6.22                          |          |        |        |
| WBCs (x10³/µl)                                 |                               |          | 7.253  | 0.007* |
| Low WBCs counts (≤ 10 x10³/µl)                 | 11.2                          |          |        |        |
| High WBCs counts (> 10 x10³/µl)                | 6.4                           |          |        |        |
| PTEN gene expression                           |                               |          | 5.808  | 0.016* |
| Low PTEN gene expression (>0.5)                | 10                            |          |        |        |
| Very low PTEN gene expression (≤0.5)           | 6.88                          |          |        |        |
| P53 (nmole/ml)                                 |                               |          | 5.022  | 0.025* |
| Low serum P53 levels (≤5 nmole/ml)             | 6.44                          |          |        |        |
| High serum P53 levels (>5nmole/ml)             | 11.03                         |          |        |        |

CD7, cluster of differentiation 7; WBCs, white blood cells; ALC, absolute lymphocyte count; ALC/AMC, absolute monocyte count/absolute monocyte count ratio; BM, bone marrow; PTEN, Phosphatase and tensin homolog on chromosome 10; *, Statistically significant at p ≤ 0.05

6. Correlation Studies

6.1. Correlation Between PTEN Gene Expression P53 Concentration And All The Parameters (Table 4).

Table (4): Correlation between PTEN gene expression, P53 concentration and all the parameters.
Discussion

The absolute lymphocyte count is a simple yet statistically powerful estimate in patients with acute leukemia [21]. Mando et al., (2018) stated that both T lymphocytes and natural killer (NK) cells can kill tumor cells and that the tumor infiltration by lymphocytes shows immune response against tumor cells which suggests better prognosis [22]. Behl et al., (2006) reported that ALC after induction chemotherapy can predict the survival in AML patients as it is an effective defense against leukemic blasts which agrees with our finding where the mean overall survival of patients with lymphocyte percentage ≤ 25 had a mean overall survival of 6.70 months compared to those with high lymphocyte percentage (>25%) whose mean survival was 10.45 months ($\chi^2=5.808$, $P=0.016$) [23]. In addition, AML patients with lower levels of ALC had shorter overall survival than patients with higher levels ($\chi^2=4.587$, $P=0.032$). This further stresses the role of lymphocytes in patients’ immunity to acute leukemia.

Le Jeune et al., (2014) assessed the prognostic value of ALC among AML patients before and after induction chemotherapy, they found that patients with low initial ALC<$1X10^9$/L showed poor prognosis [24].

Regarding the serum albumin level, in the present study AML patients with low serum albumin were of low overall survival compared to those with normal level ($\chi^2=8.698$, $P=0.003$). This agrees with Girault et al., (2017) who concluded that AML patients with good nutrition had short hospital stays had better survival highlighting the prognostic value of serum albumin [25].

The aberrant expression of CD markers not specific to a certain lineage creates ambiguity for lineage assignment and leukemia diagnosis. Elyamany et al., (2013) reported that aberrant antigen expression indicated poor prognosis and aggressiveness of the disease [26]. Also, Tiftik et al., (2004) reported that CD7 expression at diagnosis among AML patients showed low remission rate which agrees with our findings [27].

In this study, 7/9 patients expressing CD7 died during induction, which agrees with Li et al., (2011) [28]. These patients had the lowest level of serum albumin and a high percentage of bone marrow blasts whereas, they had a low total leukocytic count. Furthermore, these patients had the lowest PTEN and p53 levels reflecting more aggressive nature of the disease. Besides, 2/35 patients had aberrant expression of CD19. These patients’ attained remission from the second cycle indicating poor prognosis and low remission rate which agrees with Momani et al., (2016) who found a significant correlation between

### Figure 3. Kaplan-Meier Overall-survival Curve According to Lymphocyte % in AML Patients

The Kaplan-Meier overall-survival showed that there was statistical significant difference ($P=0.016$) in the overall survival among patients with low lymphocyte percentage than the cutoff point and they had 5.808 times more probability of death than those with high lymphocyte percentage.

### Figure 4. Kaplan-Meier Overall-survival Curve According to ALC in AML Patients

Kaplan-Meier survival curve for AML patients revealed that, patients with lower levels of ALC ($x10^9$/l) than its corresponding cut-off point had shorter overall survival time than patients with higher levels the difference was statistically significant according to the log rank test ($p<0.05$).

### Figure 5. Kaplan-Meier Overall-survival Curve According to ALC/AMC Ratio in AML Patients

The Kaplan-Meier overall-survival showed that there was no statistical significant difference ($P=0.395$) in the overall survival among patients with low ALC/AMC ratio than the cutoff point and patients with high ALC/AMC ratio.
the presence of aberrant phenotypes and prediction of remission in AML patients [29].

As regards to PTEN gene expression, it was significantly lower in AML patients compared to the control group (P=0.000) which agrees with Zayed et al., (2017) but we disagreed with them in that they couldn’t find a significant relation between PTEN expression and overall survival in patients with AML as we did [30].

On the other hand, Wu et al., (2018) didn’t find a significant difference in the level of PTEN protein (measured by ELISA) between the control group and the incomplete remission group illustrating that real time PCR is more accurate and specific than ELISA [31].

Patients with very low PTEN gene expression couldn’t achieve complete remission (CR) from the first cycle, but from the second. Also, a negative correlation was found between PTEN gene expression and bone marrow blasts percentage (r=−0.246, P=0.154) indicating poor prognosis, yet the difference was not significant. Additionally, patients pertaining to very low PTEN expression were expressing CD7 on their blasts and died during induction.

Table 4. Correlation between PTEN Gene Expression, p53 Concentration and all the Parameters

| Parameter                  | PTEN gene expression | P53 concentration |
|----------------------------|----------------------|-------------------|
| Lymphocytes (%)            | r=0.730              | r=0.781           |
|                           | P=0.000*             | P=0.000*          |
| Monocytes (%)              | r=−0.500             | r=−0.527          |
|                           | P=0.002*             | P=0.001*          |
| ALC/AMC ratio              | r=0.443              | r=0.521           |
|                           | P=0.008*             | P=0.001*          |
| Serum Albumin (g/dl)       | r=0.718              | r=0.596           |
|                           | P=0.000*             | P=0.000*          |
| P53 (nmole/ml)             | r=0.672              | -                 |
|                           | P=0.000*             |                   |
| PTEN gene expression       | -                    | r=0.672           |
|                           |                      | P=0.000*          |
| Hb (g/dl)                  | r=−0.019             | r=0.008           |
|                           | P=0.916              | P=0.965           |
| WBCs (x10^3/µl)            | r=0.168              | r=0.182           |
|                           | P=0.335              | P=0.296           |
| RBCs (x10^6/µl)            | r=0.032              | r=−0.103          |
|                           | P=0.854              | P=0.557           |
| Platelets (x10^3/µl)       | r=−0.003             | r=−0.029          |
|                           | P=0.984              | P=0.866           |
| ALC (x10^9/l)              | r=0.166              | r=0.232           |
|                           | P=0.340              | P=0.179           |
| AMC (x10^9/l)              | r=0.053              | r=0.095           |
|                           | P=0.761              | P=0.588           |
| Blasts in BM (%)           | r=−0.246             | r=−0.398          |
|                           | P=0.154              | P=0.018*          |
| Blasts in peripheral blood (%) | r=0.122         | r=0.122           |
|                           | P=0.484              | P=0.484           |

ALC/AMC, absolute monocyte count/absolute monocyte count ratio; PTEN, Phosphatase and tensin homolog on chromosome 10; Hb, hemoglobin; WBCs, white blood cells; RBCs, red blood cells; ALC, absolute lymphocyte count; AMC, absolute monocyte count; BM, bone marrow; r, correlation coefficient; *, Statistically significant at p ≤ 0.05

Figure 6. Kaplan-Meier Overall-survival Curve According to PTEN Gene Expression in AML Patients.

The Kaplan–Meier overall survival showed that AML patients with very low PTEN gene expression than its corresponding cut-off point (≤0.5) had significantly lower survival those with low levels.
expression obviates that both pathways are interrelated. The positive correlation between p53 level and PTEN gene predicts the unresponsiveness to standard chemotherapy.

Sadrzadeh et al., (2017) [36]. Similarly, Baltz et al., (2016) is a known prognostic maker of bad omen as stated by positive correlation found between PTEN gene expression indicates also poor prognosis. In contrast, the significant negative correlation found between p53 protein level and bone marrow blasts (r= -0.527, P=0.001) reflects the aggressiveness of the disease in patients with lower p53 levels as well as loss of myeloid differentiation.

The significant negative correlation found between monocyte percentage and p53 level (r= -0.527, P=0.001) indicates also poor prognosis. In contrast, the significant positive correlation found between PTEN gene expression on p53 concentration (r= 0.672, P=0.000) reflects their interrelationship which was hypothesized by Brito et al., (2015) who showed that p53 is an activator of PTEN transcription by binding directly to PTEN promoter region and that PTEN mRNA and protein levels increases in response to stimuli that result in p53 induction [35].

Additionally, our results confirmed the poor prognostic values of low levels of PTEN and p53 as they were positively correlated with low serum albumin level which is a known prognostic maker of bad omen as stated by Sadrzadeh et al., (2017) [36]. Similarly, Baltz et al., (2016) said that PTEN deficiency is frequently found in patients in the late stages of cancer [37].

In conclusion, Aberrant CD7, CD19 expression, very low expression of PTEN and low level of p53 could predict the unresponsiveness to standard chemotherapy. The positive correlation between p53 level and PTEN gene expression obviates that both pathways are interrelated. The decrease in ALC, lymphocyte percentage, serum albumin concentration and the increase in monocyte percentage indicate bad prognosis in AML patients.

We recommend the following:

1) P53 and PTEN level should be estimated in all AML patients at least at diagnosis to individualize patient’s treatment.

2) The absolute lymphocyte count, lymphocyte percentage and monocyte percentage are simple valuable prognostic markers and should be extrapolated from the CBC.

3) Serum albumin should be measured in AML as a valuable prognostic marker.

4) Flow cytometric determination of aberrant lymphoid markers (CD7, CD19) should be included in the diagnostic workup of AML patients as they are strong predictors of overall survival and response to treatment.

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Compliance with ethical standards

The study protocol was approved by the ethical committee of the MRI, Alexandria, Egypt. A signed consent was obtained from all participants before enrollment in the study.

Competence of interest

The authors declare no conflict of interest regarding this manuscript.

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Figure 7. Kaplan-Meier overall-survival Curve According to Serum P53 Concentration (µgm/ml) in AML Patients. Kaplan-Meier survival curve for AML patients revealed that, patients with lower levels of serum P53 than its corresponding cut-off point (≤5 µgm/ml) had shorter overall survival time than patients with higher levels. The difference is statistically significant according to the log rank test (p<0.05).
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