

Clinical Significance of Leukemia Stem Cells Immunophenotype Expression in Patients with Acute Leukemia

Mingfeng Zhao, Haibo Zhu, Rajbhandary Sajin, Xia Xiao, Qi Deng
Department of Hematology, Tianjin First Central Hospital, Tianjin 300192, China
zmfzmf@hotmail.com

Abstract: Background Leukemia stem cells (LSCs) are thought to originate either from normal HSCs or from more differentiated progenitor cells that have acquired malignant features. LSCs possess self-renewal capacity but are relatively quiescent compared to more mature progenitor cells. **Objectives** To detect LSCs immunophenotype of patients with acute leukemia, and to explore the relationship between the expression of LSCs immunophenotype and the prognosis of acute leukemia. **Methods** 86 patients with initial acute leukemia were studied. Using flow cytometry, the expression of LSCs immunophenotype (CD34⁺/CD38⁻/CD96⁺, referred to as CD96⁺ and CD34⁺/CD38⁻/CD123⁺, referred to as CD123⁺) in the patients were detected. **Results** 29 cases (33.7%) expressed CD96; and 35 cases (40.7%) expressed CD123; Only 48.3% of the patients with CD96 expression acquired CR or PR within two courses of chemotherapy, which was 71.9% in the patients without CD96 expression. Only 51.4% of the patients with CD123 expression acquired CR or PR within two courses of chemotherapy, which was 72.5% in the patients without CD123 expression. The patients with CD96 or CD123 expression had a high rate of hyper-leukocytes and more cases with chromosomal cytogenetics of poor prognosis. The survival rate with CD96 and CD123 expression was shorter. **Conclusions** Both CD96 and CD123 were markers of LSCs, CD96 might be more specific. Patients with expression of LSCs immunophenotype especially with the expression of CD96 had a lower rate of remission, shorter survival time and poor prognosis.

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Key words: Leukemia stem cells; Immunophenotype; Prognosis

Introduction:

Acute leukemia is one of the refractory hematological malignant diseases described as an acute disorder of diverse clonogenic disorders within the population. It is now considered to be a stem cell disease with its characteristic refractory nature being blamed on a rare population of CD34⁺/CD38⁻ leukemia stem cells. Leukemia stem cells (LSCs) are named so for their ability to survive and divide continually within the stromal microenvironment. Furthermore LSCs are immune to most present day therapeutic measures, which makes it an important area of research and a possible target to weaken acute leukemia's ability to relapse and remain refractory to treatment.

LSCs arise from a pool of mutated CD34⁺ stem cells. However, the exact immunophenotype of these cells have not yet been accurately identified. Primitive human LSCs populations can be selected by cell surface CD34⁺/CD38⁻/CD123⁺ antigens. It could lead the leukemia when transplanted the CD123⁺ populations into the nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, which indicated that CD123 was one of the LSCs immunophenotype⁽¹⁾. Similar result was achieved while xenografting CD34⁺/CD38⁻/CD96⁺ phenotypes of the AML cell population in SCID mice⁽²⁾, suggesting CD123/CD96 are important immunophenotypes in identification of LSCs. In our studies we have used these two markers to identify LSCs and explore the relationship between

expression of LSCs immunophenotype and prognosis in acute leukemia.

METHODS

Patients

The study was retrospectively performed on 86 patients diagnosed with acute leukemia at Department of Hematology, Tianjin First Central Hospital, Tianjin, China, from August 2008 to May 2012. There were 40 males and 46 females patients, with a median age of 54 years (age range, 11-86 years). Of all the patients, 70 cases were acute myelocytic leukemia (AML) (4 cases were AML-M1, 5 cases were AML-M2a, 4 cases were AML-M2b, 18 cases were AML-M3, 5 cases were AML-M4a, 6 cases were AML-M4b, 11 cases were AML-M5a, 9 cases were AML-M5b, and 8 cases were AML-M6), and 16 cases were acute lymphoblastic leukemia (ALL) (10 cases were B-ALL, 6 cases were T-ALL).

All the patients were diagnosed according with the WHO diagnostic criteria, and underwent immunophenotypic and karyotypic analysis for acute leukemia.

Detection of LSCs immunophenotype

2-5 ml of fresh bone marrow (BM) samples were collected in a heparinised tube on day of diagnosis of acute leukemia. Leukemic cells were isolated by

Ficoll gradient separation.

Using three-color labeling method (FITC, PE and PerCP, respectively) (BD Company, USA and PharMingen Company, USA), the leukemia cells were gated by the parameters of SSC/CD45, and the percentage of CD34⁺ cells among the leukemic group was detected.

The LSCs immunophenotype of CD34⁺/CD38⁻/CD96⁺ (referred to as CD96⁺) and CD34⁺/CD38⁻/CD123⁺ (referred to as CD123⁺) was detected according with the reference(3) with minor modification. In short, the leukemic cells were gated by the parameters of SSC/CD45, and the expression of CD38⁻/CD123⁺ and CD38⁻/CD96⁺ were detected among the CD34⁺ cells. Positive result was defined when the expression of LSCs immunophenotype was more than 1%.

Analyse of chromosomal karyotype

Samples of BM were collected in a heparinised tube. The BM mononuclear cells were harvested after culturing for 24 hours and chromosomal karyotype was done by G-banding analysis. The chromosomal aberrations was determined according to the international standards of human cytogenetics nomenclature.

Treatment of acute leukemia

Treatment of AML, consisted of induction therapy that included HAD regimen (Homoharringtonine, 2mg/m² per day, days 1-7; Cytarabine, 100mg/m² per 12 hours, days 1-7; Daunorubicin, 40mg/m² per day, days 1-3) or DA regimen (Daunorubicin, 40mg/m² per day, days 1-3; Cytarabine, 100mg/m² per 12 hours, days 1-7) or MA regimen (Mitoxantrone, 8mg/m² per day, days 1-3; Cytarabine, 100mg/m² per 12 hours, days 1-7) which was followed by consolidation therapy that included medium dose Cytarabine regimen (Cytarabine, 1g/m² per 12 hours, days 1-5). For elderly patients who couldn't tolerate high dose chemotherapy, CAG regimen (Cytarabine, 10mg/m² per 12 hours, days 1-14; idarubicin, 10mg per day, days 1-8; and granulocyte colony-stimulating factor, 200mg/m² per day, days 1-14) or reduced-dose DA/MA regimen was given. Treatment regimen for ALL, included an induction/remission program that consisted of VDCP regimen (Vincristine, 2mg per day, days 1,8,15,22; Daunorubicin, 40mg/m² per day, days 1-3,15,16; Cyclophosphamide, 0.8g/m² per day, days 1; Prednisone, 1mg/kg per day, days 1-28) or VDCLP regimen (The same regimen as the VDCP; L-Asparaginase, 6000u/m² per day, days 19-28) which was followed by consolidation therapy that included high dose Methotrexate (3g/m² per day, days 1). Patients were adequately supported with blood transfusion, antibiotics and other supportive treatment.

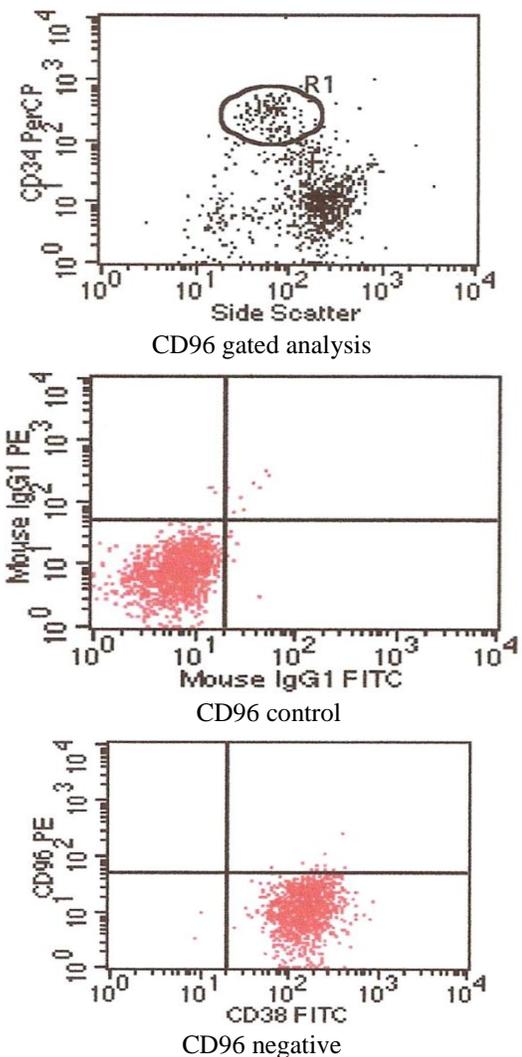
Statistical analysis

Statistical analysis was performed with SPSS (version 15.0) software. Count data were compared with χ^2 test. The probability of disease free survival was calculated by the Kaplan-Meier method, and differences between curves were compared by log-rank test. p values ≤ 0.05 were considered significant.

RESULTS

Expression of LSCs immunophenotype in acute leukemia

Of the 86 patients with initial acute leukemia, 29 cases were positive (33.7%) for CD96 expression (ranged, 0-89.1%), and the average expression level was 11.3%; 35 cases were positive (40.7%) for CD123 expression (ranged, 0-89.6%), and the average expression level was 33.1%. All the CD96 positive cases were also CD123 positive, however, 6 cases among the CD123 positive patients didn't express CD96. Figure 1 and 2 showed the flow cytometric pictures for CD96 and CD123 expression.



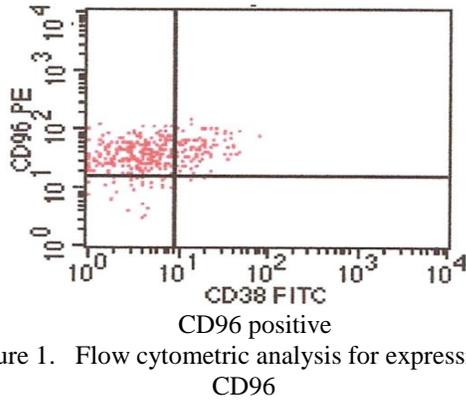


Figure 1. Flow cytometric analysis for expression of CD96

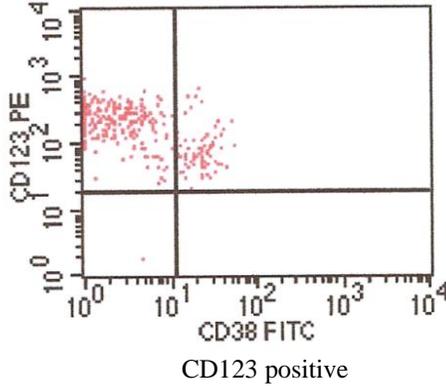
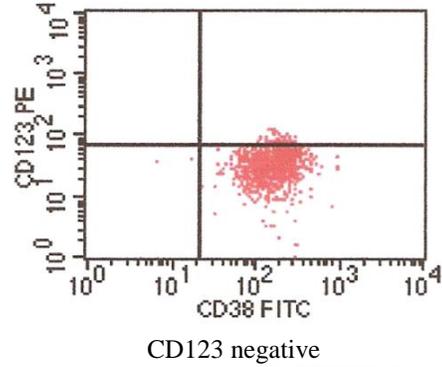
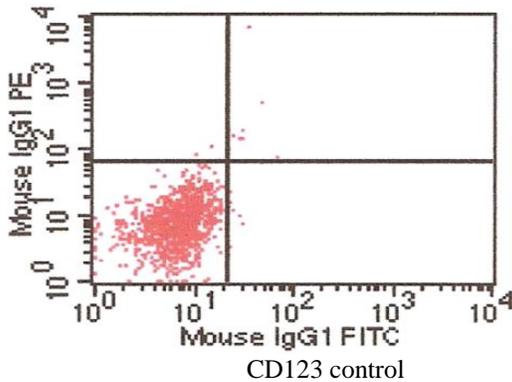
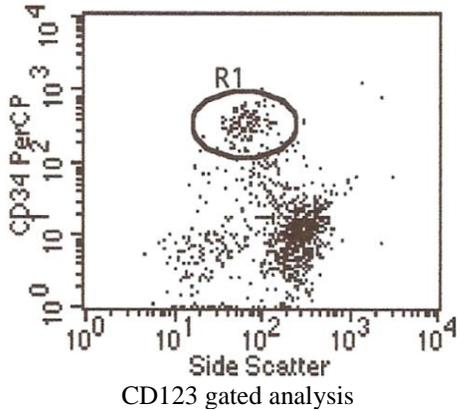


Figure 2. Flowcytometric analysis for expression of CD123



Among the subtypes of acute leukemia, much difference was observed in the expression of both CD96 and CD123. The expression of CD96/CD123 was lower on AML-M3 subtype, but higher on AML-M4,M5,M6 and T-ALL. Statistical difference was observed between the AML-M5 subtype and the expression of both CD96 and CD123 in other subtypes (for CD96 expression: $p=0.030$ and for CD123 expression: $p=0.049$). The statistical difference was most obvious when the expression of both CD96 and CD123 was compared between AML-M5 with AML-M3 (for CD96 expression: $p=0.002$ and for CD123 expression: $p=0.003$). Table 1 showed the different expression of CD96/CD123 in the subtype of acute leukemia.

Table 1. The expression of LSCs immunphenotype in the subtype of acute leukemia

Subtype	CD96		p1 Value	CD123		p2 Value
	Positive(%)	Negative(%)		Positive(%)	Negative(%)	
M1	1(25.0%)	3(75.0%)	0.718	2(50.0%)	2(50.0%)	0.712
M2	2(22.2%)	7(77.8%)	0.484	3(33.3%)	6(66.7%)	0.668
M3	2(11.1%)	16(88.9%)	0.057	3(16.7%)	15(83.3%)	0.054
M4	4(36.4%)	7(63.6%)	0.862	5(45.5%)	6(54.5%)	0.763
M5	12(60.0%)	8(40.0%)	0.030	13(65.0%)	7(35.0%)	0.049
M6	3(37.5%)	5(62.5%)	0.829	3(37.5%)	5(62.5%)	0.860
B-ALL	3(30.0%)	7(70.0%)	0.813	3(30.0%)	7(70.0%)	0.513
T-ALL	2(33.3%)	4(66.7%)	0.985	3(50.0%)	3(50.0%)	0.655
Total	29(33.7%)	57(66.3%)		35(40.7%)	51(58.3%)	

Note: p1 value is the comparison of subtype and total group on the CD96 expression; p2 value is the comparison of subtype and total group on the CD123 expression

The relationship between LSCs immunophenotype expression and induction remission in acute leukemia

Among the 29 cases with CD96 expression, only 14 cases (48.3%) acquired CR or PR within two courses of chemotherapy. On the contrary, among the 57 cases without CD96 expression, 41 cases (71.9%) acquired CR or PR, the statistical difference was

significant ($p=0.031$). Similarly, among the 35 cases with CD123 expression, only 18 cases (51.4%) acquired CR or PR. On the contrary, among the 51 cases without CD123 expression, 37 cases (72.5%) acquired CR or PR, the statistical difference was significant ($p=0.045$). Table 2 showed the relationship between expression of CD96/CD123 and efficacy of acute leukemia.

Table 2. The relationship between expression of CD96/CD123 and efficacy of acute leukemia

Immunophenotype Efficacy	CD96		CD123	
	Positive(%)	Negative(%)	Positive(%)	Negative(%)
CR+PR	14(48.3%)	41(71.9%)	18(51.4%)	37(72.5%)
NR	15(51.7%)	16(28.1%)	17(48.6%)	14(27.5%)
Total	29(33.7%)	57(66.3%)	35(40.7%)	51(58.3%)
p Value	0.031		0.045	

Note: CR: complete remission; PR: partial remission; NR: not remission

The relationship between LSCs immunophenotype expression and age of acute leukemia

The median age was 48 years for CD96 positive patients and 58 years for CD96 negative patients, the statistical difference was not significant ($p=0.435$). The median age was 48 years for CD123 positive patients and 59 years for CD123 negative patients, the statistical difference was not significant ($p=0.589$).

The relationship between LSCs immunophenotype expression and hyper-leukocytosis of acute leukemia

Among the 29 cases with CD96 expression, 16 cases (55.2%) had hyper-leukocytosis ($>30 \times 10^9/L$). On the contrary, among the 57 cases without CD96 expression, only 16 cases (28.1%) had hyper-leukocytosis, the statistical difference was significant ($p=0.014$). Similarly, among the 35 cases with CD123 expression, 17 cases (53.1%) had hyper-leukocytosis. On the contrary, among the 51 cases without CD123 expression, only 15 cases (33.3%) had hyper-leukocytosis, although the statistical difference was not significant ($p=0.071$).

The relationship between LSCs immunophenotype expression and chromosomal karyotype of poor prognosis

77 cases among 86 patients acquired the results of chromosomal karyotype, and were screened for chromosomal karyotype of poor prognosis for example $-5, 3q^-,$ hypodiploid, $t(9;22), t(4;11), t(1;19)$.

Among the 27 cases with CD96 expression who had the karyotypes done, 9 cases (33.3%) had the chromosomal karyotype of poor prognosis. Among the 57 cases without CD96 expression, only 5 cases (12.0%) had the chromosomal karyotype of poor prognosis,

there was significant statistical difference ($p=0.024$) between the groups. Among the 34 cases with CD123 expression, 8 cases (23.5%) had the chromosomal karyotype of poor prognosis, and among the 43 cases without CD123 expression, only 7 cases (16.3%) had the chromosomal karyotype of poor prognosis, the statistical difference was not significant ($p=0.425$).

The relationship between LSCs immunophenotype expression and relapse of acute leukemia

Of the 43 patients who acquired CR or PR, 7 cases (70.0%) relapsed among 10 patients with CD96 expression. On the contrary, only 11 cases (33.3%) relapsed among 33 patients without CD96 expression, the statistical difference was significant ($p=0.039$). 8 cases (61.5%) relapsed among 13 patients with CD123 expression. On the contrary, only 10 cases (33.3%) relapsed among 30 patients without CD123 expression, the statistical difference was not significant ($p=0.085$).

The survival analyse of the patients with or without LSCs immunophenotype expression

The follow-up data were acquired from 67 patients, and the follow-up time was from August 2008 to May 2012, the follow-up rate was 77.9%. One-year survival rate was 47.8% in the patients with CD96+/CD123+ expression, and 94.4% in the patients with CD96-/CD123- expression. Three-year survival rate was 26.1% in the patients with CD96+/CD123+ expression, and 86.1% in the patients with CD96-/CD123- expression. In addition, the mean survival time was 14.57 ± 2.29 months in the patients with CD96+/CD123+ expression, and 34.69 ± 2.89 months in the patients with CD96-/CD123- expression. The statistical difference was significant ($p=0.000$). These data indicated that the former had worse prognosis than the latter.

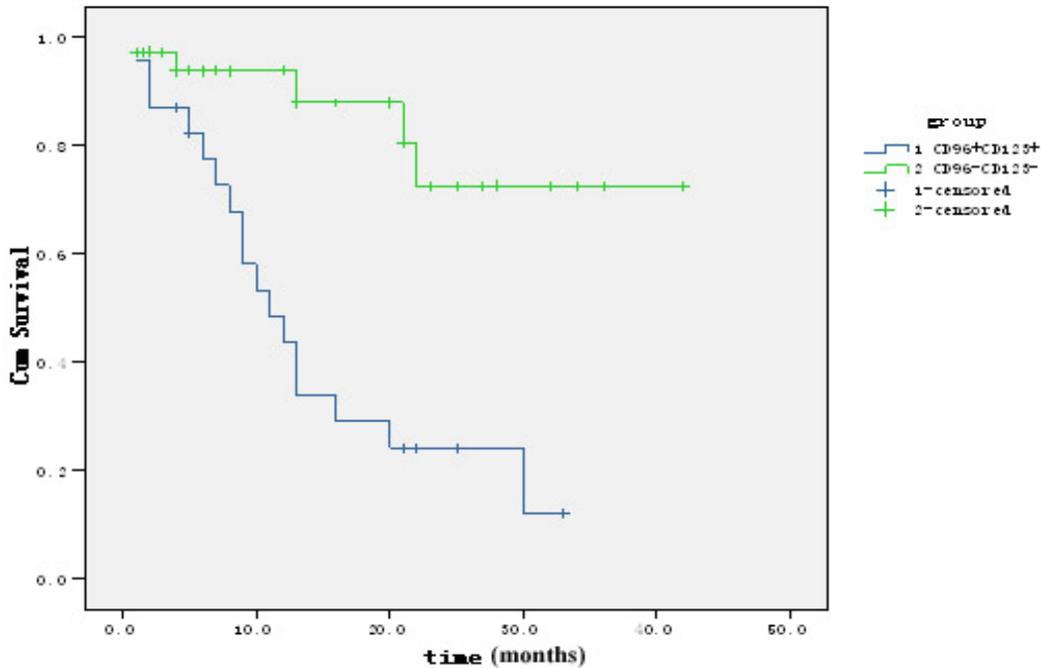


Figure 1. The Kaplan-Meier survival curves of the patients with CD96⁺CD123⁺ expression and with CD96⁻CD123⁻ expression (n=67)

Discussion:

LSCs are described as CD34⁺/CD38⁻ cells of the AML cell population which when xenografted in NOD/SCID mice are capable of generating leukemic cell lines similar to its donor⁽⁴⁾. LSCs are capable of indefinite division and differentiation and is associated with relapse and refractory nature of acute leukemia. Hence LSCs have important therapeutic and prognostic implications. LSCs are identified according to unique cell surface receptors such as CD123, CD33, CD44, CD47, CLL-1 and CD13⁽⁵⁾. The therapeutic benefit in targeting these receptors is currently being assessed in clinical trials with the use of monoclonal Anti-CD123⁽⁶⁾ and Anti-CD33⁽⁷⁾ on patients with acute leukemia. Analysis of genetic markers of LSCs has also proven to be an effective prognostic tool for acute leukemia. Using this information, our study performed an immunophenotypic analysis of CD123 and CD96 markers in different subtypes of AML patients and characterised its relation to various parameters associated with disease outcomes such as leukocyte count, age, and chromosomal aberrance.

Our results showed when used together for prognostic evaluation, higher CD96/CD123 expression was associated with hyperleukocytes, bad prognostic chromosomal aberrations, increased probability of relapse and decreased survival in AML patients. Our findings agreed with previous findings which has associated the increased expression of LSCs population

with MRD and bad prognosis in AML⁽⁸⁾. Although our study couldn't effectively determine whether other markers of LSCs namely CD33, CD44, CD47, CLL-1 and CD13 fare worse or better compared to CD123 and CD96 we could nevertheless conclude CD96 is a more specific marker compared to CD123; both are markers for bad prognosis and there was differential activation of the immunophenotypes among different subtypes of AML.

CD123 is the alpha subunit that heterodimerizes with the IL-3 β chain to form the interleukin-3 receptor and is constitutively expressed on HSCs, where it is involved in proliferation and differentiation. CD38⁺/CD34⁻/CD123⁺ cells when xenografted into NOD/SCID mice resulted in engraftment and production of a leukemic white blood cell population within the subject, studies done thereafter have highlighted the importance of CD123 in identifying MRD^(9,10) and initiating AML in patients with predisposing illnesses such as MDS⁽¹¹⁾, Fanconis Anemia⁽¹²⁾, and its association with induction failure⁽¹³⁾. Similarly CD96 is a marker which is generally not detectable on the majority of cells in the normal adult BM HSC-enriched population making it a more specific and potential target marker for therapy in AML. Correlation of these markers to generally accepted unfavourable prognostic parameters offers evidence that these markers have clinical implication. Although these markers might not prove superior to well accepted

parameters in screening high risk and low risk groups they can come in handy when tailoring individual therapies by identifying patients at increased risk of relapse and treatment failure. Furthermore the lower expression of CD123 and CD96 observed in CR patients supports its significance in predicting patient prognosis. Heterogeneity within the malignant cells also renders classical prognostic markers of acute leukemia incapable of accurately asserting patients who are at high risks. Markers such as CD123 and CD96 that identify LSCs that correlate well with known prognostic parameters can be an invaluable in these cases.

Thus, our preliminary results showed that both CD96 and CD123 were the markers of LSCs, and CD96 a more specific marker. Patients with expression of LSCs immunophenotype especially with the expression of CD96 had a higher rate of remission, decreased survival rate and poor prognosis. Although the use of these LSCs immunophenotype for therapy and prognosis can be limited as HSCs and LSCs can both express the same receptors⁽¹⁴⁾. These markers can be useful in identifying patients at increased risk of relapse, developing MRD or treatment failure information which can be useful in timely intervention and result in better patient outcomes.

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