

Original Article

Expression of indoleamine 2,3-dioxygenase in acute leukemic cells and the clinical significance

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Abstract: Objective: We discussed the relationship between expression of indoleamine 2,3-dioxygenase (IDO) in leukemic cells, clinicopathologic parameters and prognosis in acute monocytic leukemia (M_5). Method: IDO protein and RNA expressions in leukemic cells from 60 patients with acute leukemia(AL) were detected using immunohistochemistry technique and real-time fluorescence quantitative RT-PCR, respectively. Correlation analysis was performed with clinical data. Results: The positive expression rate of IDO protein was 63.3% (38/60) in patients with AL, and the positive rate in acute myeloid leukemia (AML) was 69.4% (34/49). The positive rate among M_5 patients (82.9%, 29/35) was obviously higher than that among patients with acute lymphocytic leukemia (ALL) (36.4%, 4/11). IDO was negatively expressed in peripheral blood mononuclear cells (PBMCs) in healthy subjects. IDO RNA expression in M_5 patients was higher than that in non- M_5 patients ($P<0.05$) and healthy subjects ($P<0.001$); the IDO RNA expression in non- M_5 patients was significantly higher than that in healthy subjects ($P<0.001$). IDO RNA expression was not correlated with age, gender and drug sensitivity of patients, but with combined pulmonary infection. Positive IDO expression usually predicted poor prognosis, but IDO was not an independent indicator of poor prognosis in M_5 patients. Conclusion: IDO expression was obviously increased in M_5 patients than in non- M_5 patients and healthy subjects. Positive IDO expression may predict poor prognosis in M_5 patients, but it was not an independent indicator of poor prognosis.

Keywords: Leukemia, acute, indoleamine 2,3-dioxygenase, prognosis

Introduction

Leukemia is a hematopoietic malignancy derived from pluripotent stem cells or early progenitor cells (myeloid or lymphoid). In China, acute leukemia (AL) has a higher incidence than chronic leukemia; among adults, acute myeloid leukemia (AML) is more common than acute lymphocytic leukemia (ALL), while in children, ALL is more common. AML- M_5 shows an increasing incidence in recent years and has now becomes one of the most common types of leukemia. Chemotherapy is the major treatment, but the response rate is low and the relapse is high for M_5 patients after chemotherapy. Moreover, the response rate is even lower after secondary chemotherapy. Therefore, it is urgent to find new treatments (e.g., immunotherapy and targeted therapy) that can increase response rate, reduce relapse, prolong disease-free survival and improve prognosis.

Materials and method

Clinical data

Sixty patients with AL hospitalized at Department of Hematology of He'nan Province People's Hospital from September 2009 to June 2012 were recruited. Standard chemotherapy was administered: DA, IA or MA chemotherapy for AML; after achieving remission, MA, IA, DA, HAA and MEA chemotherapy were administered. Large-dose cytarabine was administered as consolidation therapy. For ALL, VDLP chemotherapy was used, followed by CAM, HD-MTX, Hyper-CVAD-A/B and MEA as consolidation therapy.

Reagents

Ficoll-I-lypaque separated human peripheral blood lymphoid cells (Tianjin Haoyang Biological

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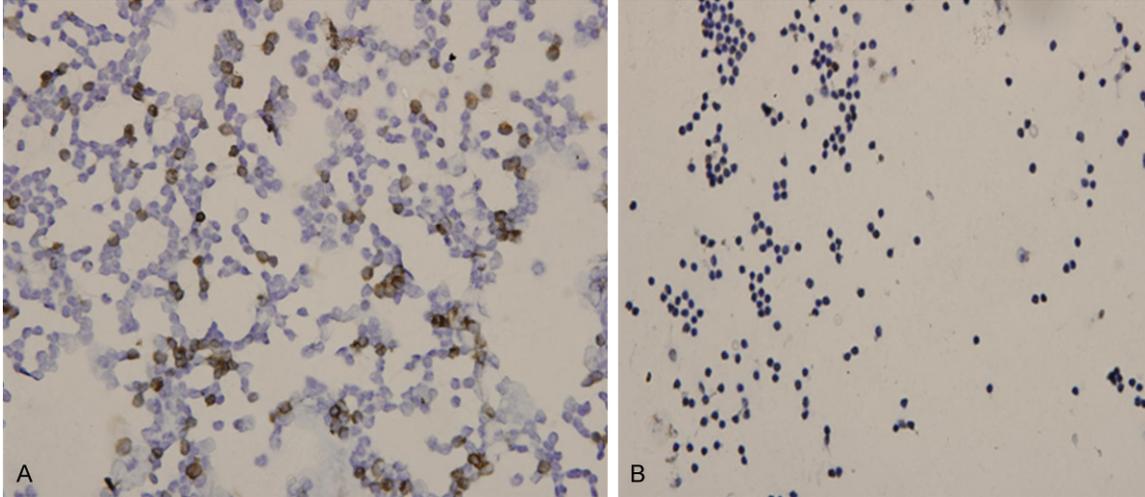


Figure 1. Acute leukemia IDO immunohistochemical staining results. A: Acute myelogenous leukemia (M_5) IDO expression ($\times 200$); B: IDO expression in patients with acute lymphoblastic leukemia ($\times 200$).

Company); TRIZOL (Invitrogen, USA); mouse monoclonal antibody against IDO (10.1) (CHEMICON international, Canada); S-P super sensitive kit, DAB color development kit (Fuzhou Maixin Biotech. Co., Ltd.); cDNA reverse transcription kit (MBI, Lithuania); 2 \times SYBR Green I PCR Mix (TOYOBO, Japan).

Specimen collection and separation of PBMCs

From AL patients and healthy subjects 3 mL of bone marrow or 5 mL of peripheral venous blood was collected. PBMCs were separated by Ficoll-I-lypaque centrifugation. The cells were counted, and some were used for immunohistochemistry and the remaining for reverse transcription.

Immunohistochemistry detection of IDO protein expression in leukemic cells

IDO protein expression was detected according to the manufacturer's instruction of S-P super sensitive kit. The percentage of positive cells in each slice was calculated, and percentage above 5% was considered positive expression according to the literature.

Detection of IDO RNA expression by real-time fluorescence quantitative RT-PCR

Leukemic cells were lysed with Trizol reagent, and RNA extraction was performed. The total RNA was reversely transcribed into cDNA, and IDO mRNA expression was detected using

GAPDH as internal reference. Upstream primer of IDO: 5-TGACGCCTGTGTGAAAGC-3, downstream primer 5-AATCAGTGCCTCCAGTTCC-3, length of amplified fragment 155 bp; upstream primer of GAPDH: 5-TCATCCCTGCCTCTACTG-3, downstream primer 5-TGCTTACCACCTTCTTG-3, length of amplified fragment 175 bp. Real-time fluorescence quantitative RT-PCR was performed using the following system: total volume 25 μ L, 2 \times SYBR Green Realtime PCR Master Mix 12.5 μ L, upstream and downstream primer 0.5 μ L each, cDNA 1 μ L, ddH₂O 10.5 μ L. PCR conditions: predenaturation at 95 $^{\circ}$ C for 60 s, denaturation at 95 $^{\circ}$ C for 45 s, annealing at 62 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 30 s, 39 cycles. The dissolution curve was plotted from 60 $^{\circ}$ C to 90 $^{\circ}$ C. $2^{-\Delta\Delta C_t}$ method was used to analyze the relative expression of IDO mRNA in each group. Finally, the correlations between IDO expression, clinical parameters and prognosis among M_5 patients were analyzed.

Statistical process

Statistical analysis was performed using SPSS 18.0 software. The mRNA expression data did not obey normal distribution, and therefore nonparametric rank sum test was adopted. According to immunohistochemistry detection, PCR result above 2 was defined as positive expression of mRNA. After conversion into count data, the analysis was performed using χ^2 test on a fourfold table. To determine the

The expression of leukemia cells

Table 1. Correlation between IDO mRNA expression and clinicopathologic parameters of AML-M₅ cases

Clinicopathologic parameters	Cases	Average rank sum	Rank sum	Mann-Whitney U	Z value	P value
Gender						
Male	13	17.31	225.0	134.0	-0.307	0.759
Female	22	18.41	405.0			
Disease status						
First diagnosis	21	16.33	343.0	112.0	-1.179	0.239
Relapsed or refractory	14	20.5	287.0			
Drug sensitivity						
Sensitive	20	20.0	400.0	110.0	-1.333	0.182
Insensitive	15	15.33	230.0			
Pulmonary infection						
Yes	7	26.29	184.0	40.0	-2.392	0.017
No	28	15.93	446.0			
Immunohistochemistry						
Positive	29	20.86	605.0	4.0	-3.633	0.000
Negative	6	4.17	25.00			

Table 2. Prognostic value of each indicator in AML-M₅ using Cox regression model

Group	B (regression coefficient)	S.E (standard error)	Wald (Chi-square value)	P value
Gender	0.457	0.455	1.010	0.315
Disease status	0.199	0.566	0.124	0.725
Drug sensitivity	-0.169	0.458	0.136	0.713
Pulmonary infection	0.887	0.619	2.054	0.152
IDO expression	0.885	0.626	1.997	0.158

It can be seen from **Table 2** that IDO expression was not the independent prognostic value in AML-M₅ patients.

consistency in positive rate of mRNA expression in immunohistochemistry and after conversion, Kappa statistics was adopted; the effect of each indicator on survival was compared by log-rank test; the prognostic value of each indicator was evaluated using Cox regression model. $P < 0.05$ indicated statistical significance.

Results

Comparison of general data

Of 60 AL patients, there were 24 males and 36 females; 39 cases were first diagnosed, and 21 cases were relapsed or refractory with a median age of 35 years (6-68 years). There were 11 cases of ALL, 7 cases of AML M₁+M₂, 4 cases of

M₃, 3 cases of M₄ and 35 cases of M₅. All M₅ cases were followed up and the last follow-visit was in December 2013. The control group had 15 healthy subjects (7 males, 8 females), which had a median age of 29 years (19-37 years).

IDO protein expression

Positive expression of IDO protein was defined as appearance of brownish yellow particles in the cytoplasm (**Figure 1**). The positive expression of IDO was 63.3% (38/60) in AL cases and 69.4% (24/49) in AML cases; both were significantly higher than in ALL (36.4%, 4/11). The positive rate was 82.9% (29/35) in AML-M₅ cases. IDO was negatively expressed in PBMCs of healthy subjects.

IDO mRNA expression

The concentration of extracted mRNA was 300-1000 µg/mL with purity of 1.8-2.1. The R² values for standard curve were both above 0.99 for GAPDH and IDO genes, and the relative expression was analyzed by 2^{-ΔΔCT} method. The amplification curves were smooth for both genes, and the dissolution curves were unimodal, indicating specific amplification. The dissolution peak occurred at 90.0°C and 85.5°C for GAPDH and IDO, respectively. Of 35 cases with AML-M₅, the relative expression of IDO mRNA was 2.955 7 (0.90-7.02) on average; for 25 non-M₅ cases, the relative expression of IDO mRNA was 2.265 5 (0.65-3.97) on average; for 15 healthy subjects, the relative expression was 0.963 0 (0.55-1.72) on average. The IDO mRNA expression was higher in AML-M₅ cases than in non-M₅ cases ($P < 0.05$) and healthy subjects ($P < 0.001$); IDO mRNA expression was higher in non-M₅ cases than in healthy subjects ($P < 0.001$). IDO mRNA expression was correlated with combined pulmonary

infection, but not with gender and drug sensitivity [1] of AL patients (**Table 1**). After converting IDO mRNA expression into count data, the positive rate was 85.7% (30/35) in AML-M₅ cases; IDO mRNA expression was positively correlated with IDO protein expression ($P < 0.001$).

Correlation between IDO expression and prognosis in AML-M₅ cases

All 35 AML-M₅ cases were followed up with a median follow-up of 15.7 months (5-35 months). IDO protein and mRNA expressions were highly consistent and showed strong correlation ($\kappa = 0.677$, $P < 0.001$). Therefore, survival analysis was performed based on positive rate of IDO mRNA expression; the effect of each indicator on survival was compared using log-rank test. The results showed that the median survival was 13.9 months in cases positive for IDO mRNA expression, which was much shorter than in cases negative for IDO mRNA expression (29.0 months) ($P = 0.045$). The median survival was 18.5 months in male cases and 14.2 months in female cases ($P = 0.212$); the median survival was 18.3 months in the first diagnosed cases and 12.4 months in relapsed or refractory cases ($P = 0.159$). For drug-sensitive cases, the median survival was 14.3 months, while for drug-insensitive cases, the median survival was 16.8 months ($P = 0.838$); the median survival for cases combined with pulmonary infection was 9.3 months, and for cases not combined with pulmonary infection, the median survival was 16.8 months ($P = 0.011$). Thus IDO expression and combined pulmonary infection were the prognostic factors, while gender, disease status (the first diagnosis, relapsed or refractory) and drug sensitivity were not ($P > 0.05$). The prognostic value of each indicator was assessed using Cox regression model, with results given in **Table 2**.

Discussion

IDO is an intracellular enzyme with high expression in various tumor cells [2-4]. By catalyzing the degradation of tryptophan, IDO can lead to a low-tryptophan microenvironment for tumor cells. The deficiency of tryptophan will result in reduced protein synthesis, thereby inhibiting the proliferation of cytotoxic T lymphocytes (CTL). The metabolites of tryptophan catabolism can induce lymphocyte apoptosis by inducing the generation of oxygen free radicals. The

question is whether the tumor cell proliferation will be affected by deficiency of tryptophan and the accumulation of the metabolites. There is a checkpoint in the middle of G₁ phase for T cells that is very sensitive to tryptophan deficiency. Therefore, the T cells will neither proliferate massively nor achieve clonal expansion in the presence of IDO [5]. The T cells in the resting state are more vulnerable to apoptosis, leading to deficiency of T cells and hence cell-mediated immune disorder. The kynurenine (Kyn), the metabolite of tryptophan degradation catalyzed by IDO, has cytotoxicity on T cells, but this effect is different from that due to tryptophan deficiency. Besides, host T cells are more sensitive to the cytotoxicity of IDO than tumor cells [5]. Thus activated lymphocytes are sensitive to both low-tryptophan environment and accumulated Kyn. IDO expression in tumor cells can significantly inhibit the activation; proliferation and cytotoxicity of T cells, while the tumor cells itself are little affected. IDO is the rate-limiting enzyme in extrahepatic metabolism of tryptophan and consumes tryptophan to produce Kyn. IDO expressed in tumor cells or in antigen presenting cells can lead to incompetence and apoptosis of T cells in the lymph nodes near the tumor through the mechanism of tryptophan deficiency and accumulation of immunosuppressive metabolites of tryptophan. Or it is involved in immune tolerance and immune escape of tumors by inducing the differentiation of naïve T cells to Treg cells [6-9]. We found through animal experiments that 1-methyl-tryptophan (1-MT), the competitive inhibitor of IDO, improved the effect of immunotherapy with leukemia vaccine. For relapsed mice administered with leukemia vaccine alone, the growth of neoplastic nodules was also inhibited effectively by the injection of 1-MT [10]. Thus the effect of immunotherapy can be improved significantly by silencing IDO in dendritic cells or injecting IDO inhibitor (small-molecule 1-MT) [7, 11].

In the present study, IDO protein was highly expressed in leukemic cells with positive rate of 63.3%; a particularly high expression was noted in M₅ patients (82.9%). Tang et al. [12] found that the positive expression rate of IDO protein in AML was 74% (17/23), which agreed with our results. It was confirmed that IDO expression in hematologic cancer cells could indirectly inhibit the proliferation of T cells by degrading Trp in the microenvironment. By this mechanism, the signal transduction for the activation of T cells is blocked, thus promoting

immune escape of leukemic cells. The average survival of M₅ patients positive for IDO expression was 13.9 months, which was much shorter than that of patients negative for IDO expression (29.0 months). It can be inferred that IDO expression influences the prognosis of M₅ patients. Brandacher et al. [2] found that IDO was highly expressed in colon cancer and IDO expression was the independent indicator of poor prognosis. However, we only confirmed that IDO expression affected the prognosis of M₅ patients. As indicated by Cox regression model, IDO expression was not the independent prognostic indicator for M₅ patients. This finding may be explained by the action of other factors, such as other rate-limiting enzymes of tryptophan (eg., tryptophan 2,3-dioxygenase (TDO)). More studies are needed to prove this.

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Disclosure of conflict of interest

None.

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