High *INDO* (indoleamine 2,3-dioxygenase) mRNA level in blasts of acute myeloid leukemic patients predicts poor clinical outcome

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ABSTRACT

Indoleamine 2,3-dioxygenase degrades the amino acid tryptophan which is essential for T cells. Tryptophan depletion causes T-cell cycle arrest and solid tumors that express high levels of indoleamine 2,3-dioxygenase can create immune suppression. Recently, blasts of patients with acute myeloid leukemia were shown to express indoleamine 2,3-dioxygenase. We determined *INDO* (encoding gene for indoleamine 2,3-dioxygenase) mRNA expression in leukemic blasts of 286 patients with acute myeloid leukemia by gene-expression profiling. Results were validated by quantitative polymerase chain reaction analysis in blasts of an independent cohort of 71 patients. High *INDO* expression was correlated to significantly shortened overall and relapse-free survival. Correlation of *INDO* expression to relevant known prognostic factors and survival identified high *INDO* expression as a strong negative independent predicting variable for overall and relapse-free survival. Inhibition of indoleamine 2,3-dioxygenase expressed by myeloid leukemic blasts may result in breaking immune tolerance and offers new therapeutic options for patients with acute myeloid leukemia.

Key words: indoleamine 2,3-dioxygenase, *INDO*, acute myeloid leukemia, immunesurveillance, immune-therapy.

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Introduction

Successful immunotherapy requires not only the development of effector T cells, but also the break-through of the local state of immune tolerance that tumors can create. Besides several cellular and soluble factors (like the presence of regulatory T cells and transforming growth factor β),[1] overexpression of the enzyme indoleamine 2,3-dioxygenase can induce tolerance. Indoleamine 2,3-dioxygenase degrades the essential amino acid tryptophan into kynurenine. High levels of indoleamine 2,3-dioxygenase result in immune suppression because T cells undergo cell cycle arrest in G1 phase at low tryptophan levels.[2] Moreover, kynurenine and its derivates are directly toxic for T cells.[3] Biologically relevant indoleamine 2,3-dioxygenase to limit T-cell activation[4] is expressed by interferon-α stimulated antigen presenting cells, in lower intestinal epithelial cells in which non-pathogenic bacteria are frequently present, and in trophoblast cells in which it protects the fetus from attack by maternal cytotoxic T cells.[5]

Tumor induced overexpression of indoleamine 2,3-dioxygenase causes immune-suppression at two levels. First, inhibition of effective T-cell *priming* by antigen presenting cell derived indoleamine 2,3-dioxygenase has been demonstrated in tumor-draining lymph nodes.[6] Second, the effector phase of an anti-tumor immune response is hampered because many human solid tumors themselves express indoleamine 2,3-dioxygenase.[7-9]

In acute myeloid leukemia, acquired mutations of the hematopoietic stem cells block differentiation. The result is accumulation of immature cells in the bone marrow and peripheral blood, often accompanied by suppression of the normal blood cells. With chemotherapy and stem cell transplantation about 70% of patients achieve complete remission but approximately half of these patients relapse from the status of minimal residual disease.[10] The immune system as surveillant in acute myeloid leukemia is unlikely to play a role at the moment of diagnosis (when an enormous tumor burden exists) but this is more likely during the period of minimal residual disease (when the patient has achieved morphological complete remission). We hypothesize that, in this phase of the disease, overexpression of indoleamine 2,3-dioxygenase by myeloid leukemic blasts could hamper immunesurveillance resulting in immune escape and shortened relapse-free and overall survival. With regard to hematologic malignancies, Curti *et al*. reported active indoleamine 2,3-dioxygenase protein in 52% of acute myeloid leukemic samples.[11] They

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also clearly demonstrated that indoleamine 2,3-dioxygenase expressed by human myeloid leukemic cells induces the expansion of $CD4^+CD25^+FOXP3^+$ regulatory T cells in mice.[12] These data presume an important role for indoleamine 2,3-dioxygenase in immune escape. However, negative influence of high indoleamine 2,3-dioxygenase expression on overall survival of patients has only been demonstrated for endometrial and colorectal cancer.[8,9] In the present study we investigated whether the expression of *INDO* (the gene encoding for indoleamine 2,3-dioxygenase) correlates to clinical outcome of patients with acute myeloid leukemia. We determined *INDO* expression in myeloid leukemic blasts of 285 patients by gene-expression analyses and in 71 patients by real-time quantitative polymerase chain reaction (qPCR) and correlated these data to relevant known prognostic factors and survival.

Design and Methods

Patients' characteristics

After informed consent and according to the recommendations as defined in the declaration of Helsinki in 1989, bone marrow derived samples were collected from patients with primary acute myeloid leukemia. Patients were classified according to the French-American-British (FAB) classification.[13] Clinical characteristics of the patients that were selected for gene-expression profiling analysis have been previously described[14] and shown in Table 1. For patients analyzed by qPCR, clinical characteristics are comparable to the clinical characteristics of patients that were analyzed by gene-expression profiling (Table 1).

Cytogenetic risk group was defined as favorable (t(8;21), inv(16) or t(15;17)), standard (neither favorable nor adverse) or adverse (complex karyotype, -5, -7, del(5q), del(7q), abn (3q), t(6;9) or abn (11q23)).

Patients of both groups received therapy according to HOVON (Dutch-Belgian Hematology-Oncology Co-operative Group) protocols. Most patients received 2 cycles of chemotherapy (containing cytarabin combined with idarubicin or amsacrine) followed by an autologous stem cell transplantation or a third cycle of chemotherapy (mitoxantrone and etoposide). Only 3 patients received allogeneic stem cell transplantation. Protocols are available at *www.hovon.nl*. Overall survival was defined as the time period from inclusion to death or last date of follow-up; relapse-free survival was defined as the time period from achievement of complete remission to relapse.

Sample characteristics

Blood and bone marrow derived mononuclear cell fractions from patients with primary acute myeloid leukemia were obtained by Ficoll centrifugation (purity of blasts >80%), cryopreserved and used for gene-expression profiling. For qPCR analysis, $7AAD^-$/AnnexinV^-/$CD45^{dim}$ cells from patients with primary acute myeloid leukemia were FACS sorted after thawing (purity of blasts >95%). For comparison, bone marrow from 5 healthy donors was withdrawn and $7AAD^-$/AnnexinV^-/$CD45^{dim}$ normal blasts (purity >95%) were FACS sorted.

Table 1. Comparison of clinical characteristics of patients with acute myeloid leukemia whose blasts were analyzed for INDO expression by gene-expression profiling and by qPCR.

Type of analysis	*Gene-expression profiling*	*qPCR*
Number	286	71
Median age in years at diagnosis (range)	44 (15-78)	54 (16-75)
Median white blood cell count at diagnosis ($10\times^9$/L, range)	28 (0.3-582)	42 (0.4-282)
Complete remission rate, number (%)	203 (79.6)	55 (78)
Median overall survival in months (range)	13 (0-166)	14.4 (0.03-174)
Median relapse free survival in months (range)	11 (0-166)	16.6 (0.23-173)
FAB classification, number (%)		
AML M0	6 (2)	4 (6)
AML M1	63 (22)	9 (13)
AML M2	66 (23)	20 (19)
AML M3	19 (7)	3 (4)
AML M4	53 (19)	12 (17)
AML M5	65 (23)	20 (28)
AML M6	3 (1)	3 (4)
not determined	10 (3)	
Cytogenetic risk group, number (%)		
Favorable	57 (20)	4 (6)
Standard	176 (62)	40 (56)
Adverse	39 (14)	6(9)
No metaphasis	13 (4)	6 (9)
Not done		15 (21)
FLT3 status, number (%)		
FLT3 ITD pos	78 (27)	19 (27)
FLT3 ITD neg	207 (73)	52 (73)
FLT3 TKD pos	33 (12)	5 (7)
FLT3 TKD neg	252 (88)	66 (93)

Gene-expression profiling and real-time qPCR

Gene-expression levels for *INDO* were determined by using Affymetrix U133A GeneChips[14] (Affymetrix-id for *INDO* 210029_at, M34455 NCBI). For qPCR, RNA was isolated using RNA-Bee solution (Tel-Test Inc, Friendswood, TX, USA). Total RNA was stored at -80°C. cDNA synthesis was performed according to a standardized Europe Against Cancer protocol.[15] PCR amplification was performed with a LightCycler real-time PCR machine (Roche Diagnostics, Almere, the Netherlands). Reaction volumes were 20 μL, consisting of 2 μL cDNA, 2 μL of LightCycler Fast Start DNA SYBR Green Mastermix (Roche) and 0.5 μM reverse and forward primers. $MgCl_2$ was added to a final concentration of 3.5 μM (for *GUS* 4 μM). qPCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 45 cycles, each for 15 sec at 95°C, 10 sec at 58°C (for *GUS* 59°C) and 10 sec at 72°C. Relative quantitation of gene-expression was determined using the LB1610 cell line (*see Acknowledgments*). All results were normalized with respect to the β-glucuronidase (*GUS*)

control. Primer sequences were: *INDO* forward: 5'-GTGTTTCACCAAATCCACGA-3', reverse: 5'-CTG-ATAGCTGGGGGTTGC-3'; *GUS* forward: 5'-GAA-AATATGTGGTTGGAGAGCTCATT-3', reverse: 5'-CCGAGTGAAGATCCCCTTTTTA-3 (Biolegio BV, (Nijmegen, the Netherlands).

Western blot

For Western Blot analysis, human peripheral blood derived monocytes selected by magnetic separation (positive selection with CD14 microbeads, Miltenyi Biotec, Bergisch Gladbach, Germany) were used. Purity was greater than 97%. Monocytes were incubated for 18 hours with interferon-α. Snap frozen cells were prepared by resuspending in lysis buffer (50 mM Tris-HCl (pH 7.6), 5 mM dithiotreitol, 20 μL PIC (Protease Inhibitor Cocktail; 1 tablet/mL H_2O), 20% glycerol and 0.5% NP-40). Protein content of the supernatant was determined by the Bio-Rad protein assay. Twenty micrograms of total cell lysates were fractionated on a 12.5% polyacrylamide gel containing SDS and transferred onto a PVDF membrane. Indoleamine 2,3-dioxygenase was visualized by luminescence (ECL Kit, Amersham) using Hyperfilm ECL (Amersham) after incubation with anti-human indoleamine 2,3-dioxygenase antibody (clone 10.1, Chemicon, 1:1000) and horseradish peroxidase–coupled rabbit anti-mouse IgG. α-tubulin was detected by incubation with a mouse anti-human α-tubulin monoclonal antibody (Sc-5286 Santa Cruz) by luminescence as above.

Statistical analysis

Statistical analyses were conducted using a SPSS 12.0.1 software program. To determine associations between variables, Spearman's correlation coefficient was used. Differences between patients' characteristics were analyzed with the Mann-Whitney U test. For survival data, Kaplan-Meier curves were compared by means of the log-rank test. To explore the simultaneous effect of several variables on overall and relapse-free survival the Cox regression model was used.

Results and Discussion

Two hundred and eighty-six patients were analyzed for the expression of *INDO* by gene-expression profiling. *INDO* mRNA expression was variable and not significantly associated with any one of the 16 subtypes of acute myeloid leukemia, which were identified on their specific gene-expression signatures,[14] presuming independency of these prognostic clusters (Figure 1A). Moreover, no significant differences were found between INDO mRNA expression of different FAB subtypes and cytogenetic risk profiles. Correlation of *INDO* expression to survival data of 262 patients revealed that all patients with prolonged overall survival had low *INDO* expression. We decided to validate these data by qPCR. Correlation between microarray and qPCR is generally strong, although there is non-concordance in 13-16% of genes.[16] *INDO* mRNA expression levels relative to *GUS* by qPCR nicely correlated to protein levels as determined by Western blot on human peripheral blood interferon-α stimulated monocytes (Figure 1B). We then determined *INDO* mRNA expression levels in 71 patients and 5 healthy donors. *INDO* expression relative to *GUS* in blasts of healthy donors was low (mean 0.538, median 0.32, range 0.06-1.18). *INDO* expression relative to *GUS* in blasts of patients with acute myeloid leukemia was highly variable, but mean and median expression was higher (mean 5.68, median 0.51, range 0-304) than *INDO* expression in blasts of healthy donors.

As in the patient group analyzed by gene-expression profiling, no significant differences in *INDO* expression analyzed by qPCR were found between FAB subtypes and cytogenetic risk groups. As the white blood cell count in this patient group was high (median 42×10^9/L) and *FLT3* (fms-related tyrosine kinase 3) mutations are frequently found in patients with a high white blood cell count, we also analyzed *FLT3* internal tandem duplication (*ITD*) and tyrosine kinase domain (*TKD*) mutations, but *INDO* expression was not significantly different in patients with the mutations of *FLT3* (mean *INDO* expression: *FLT3-ITD* positive patients 11.5, *FLT3-ITD* negative patients 11.6).

When correlating *INDO* expression levels to clinical outcome, patients with prolonged overall survival had low *INDO* levels (Figure 1C). As the maximum *INDO* level in healthy donors was 1.2 relative to *GUS*, we divided the patients into 2 groups; those having *INDO* greater than 1.2 (n=15) or lower than or equal to 1.2 (n=56). There was no difference in median age and white blood cell counts at diagnosis between these groups. However, patients with *INDO* level > 1.2 had a significantly decreased overall survival as compared to patients with *INDO* level lower than 1.2 [7.4 months *vs.* 21.4 months respectively, p=0.01 (Mann-Whitney U)]. Also, relapse-free survival was significantly shortened in patients with *INDO* level gretear than 1.2 as compared to patients with *INDO* level lower than 1.2 (6.1 months *vs.* 24.5 months respectively, p=0.025 (Mann-Whitney U)). Note that other cut-off levels such as 0.5 (median of healthy controls) or 1.5 resulted in comparable significant results. *INDO* expression more or less than 1.2 resulted in strongly diverging Kaplan Meier survival curves for overall and relapse-free survival (Figures 1D and 1E).

In a univariate regression model, *INDO* expression level greater than 1.2 was the strongest predictor for overall survival (p<0.001 odds ratio 3.2), as compared to white blood cell count (p=0.038, odds ratio 1.004), age (p=0.012, odds ratio 1.019) and *FLT3-ITD* mutation (p=0.058, odds ratio 1.8). In a multivariate regression model with the same variables, *INDO* expression level greater than 1.2 remained the strongest independent factor predicting survival (p=0.001 vs. p=0.29 (white blood cell count), p=0.005 (age) and p=0.055 (*FLT3-ITD* mutation).

As the groups of patients with good and poor cytogenetic risk profile were too small to use in a multiple regression model, the group with an intermediate cytogenetic risk profile was analyzed (n=40). Also in this group *INDO* expression was a strong predictor for sur-

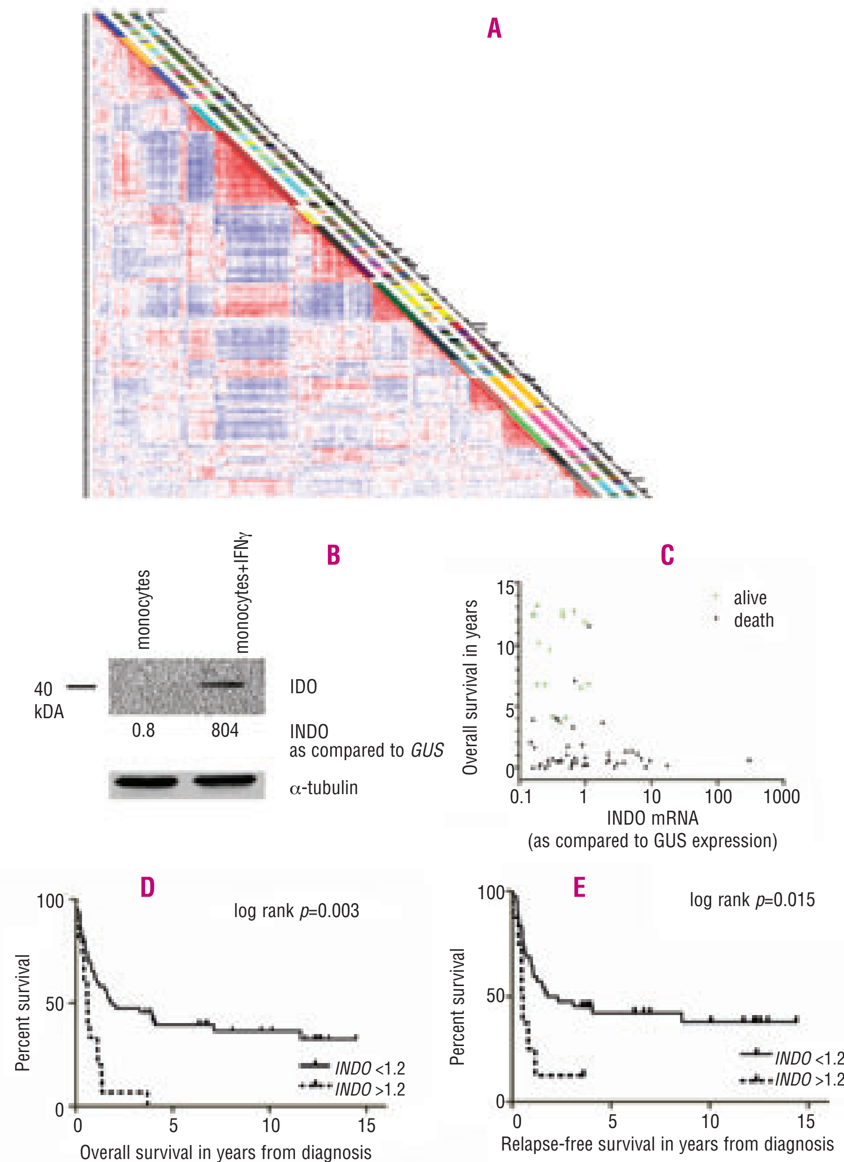

Figure 1. (A) *INDO* expression by microarray and by qPCR correlated to clinical outcome. Unsupervised cluster analyses on the basis of gene-expression profiles of 285 blasts of patients with acute myeloid leukemia. The columns adjacent to the heatmap indicate sample characteristics (column 1: the 16 gene-expression cluster (distinct colors 1-16); column 2: FAB (M0-red, M1-green, M2-purple, M3-orange, M4-yellow, M5-blue, M6-grey); column 3: karyotype (normal-green, inv(16)-yellow, t(8;21)-purple, t(15;17)-orange, 11q23 abnormalities-blue, 7(q) abnormalities-red, +8-pink, complex-black, other-gray)); column 4: *INDO* mRNA expression (probe set ID 210029_at, size of histogram proportional to level of expression). *INDO* expression does not correlate to one of the 16 previously described gene-expression subgroups,[14] the FAB classification or karyotype of the acute myeloid leukemia cases. (B) *INDO* mRNA expression levels (relative to *GUS*) by qPCR nicely correlated to protein levels of indoleamine 2,3-dioxygenase (IDO) as determined by Western Blot on human peripheral blood interferon-γ (IFN-γ) stimulated monocytes. (C) *INDO* expression analyzed by qPCR in 71 patients with acute myeloid leukemia. Patients with prolonged overall survival have low *INDO* expression level. D. and E. *INDO* expression levels analyzed by qPCR; *INDO* expression >1.2 (relative to *GUS*) correlates to significantly shortened overall and relapse-free survival (x- axis is log transformed).

vival (p=0.009, odds ratio 1.146). Myeloid leukemic blasts do express an active form of indoleamine 2,3-dioxygenase protein as previously demonstrated in human myeloid leukemic samples and *in vivo* in mice.[11,12] Moreover, Curti *et al*. demonstrated good correlation between *INDO* mRNA level, indoleamine 2,3-dioxygenase protein expression levels and function in myeloid leukemic samples.

Our findings show that all patients with prolonged overall survival had low *INDO* expression levels and that high *INDO* expression levels were a strong negative predicting variable for worse outcome of patients with acute myeloid leukemia. In a multivariate regression model, a high *INDO* expression level was a stronger predicting variable than white blood cell count, age and *FLT3-ITD* mutational status.

High *INDO* expression was also significantly correlated to a lower complete remission rate (53% for patients with *INDO* expression level greater than 1.2 (p=0.01 (Mann-Whitney U) versus 84% for patients with *INDO* expression level lower than 1.2, p=0.01 (Mann-Whitney U)). This was surprising as it is not expected that the amount of indoleamine 2,3-dioxygenase in blasts is affecting drug sensitivity. In this study, we cannot exclude that high *INDO* expression is a part of a more complex phenotype associated with chemoresistance. However, when only analyzing patients that achieved complete remission, high *INDO* expression was correlated to shortened relapse-free survival.

In the treatment of patients with acute myeloid leukemia, there is a need for additional therapies to prevent relapse in patients that have achieved complete remission. The success of allogeneic stem cell transplantation has demonstrated clearly that immune surveillance plays a role by eradicating minimal residual leukemic cells resulting in prolonged complete remission rates for patients with acute myeloid leukemia. However, allogeneic stem cell transplantation is not available for many patients. For these patients, rehabilitation of autologous immune surveillance, for example

by inhibition of tumor induced indoleamine 2,3-dioxygenase expression could offer new treatment possibilities. As inhibition of indoleamine 2,3-dioxygenase by orally available inhibitors like 1-methyl–tryptophan is effective in mice and synergistic with chemotherapy,[17,18] we now provide further reason for rapid exploration of the introduction of indoleamine 2,3-dioxygenase inhibition in the treatment of patients with acute myeloid leukemia. Besides these opportunities for new immune modulating therapies, measuring *INDO* levels provides useful prognostic information, as a high *INDO* expression level is correlated to a lower complete remission rate, and shorter overall and relapse-free survival of patients with acute myeloid leukemia.

Authorship and Disclosures

MC performed experiments, analyzed results and wrote the paper; AvdL designed research; CH collected samples; JJ performed experiments and analyzed results; AZ performed experiments; RD and PV performed experiments and analyzed results; BL and GO designed research. The authors reported no potential conflicts of interest.

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