

The role of plasma IDO activity as a diagnostic marker of patients with colorectal cancer

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Abstract High levels of indoleamine 2,3-dioxygenase (IDO) are involved in tumour escape mechanisms. The aim of this study is the evaluation of L-kynurenine of plasma as marker of diagnostic and prognostic in patients with colorectal cancer. The study included 78 patients with colorectal cancer, of whom 15 % were in stage I/II, 30 % in stage III, and 55 % in stage IV, and was compared with a control group of 70 healthy subjects. The receiver operating characteristic (ROC) curve analysis showed an area under the curve of 0.917, with a specificity of 100 % and with a sensitivity to detect cancer of the colon of 85.2 %, taking 1.83 μM as a cut-off point. The overall survival analysis also indicated that patients with low levels of L-kynurenine in plasma increased survival rate after 45 months of follow-up ($P = 0.032$). These results show that the plasma levels of L-kynurenine could be a good biomarker to differentiate individuals with colorectal cancer from healthy individuals.

Keywords Biomarker · Colorectal cancer · Indoleamine 2,3-dioxygenase · L-Kynurenine

Introduction

The early detection of colorectal cancer (CRC) is one of the great challenges in combating this disease. There are almost 1 million new cases every year all over the world, and almost half a million die of CRC [1]. The disease is asymptomatic in the initial stages in the majority of cases; around 45 % of cases are detected in an advanced phase. Thus, along with the advances in treatment, the development of new tools to diagnose CRC in the early stages could lead to a great reduction in the mortality due to CRC.

One of the current objectives in the field of cancer is the study of biomarkers for the early detection of colorectal cancer. However, the biomarkers used generally have a low sensitivity and specificity in the early stages of the disease [2, 3]. Thus, a great variety of markers, such as CEA, CA 19.9, MSI, KRAS, and p53, have been proposed for the diagnosis of CRC in the last few years [4–9]. Any molecule produced specifically by tumour cells, or is closely associated with these cells, would be an ideal marker for CRC [7]. Furthermore, it must be taken into account that for clinical applications blood is the body fluid of choice for the evaluation of biomarkers since its composition is normally stable.

It is currently known that the expression of immunosuppressive molecules has an influence on the prognosis, the appearance of metastasis, and the response to treatment with cytostatics. Indoleamine 2,3-dioxygenase (IDO) is one of these molecules, and is overexpressed in different types of cancer such as, gynaecological cancers, melanomas, lymphomas, leukaemia and colorectal cancer [10–16]. Furthermore, its expression by tumour cells or by immune system cells facilitates the escape from immune attack which could induce immunotolerance in other parts of the body, as well as influence the effectiveness of the

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treatment. IDO is an inducible enzyme that catalyses the first step in the tryptophan degradation pathway and gives rise to the production of N-formylkynurenine, which is rapidly metabolised to L-kynurenine [17]. Several studies have already demonstrated that IDO is expressed in cancer of the colon [15, 18]. These studies showed the potential to use the IDO activity or their metabolites (L-kynurenine) as a cancer biomarker. In this respect, recently we observed that the L-kynurenine levels are increased in patients with CRC and the treatment with monoclonal antibodies decreased L-kynurenine levels to those of the control group of healthy subjects [19].

The main objective of this study was to assess whether plasma levels of L-kynurenine may be useful as a biomarker for prognosis in colorectal cancer patients through.

Materials and methods

Subjects

This study was approved by the Clinic Research Ethics Committee of Hospital General Yagüe (Burgos, Spain) and the patients gave their informed consent to participate, after having been fully informed on the purpose of the study. The study included 78 patients with colon cancer diagnosed between 2008 and 2010 in the Oncology Unit of Hospital Yagüe in Burgos (Spain). Of these, 15 % (12/78) were classified as being in stage I/II, 30 % (24/78) were shown to be in stage III, and the remaining 55 % (43/78) were designated as stage IV. The reference diagnosis for each patient was obtained from histological analysis of surgically extracted specimens or a biopsy. The clinical pathological stages were determined according to the classification of malignant tumors by International Union against Cancer Tumor-Node-Metastasis (TNM) staging system. Seventy healthy volunteers, who were of similar age to the cancer patients and confirmed to be cancer-free through the use of clinical and imaging examinations, were taken as controls in the study.

None of patients had any preoperative chemotherapy or radiotherapy. However, after surgery, all patients were treated with various regimens of adjuvant chemotherapy. Of the 78 patients, 32 (41 %) received adjuvant chemotherapy, using a fluoropyrimidine-based regimen or/and starting 4 weeks after surgery. Capecitabine was administered to 8 patients, FOLFIRI (5-FU/leucovorin/irinotecan) to 1, FOLFOX (5-FU/leucovorin/oxaliplatin) to 5 and XELOX (capecitabine/oxaliplatin) to 18 patients. Thirty-two (41 %) patients were treated using a fluoropyrimidine plus monoclonal antibodies (bevacizumab or cetuximab) based regimen. The remaining 14 patients

(18 %) had not received chemotherapy at the time of sample collection.

All patients who had similar adjuvant treatment after surgery were available for follow up for a period of 120 months or until death. The mean follow-up time was 67.5 months (range 4.6–80.4 months), and during that time, there were 31 cancer-related deaths (38.7 %).

Indoleamine 2,3-dioxygenase activity assay (IDO)

Heparinized blood samples were obtained and then centrifuged at $800 \times g$ 10 min and the plasma was frozen at -80°C .

IDO activity was evaluated by measuring L-kynurenine in the plasma according to Alegre et al. [20]. Briefly, 100 μL of trichloroacetic acid (30 %, w/v) were added to 100 μL of sample to precipitate proteins, boiled for 10 min and centrifuged at $2,500 \times g$ for 10 min. L-kynurenine in the supernatant was measured in an HPLC system with a Waters C18 column (4.5 mm \times 15 cm). The mobile phase was 15 mM acetic acid–sodium acetate (pH 4.0) containing 27 mL/L acetonitrile, and kynurenine was detected by absorbance at 360 nm. The inter-assay coefficient variations of the method were 2.8 % and intra-class coefficient correlation (ICC) = 0.955 IC 95 % (0.937–0.969).

Tumor markers assays

Serum CEA and CA19-9 were measured using the electrochemiluminescence immunoassay on Modular Analytics E170 immunoassay analyser (Roche Diagnostics, Mannheim, Germany). The recommended cut-off levels of serum CEA and CA19-9 were 5 ng/mL and 37 U/mL, respectively. These assays were done by the clinical analyses service of the Burgos University Hospital.

Statistical analysis

Statistical analysis was performed using the SPSS software package (IBM SPSS Statistics 19). The samples were analyzed by triplicate. The Pearson Chi square test and Student's *t* test were used to determine the association between L-kynurenine levels and various clinicopathological parameters. Differences in L-kynurenine levels between cancer patients and healthy controls were compared using one-way analysis of variance (ANOVA). ROC curve analysis was employed to assess the feasibility of using L-kynurenine levels as a diagnostic tool. The Kaplan–Meier method was used to estimate the overall survival rate as a function of time. The differences between survival curves were assessed using the log-rank test. Values of $P < 0.05$ were considered significant.

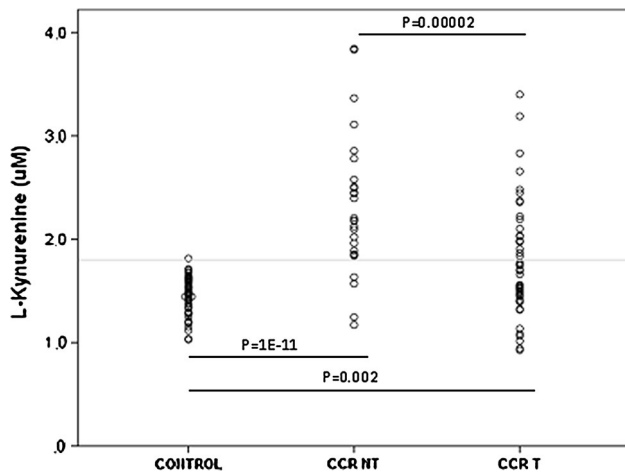


Fig. 1 Scatter plot indicates values of L-kynurenine in healthy controls and patients with colon cancer non treated (CCR NT) and treated (CCR T). Subgroup analysis revealed significant differences ($P < 0.05$) between controls and colon cancer patients non treated and treated. Cut off value for L-kynurenine levels is $1.827 \mu\text{M}$ (represented by line across each graph)

Table 1 Correlation between plasma IDO status and clinicopathological parameters of patients with colorectal cancer

Clinical parameters	No. of patients (%)	Kynurenine (μM)	P value
Age (years)			
≤67	39 (50)	1.814 ± 0.710	0.186
>67	39 (50)	2.035 ± 0.750	
Gender			
Male	54 (69)	1.844 ± 0.701	0.146
Female	24 (31)	2.106 ± 0.787	
Histological grade			
Well	9 (11)	2.404 ± 1.033	0.129
Moderate	59 (76)	1.916 ± 0.678	
Poor	10 (13)	1.714 ± 0.729	
Disease stage			
I/II	11 (14)	1.953 ± 0.619	0.317
III	24 (31)	2.102 ± 0.694	
IV	43 (55)	1.818 ± 0.776	
Metastases			
No	34 (44)	2.090 ± 0.625	0.200
Yes	44 (56)	1.831 ± 0.771	
Previous chemotherapy			
No	50 (64)	1.933 ± 0.745	0.900
Yes	28 (36)	1.911 ± 0.728	
CEA			
≤5 ng/mL	44 (56)	1.895 ± 0.681	0.640
>5 ng/mL	34 (44)	1.974 ± 0.816	
CA 19.9			
≤37 U/L	50 (65)	1.975 ± 0.688	0.349
>37 U/L	28 (36)	1.809 ± 0.819	

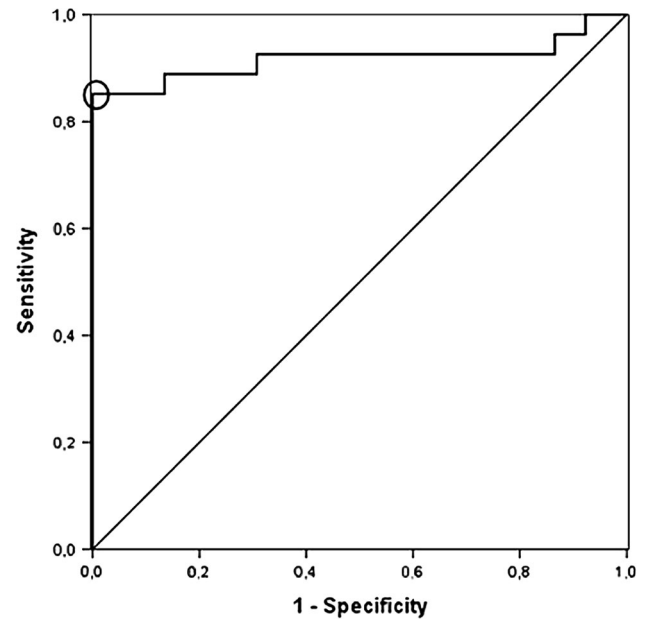


Fig. 2 ROC curve analysis for detection of colorectal cancer using measurement of kynurenine levels in plasma. The circle point was optimal cut-off value of IDO. The area under the ROC curve was 0.917 (95 % CI 0.827–1.000, $P < 0.001$)

Results

IDO activity was determined by measuring the plasma concentration of L-kynurenine in patients with cancer of the colon and the results were compared with those of healthy controls (Fig. 1). Significant differences were observed on comparing the patients with cancer with the control group of healthy individuals ($P < 0.005$). A significant difference was also observed in the L-kynurenine levels between treated and untreated patients with cancer of the colon ($P < 0.005$). To evaluate the role in cancer of the colon, the relationship was analysed between the L-kynurenine plasma concentrations and the clinical-pathological parameters such as, age, gender, and histological grade, stage of the disease, and the presence/absence of metastasis (Table 1). No significant relationship was found between the plasma L-kynurenine levels and the clinical-pathological parameters.

The analysis of the ROC curve between the control cases and the cases with cancer, obtaining an area under the curve of 0.917, is shown in Fig. 2. With a specificity of 100 %, the highest sensitivity achieved in the detection of cancer of the colon was 85.2 % with a cut-off value of $1.827 \mu\text{M}$ L-kynurenine.

With the aim of studying the influence of the L-kynurenine levels on the overall survival time, the Kaplan–Meier curves were plotted along with the log-rank test (Fig. 3). The results of the analysis showed a longer overall survival for patients with L-kynurenine levels $<1.827 \mu\text{M}$ (median survival: 80.4 months, $n = 36$) than those with

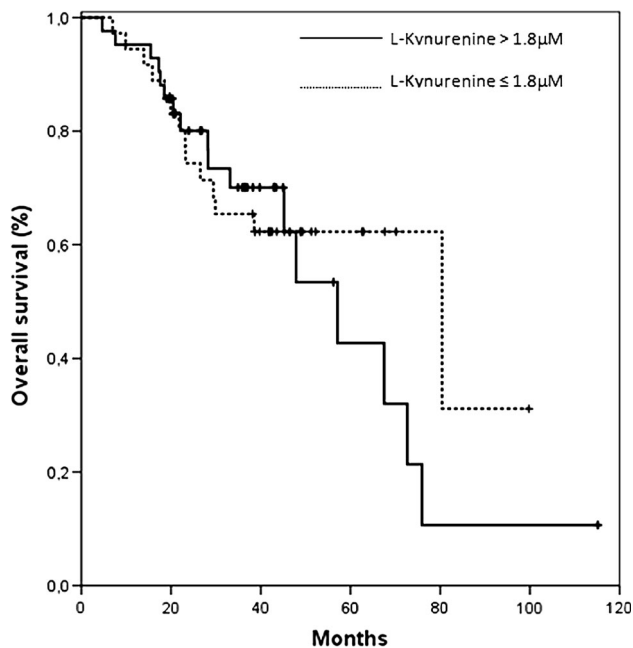


Fig. 3 Kaplan–Meier estimates of the overall survival for colon cancer patients. Patients were stratified according to the plasma levels of kynurenine, $P = 0.606$

higher levels (median survival: 57.1 months, $n = 42$), although the difference was not statistically significant ($P = 0.606$). However, when the data were divided into two parts from the crossover point obtained at 45 months a statistically significant difference was observed ($P = 0.032$) between the groups after 45 months.

Discussion

IDO appears to play a central role in the development of immunodeficiency and immunotolerance in tumours. The expression of IDO by tumour cells has been proposed as an effective tumour immuneescape strategy and an intrinsic mechanism of immune resistance of the tumour [16, 21], and is expressed by a variety of neoplasms [10, 12, 13, 18, 22, 23], thus indicating its possible use as a biomarker for the diagnosis, or for predicting the clinical results, of these diseases. IDO activity in patients with cancer is measured by determining the L-kynurenine levels in serum or tissue by high pressure liquid chromatography [24].

Recent review [25] shows that the high expression of IDO by the neoplastic epithelium correlates with poor prognosis in human colon cancer. Brandacher et al. [15] observed that the increase in the expression of the IDO protein in tissues with cancer of the colon significantly correlated with a poor prognosis, which indicated that the IDO expression in tumours could become an important prognostic variable. More recently, Gao et al. [18] detected

the presence of IDO in the cytoplasm of cancer and in normal epithelial cells in 41 cases of primary tumour of the colon and in the lymph nodes. These authors observed differences in the expression of IDO when they compared lymph glands with metastasis (low expression) and without metastasis (high expression).

Many biomarkers in blood have been investigated and proposed for the detection of colorectal cancer. Since s-CEA was described for the first time, it has become the most widely accepted and used marker all over the world to evaluate patients with colorectal cancer, despite it only having a sensitivity of 30–40 % for tumours in the early stages [26, 27]. Therefore, it is necessary to find biomarkers for the prognostic and progression of the disease.

The importance of the use for clinical diagnosis of the L-kynurenine levels is that as the analysis is performed on peripheral blood, it would appear to be an ideal source for the follow up of the progression of the disease, since the taking of specimens is relatively painless. It is known that L-kynurenine levels in the serum or plasma can be affected by different physiological and pathological factors such as, pregnancy, organ transplants, and autoimmune diseases or viral infections [24]. Ciorba [25] indicate that the serum kynurenine:tryptophan is elevated in colon cancer, suggesting this measurement may prove useful as a disease biomarker. Recently, we observed that the L-kynurenine levels in patients treated with chemotherapy are significantly lower than those patients not treated.

When the analysis on the possible use of L-kynurenine levels as a biomarker through the study of the ROC curves showed an AUC of 91.7 % at an L-kynurenine concentration of 1.83 μM , this demonstrated that the L-kynurenine levels could be a useful indicator to distinguish colorectal cancer from healthy subjects. Yoshikawa et al. [28] have already proposed that plasma L-kynurenine levels could serve as a prognostic factor in patients with diffuse large B-cell lymphoma, and established the cut-off point at 1.5 μM of L-kynurenine.

The analysis of the Kaplan–Meier showed no difference between the survival function of the two groups. However, the log-rank test could detect a difference between groups when the risk of an event is consistently greater for one group than another [29]. In our results we observed that the curves cross at 45 months. If there is a crossing problem, for check the difference we split up the data in two parts from the crossing point. The results according to the log-rank test results in a statistically significant difference between the groups after 45 month. Therefore, the curve showed a prognostic significance for L-kynurenine levels after 45 months. These results are similar to those obtained in the study by Brandacher et al. [15], who observed that the tumours that expressed high IDO levels were significantly associated with a poor prognosis after the first

45 months. In conclusion, in our study, the L-kynurenine levels in the plasma could be a novel non-invasive method to distinguish between colorectal cancer and healthy subjects.

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Conflict of interest The authors have no conflict of interest to report.

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