Quantitative expression analysis and prognostic significance of L-DOPA decarboxylase in colorectal adenocarcinoma

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BACKGROUND: L-DOPA decarboxylase (DDC) is an enzyme that catalyses, mainly, the decarboxylation of L-DOPA to dopamine and was found to be involved in many malignancies. The aim of this study was to investigate the mRNA expression levels of the DDC gene and to evaluate its clinical utility in tissues with colorectal adenocarcinoma.

METHODS: Total RNA was isolated from colorectal adenocarcinoma tissues of 95 patients. After having tested RNA quality, we prepared cDNA by reverse transcription. Highly sensitive quantitative real-time PCR method for DDC mRNA quantification was developed using the SYBR Green chemistry. GAPDH served as a housekeeping gene. Relative quantification analysis was performed using the comparative Ct method (2^(-ΔΔCt)).

RESULTS: DDC mRNA expression varied remarkably among colorectal tumours examined in this study. High DDC mRNA expression levels were found in well-differentiated and Dukes’ stage A and B tumours. Kaplan–Meier survival curves showed that patients with DDC-positive tumours have significantly longer disease-free survival (P = 0.009) and overall survival (P = 0.027). In Cox regression analysis of the entire cohort of patients, negative DDC proved to be a significant predictor of reduced disease-free (P = 0.021) and overall survival (P = 0.047).

CONCLUSIONS: The results of the study suggest that DDC mRNA expression may be regarded as a novel potential tissue biomarker in colorectal adenocarcinoma.

Keywords: colorectal adenocarcinoma; L-DOPA decarboxylase; DDC; real-time PCR; tumour biomarkers

Colorectal carcinoma (CRC) is the third most common malignant tumour and the fourth most common cause of cancer death in the world (Parkin et al., 2001a; Jemal et al., 2009). The prognosis of patients with CRC largely depends on the degree of penetration of the tumour through the bowel wall, the nodal status, and the presence or absence of distal metastases (Steinberg et al., 1986). These three characteristics are the key features of all clinical staging systems developed for this disease (Compton and Greene, 2004).

In spite of the fact that clinicopathological staging separates patients with CRC into groups with distinct outcomes, it provides little information about response to treatment in individual patients (Walther et al., 2009). In an attempt to refine prognostication and predict the benefit derived from systemic treatment, several protein and genetic markers have been evaluated in patients with CRC, including allelic loss of chromosome 18q (Jen et al., 1994; Martinez-Lopez et al., 1998; Ogunibi et al., 1998; Popat and Houlston, 2005), absence of the deleted in colorectal carcinoma (DCC) protein (Shibata et al., 1996; Reymond et al., 1998; Popat and Houlston, 2005), decreased SMAD4 mRNA expression (Boulay et al., 2002; Alazzouzi et al., 2005), expression and/or abnormalities of cytoplasmic oncoprotein p53 (TP53) (Sun et al., 1992; Munro et al., 2005; Russo et al., 2005), protein levels and/or gene haplotype of thymidylate synthetase (TYMS) (Popat et al., 2004; Suh et al., 2005; Tsourellis et al., 2008), microsatellite instability (MSI) (Gryfe et al., 2000; Popat et al., 2005), and chromosomal instability (CIN) (Walther et al., 2008). Nevertheless, none of these biomarkers has been prospectively validated and established so far in clinical practice. Hence, the identification of new, reliable prognostic and predictive biomarkers that will contribute utmost to clinical decision-making, remains an important research topic (Locke et al., 2006; Walther et al., 2009).

L-DOPA decarboxylase (DDC) is a pyridoxal 5-phosphate (PLP)-dependent enzyme that catalyses the decarboxylation of 3,4-dihydroxy-L-phenylalanine (L-DOPA) to dopamine (DA) and 5-hydroxy-L-tryptophan (5-HTP) to serotonin (5-HT) (Christenson et al., 1972). DCC is expressed in the central nervous system as well as in peripheral organs, such as the liver, kidney, pancreas and placenta (Lindstrom and Sehlin, 1983; Maneckjee and Baylin, 1983; Ichinose et al., 1989; Mappouras et al., 1990; Sieri et al., 2003). Moreover, enzymatically active DDC was recently found in human leukocytes as well as in the histiocytic lymphoma cell line U-937, thus suggesting a cross-talk between the nervous and the immune systems and raising new questions about the regulatory role of DDC in immune responses (Kokkou et al., 2009a, b). Recently, two endogenous inhibitors of the enzymatic activity of DDC have...
been identified and purified, one from human serum and another from the membrane fraction of human placental tissue, yet their biological significance remains unexplored (Vassiliou et al, 2005, 2009).

The structure of the human DDC gene has been fully determined. The single-copy gene encoding DDC maps to chromosome 7p12.2, close to the epidermal growth factor receptor (EGFR) gene, and is composed of 15 exons spanning a genomic region of more than 85 kb (Ichinose et al., 1989; Sumi-Ichinose et al., 1992). Furthermore, two other DDC mRNA transcripts encoding distinct DDC protein isoforms as well as alternative splicing in 5'-untranslated region have been identified and characterised (Krieger et al., 1991; Ichinose et al., 1992; O’Malley et al., 1995; Vasileiou et al., 2004).

It is worth mentioning that DDC is regarded as a general biomarker for neuroendocrine tumours (Gazdar et al., 1988; Gilbert et al., 1995; Ippolito et al., 2005). High DDC mRNA expression has been noticed in small-cell lung carcinoma (SCLC), neuroblastoma, and pheochromocytoma (Jensen et al., 1990; Gilbert et al., 1999; Uccella et al., 2006). Moreover, it has been suggested that DDC mRNA levels could be a potential biomarker for the detection of minimal residual disease in patients with neuroblastoma and for the discrimination of neuroblastoma from other small round-cell tumours of childhood (Gilbert et al., 1999; Bozzi et al., 2004).

Recent studies revealed that DDC is also implicated in prostate cancer neuroendocrine differentiation, accounting for abnormal prostate cell proliferation and differentiation (Wafa et al., 2007), as it is an androgen receptor (AR) coregulator protein acting at the cytoplasmic level to enhance AR activity and to differentially modulate AR-regulated genes (Wafa et al., 2003; Margiotti et al., 2007). Not surprisingly, DDC gene expression at the mRNA level has been proposed as a novel tissue biomarker in prostate cancer (Avgeris et al., 2008). DDC is also overexpressed in peritoneal dissemination of gastric carcinoma, and its quantification has been shown to be reliable and effective for the selection of patients for adjuvant intraperitoneal chemotherapy, aiming at preventing peritoneal recurrence (Sakakura et al., 2004). Still, the role of DDC in CRC remains unclear.

The above data encouraged us to analyse the DDC mRNA expression in colorectal adenocarcinoma specimens, developing an ultra-sensitive and highly accurate quantitative real-time PCR methodology using the SYBR Green chemistry, and to examine its potential prognostic significance and clinical application as a novel molecular tissue biomarker for colorectal adenocarcinoma.

MATERIALS AND METHODS

Tissue samples and RNA isolation

Included in this study were tumour specimens from 95 patients having undergone surgical treatment for primary colorectal adenocarcinoma between 2000 and 2003. The selection criteria for the specimens included the availability of sufficient tissue mass for RNA extraction and assay. Tumour tissues had been frozen in Tissue specimens were pulverised and then dissolved in TRI Reagent (Ambion (Europe) Ltd., Huntingdon, UK). Following the manufacturer’s instructions, we extracted and diluted total RNA in an RNA Storage Solution (Ambion Ltd), and stored it at −80°C until use.

Patient age ranged from 35 to 88 years with a mean ± s.e. of 67.3 ± 1.01. Other patients’ characteristics and stage of tumours are shown in Tables 1–3. Follow-up information was available for 72 patients and included survival status (alive or deceased) and disease status (disease-free or recurrence/metastasis) along with the dates of the events and cause of death.

The study was performed with respect to the ethical standards of the 1975 Declaration of Helsinki Principles, as revised in 1996, and

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**Table 1** Distribution of numerical variables of the study in 95 colorectal adenocarcinoma patients

| Variable                  | Mean ± s.e. | Range | Median | 25  | 50  | 75  |
|---------------------------|-------------|-------|--------|-----|-----|-----|
| DDC in tumours (dKc)      | 9.02 ± 1.51 | 0.04 – 91.95 | 0.17 | 3.09 | 12.82 |
| Patient age (years)       | 67.3 ± 1.01 | 35 – 88 | 62.0 | 68.0 | 75.0 |
| DFS (months)              | 36.6 ± 2.63 | 0.5 – 79.0 | 16.7 | 39.5 | 52.2 |
| OS (months)               | 38.1 ± 2.63 | 0.5 – 79.0 | 17.7 | 42.5 | 54.2 |

**Table 2** Relationships between DDC status and other clinicopathological variables

| Variable                  | Total | DDC negative* | DDC positive* | P-value |
|---------------------------|-------|---------------|---------------|---------|
| Nodal status              |       |               |               |         |
| Positive                  | 49    | 35 (71.4)     | 14 (28.6)     | 0.63*   |
| Negative                 | 41    | 32 (78.0)     | 9 (22.0)      |         |
| X                         | 5     |               |               |         |
| Stage*                    |       |               |               |         |
| A/B                       | 47    | 33 (70.2)     | 14 (29.8)     | 0.13*   |
| C/D                       | 41    | 35 (85.4)     | 6 (14.6)      |         |
| X                         | 7     |               |               |         |
| Histologic grade          |       |               |               |         |
| I                         | 6     | 2 (33.3)      | 4 (66.7)      | 0.011*  |
| II                        | 70    | 51 (72.9)     | 19 (27.1)     |         |
| III                       | 11    | 11 (100)      | 0 (0.00)      |         |
| X                         | 8     |               |               |         |

**Table 3** DDC expression and survival of patients with colorectal adenocarcinoma

| Variable                  | Disease-free survival | Overall survival |
|---------------------------|-----------------------|------------------|
|                           | HR*       | 95% CI         | P-value | HR*       | 95% CI         | P-value |
| Univariate analysis DDC   |           |               |         |           |               |         |
| Negative                  | 1.00      | 1.00           |         | 1.00      | 1.00           |         |
| Positive                  | 0.18      | 0.04 – 0.77    | 0.021  | 0.23      | 0.053 – 0.97   | 0.047  |
| As continuous variable    | 0.96      | 0.93 – 1.00    | 0.089  | 0.95      | 0.90 – 1.00    | 0.089  |
| Nodes positive            | 1.80      | 0.83 – 3.89    | 0.13   | 2.69      | 1.11 – 6.51    | 0.027  |
| Stage (ordinal)           | 2.31      | 1.42 – 3.73    | 0.001  | 2.53      | 1.48 – 4.34    | 0.001  |
| Histologic grade (ordinal)| 3.07      | 1.33 – 7.05    | 0.008  | 3.92      | 1.57 – 9.74    | 0.003  |
| Multivariate analysis DDC |           |               |         |           |               |         |
| Negative                  | 1.00      | 1.00           |         | 1.00      | 1.00           |         |
| Positive                  | 0.37      | 0.08 – 1.67    | 0.19   | 0.56      | 0.12 – 2.67    | 0.46   |
| Nodes positive            | 0.76      | 0.29 – 2.00    | 0.58   | 1.14      | 0.36 – 3.58    | 0.82   |
| Stage (ordinal)           | 2.31      | 1.30 – 4.10    | 0.004  | 2.32      | 1.14 – 4.74    | 0.021  |
| Histologic grade (ordinal)| 2.13      | 0.86 – 5.30    | 0.103  | 2.70      | 0.98 – 7.41    | 0.054  |

Abbreviations: CI = confidence interval; DDC = L-DOPA decarboxylase; HR = hazard ratio. *HR estimated from Cox proportional hazard regression model. **CI of the estimated HR. *Multivariate models were adjusted for patients’ nodal status, Dukes’ stage, and histologic tumour grade.
has been approved by the ethics committee of the University General Hospital 'Attikon'.

cDNA synthesis

First-strand cDNA was produced from total RNA by using an RNA PCR Kit Version 3.0 (TaKaRa Bio Inc., Tokyo, Japan), according to the manufacturer’s instructions. The reaction mixture contained 2 μg of total RNA diluted in 11 μl of diethylpyrocarbonate (DEPC)-treated water, 2.5 pmol Oligo dT-Adaptor primer, 2 μl RT Buffer (10 mM Tris-HCl (pH = 8.3), 500 mM KCl), 4 μl 5 mM MgCl2, 2 μl 10 mM dNTP mix, 20 U RNase inhibitor (RNase Inhibitor; TaKaRa Bio Inc.) and 1.25 U reverse transcriptase (AMV Reverse Transcriptase XL; 5 U μl–1, TaKaRa Bio Inc.). The final reaction volume was 20 μl. The reaction mixture was incubated at 30°C for 10 min, 60°C for 30 min, and the reaction was terminated by heating the mixture at 99°C for 5 min and cooling it at 5°C for 5 min.

Real-time quantitative PCR

Quantitative real-time PCR was performed using the SYBR Green chemistry in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (Figure 1A). On the basis of the information of the DDC and GAPDH cDNA sequences, two pairs of gene-specific primers were designed. The reaction mixture contained 50 ng of cDNA diluted in 2.5 μl of DEPC-treated water, 5 μl Power SYBR Green PCR Master Mix (2 μl –1) (Applied Biosystems), and 2 μl of gene-specific primers (final concentration, 50 nM each), in a final reaction volume of 10 μl. The DDC real-time PCR primers were 5'-GAACAGACTTAAAGGGAGCCTTT-3' and 5'-AATGCCGGTAGTCACTGATAAGC-3', producing a 90-bp PCR amplicon, and the GAPDH real-time PCR primers were 5'-ATGGGGAAGGTGAGGCC-3' and 5'-GGTCATTGATGGCAACAATATC-3', resulting in a 107-bp PCR amplicon. The cycling conditions were as follow: a denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, and a final step for the generation of a dissociation curve to distinguish between the main PCR product and primer-dimers (Figure 1B).

Calculations were made with the use of the comparative CT (2-ΔΔCT) method. GAPDH was used as an internal control gene to normalise the PCRs for the amount of RNA added to the reverse transcription reactions, whereas the breast adenocarcinoma epithelial cell line MCF7 was used as a calibrator for making PCRs from distinct runs comparable (Livak and Schmittgen, 2001). ΔΔCT represents the difference between the mean ΔCT value of a colon sample and the mean ΔCT of the calibrator, both calculated after the same PCR run, whereas ΔCT is the difference between the threshold cycle (CT) of the target gene (DDC) and the CT of the endogenous reference gene (GAPDH) of the same sample.

Normalised results were expressed as the ratio of DDC mRNA copies to GAPDH mRNA copies calculated for each colon tissue sample in relation to the same ratio calculated for MCF7 cells. The normalised (2-ΔΔCT) amounts of tissue sample DDC mRNA levels were then multiplied with the average ratio of DDC mRNA copies to GAPDH mRNA copies of MCF7 cells (2-13.384), calculated from the intercept of the regression line shown in Figure 1C, resulting in comparable results that do not depend on the DDC mRNA expression levels of MCF7 cells. Finally, these results were multiplied by 1000, thus yielding c/Kc (DDC mRNA copies per 1000 GAPDH mRNA copies). Each real-time PCR reaction was performed in triplicate to evaluate the reproducibility of data.

Statistical analysis

The X-tile algorithm was used to generate an optimal cut-off point for DDC (Camp et al., 2004), as there are no established cut-off points concerning its expression in colorectal adenocarcinoma.
Having corrected for the use of minimum P-value statistics, the X-tile software yielded an optimal cut-off of 12.82 c/Kc, equal to the 75th percentile, with a calculated Monte Carlo P-value < 0.05. According to this cut-off, DDC mRNA expression was classified as positive or negative, and associations between DDC status and other qualitative clinicopathological parameters were analysed using the χ²-test or the Fisher’s exact test, where appropriate.

Cox proportional hazard regression model was developed to assess the association between the prognostic markers and the relative risks for relapse and death of patients (Cox, 1972). Cox analysis was conducted at both univariate and multivariate levels. Only patients for whom the status of all variables was known were included in the multivariate regression models, which incorporated DDC mRNA expression and all other variables for which the patients were characterised. The multivariate models were adjusted for nodal status, histologic tumour grade, and Dukes’ stage (Dukes, 1932; Hamilton and Aaltonen, 2000).

Survival analyses were also performed by constructing Kaplan–Meier disease-free survival (DFS) and overall survival (OS) curves (Kaplan and Meier, 1958). The differences between the curves were evaluated by the log-rank test.

RESULTS

Validation of the comparative CT (2⁴ΔΔC_T) method for DDC mRNA quantification
For the ΔΔC_T calculation to be valid, the amplification efficiencies of the target and reference genes must be approximately equal (Livak and Schmittgen, 2001). For this purpose, the C_T values for DDC and GAPDH, corresponding to the number of cycles at which the fluorescence emission monitored in real time reached a threshold of 10 times the standard deviation of the mean baseline emission from cycles 3 to 15 (Figure 1A) (Giulietti et al., 2001), were measured in serial dilutions of a control cDNA over a 100-fold range, and the ΔC_T (namely the difference C_T,DDC–C_T,GAPDH) was plotted vs the log cDNA dilution. The absolute value of the slope of the resulting plot is close to zero, which implies similar amplification efficiencies of both amplicons (Figure 1C).

DDC expression status in colorectal adenocarcinoma tissues and its association with patients’ clinicopathological variables
mRNA expression of DDC in colorectal adenocarcinoma tissues varied from 0.04 to 91.95 c/Kc (DDC mRNA copies per 1000 GAPDH mRNA copies) with a mean ± s.e. of 9.02 ± 1.51 (Table 1).

Table 2 shows the association of DDC mRNA expression status of the tumour with various clinicopathological variables. DDC values were classified into two categories (positive or negative), as described in the Materials and Methods section. Out of 95 colon adenocarcinomas examined, 24 (25.3%) were classified as positive for DDC expression and 71 (74.7%) as negative. DDC positivity was found more frequently in well-differentiated tumours, whereas high-grade colorectal tumours were found to be DDC-negative (P = 0.011). Significant associations between DDC status and patient age, nodal status, or Dukes’ stage were not observed.

DDC expression status and colorectal adenocarcinoma survival
Complete follow-up information was available for 72 patients, among whom 27 (38%) had relapsed and 22 (31%) had died. In Cox univariate analysis, histologic tumour grade, and disease Dukes’ stage were significant predictors of DFS and OS, as expected. In addition to these established prognostic factors, DDC mRNA expression was found to be an important predictor of DFS and OS (P = 0.021 and P = 0.047, respectively). DDC-positive patients were found to have a significant lower risk to relapse (HR = 0.18) or die (HR = 0.23) (Table 3). The Kaplan–Meier survival curves also show that patients with DDC-positive tumours have remarkably longer DFS (P = 0.009) and OS (P = 0.027), compared to those with DDC-negative malignancies (Figure 2). In multivariate analysis, when all parameters were included in the Cox regression model, only Dukes’ stage retained its prognostic significance for DFS and OS of patients with colorectal adenocarcinoma (P = 0.004 and P = 0.021, respectively) (Table 3).

DISCUSSION
Cancer of the colon and rectum is an important public health issue, constituting a major cause of worldwide morbidity and mortality (Pisani et al., 2002; Jemal et al., 2009). In Europe, CRC is the second most common malignancy among women after breast cancer and the third most frequent in men after prostate and lung cancer. Moreover, CRC is the second most common cause of cancer death for both sexes, accounting for 12% of all tumour-related deaths (Parkin et al., 2001b; Ferlay et al., 2010). Early diagnosis of CRC and early detection of recurrence after surgery are critical for effective treatment and/or positive clinical outcome. Endoscopic examination of the colon remains the most reliable screening method for this type of malignancy (Newcomb et al., 1992; Lieberman et al., 2000).

Figure 2 Kaplan–Meier survival curves. Kaplan–Meier curves for disease-free survival (DFS) (A) and overall survival (OS) (B) of patients with DDC-positive and DDC-negative colorectal adenocarcinoma. DDC expression was found to have a favourable prognostic value for colorectal adenocarcinoma, as patients with DDC-positive colorectal adenocarcinoma have significantly longer DFS (P = 0.009) and OS (P = 0.027), in comparison with those whose tumours are DDC-negative.
All existing classification systems for CRC distinguish between patients with early-stage CRC and those with very advanced-stage disease; nonetheless, they are less efficient in predicting the prognosis of patients with intermediate levels of tumour burden (McLeod and Murray, 1999). On the basis of studies published over the last few years, the American Society of Clinical Oncology Tumour Marker Panel and the European Group on Tumour Markers have recently suggested that preoperative carcinomembryonic antigen (CEA) levels may be used as an independent prognostic factor, assisting in staging and surgical treatment planning. It should also be noted that CEA is the marker of choice for monitoring the response of metastatic disease to systemic therapy (Duffy et al, 2003; Locker et al, 2006). However, neither CEA nor any other biomarkers that have been proposed in the past, such as CA19-9, have enough sensitivity for colon cancer detection (van der Schouw et al, 1992; Pokorny et al, 2000; Locker et al, 2006).

1-DOPA decarboxylase is a PLP-dependent enzyme participating in the catecholamine biosynthesis pathway, responsible principally for the synthesis of the key neurotransmitters DA and 5-HT (Christenson et al, 1972). Biogenic amines are generally considered to participate in various processes, such as angiogenesis, cell proliferation, differentiation and apoptosis (Berry et al, 1996; Medina et al, 1999; Lang et al, 2005), which implies a potentially significant role of DDC in cancer pathobiology and progression. Interestingly enough, it has recently been shown that catecholamines, including DA itself, inhibit erythrocyte apoptosis by preventing scavenger receptor activation and subsequent phosphatidylserine exposure on the cell membrane (Lang et al, 2005), which in turn triggers clearance of apoptotic cells by macrophages.

DDC mRNA expression is used for the differential diagnosis of neuroblastoma from other pediatric small round-cell malignancies (Gilbert et al, 1999; Bozzi et al, 2004). Quantification of DDC mRNA expression using real-time RT-PCR has been proposed as a method useful for the prediction of peritoneal recurrence in patients with gastric carcinoma (Sakakura et al, 2004). In addition, DDC protein expression is a biomarker of neuroendocrine differentiation in SCLC cells and prostate carcinoma (Gilbert et al, 2000; Wafa et al, 2007). DDC mRNA levels were also found to be particularly elevated in cancerous prostate tissue, in comparison with benign prostate hyperplasia. High-expression levels of DDC were found to be associated with more aggressive prostate tumours (Avgeris et al, 2008).

Several peripheral cancers are characterised by an extremely high DDC activity, associated with the tumour. This is especially apparent with lung cancers of small-cell origin, although variants showing no protein expression have been observed as well (Berry et al, 1996). Remarkable increase in DDC activity, in comparison with normal tissue levels, is also seen in primary intestinal cancer and its related metastases in the spleen and liver (Gilbert et al, 1995). The significance of this increase in DDC activity and resultant monoamine synthesis by the cancer cells is still unknown (Berry et al, 1996), yet it is closely related to the implication of DDC in cancer.

In this study, we investigated the expression of the DDC gene in colorectal adenocarcinoma and its prognostic significance. Our study revealed a statistically significant, negative association between DDC mRNA expression levels and the histologic tumour grade ($P = 0.011$). Colorectal tumours of low histologic grade (I) were more frequently DDC-positive, in contrast with malignancies of high grade (II/III). These data seem to indicate that higher DDC expression is associated with well-differentiated intestinal tumours.

In accordance with the above-mentioned results, the Kaplan–Meier analysis showed significantly higher DFS and OS time for patients having positive DDC mRNA expression ($P = 0.009$ and $P = 0.027$, respectively). Cox univariate regression analysis showed that DDC-positive patients had a significantly lower risk of relapse (~5 times) and a higher probability of survival (~4 times). In the Cox multivariate regression model, the levels of DCC mRNA were adjusted for patients’ nodal status, Dukes’ stage and histologic tumour grade. When all these non-molecular parameters were included in the multivariate analysis model, DCC mRNA expression did not show statistically significant independence as a prognostic factor for DFS or OS of patients with colorectal adenocarcinoma.

In conclusion, this study revealed that higher mRNA expression levels of DDC are related with less advanced and/or aggressive tumours. Our results imply that DDC mRNA overexpression is linked to favorable prognosis in patients with colorectal adenocarcinoma and may constitute a useful tissue biomarker. Involvement of DDC in apoptosis of colon cancer cells and/or response of DCC-positive tumours to chemotherapy are two potential explanations, but further investigation is required to clarify the role of DDC in CRC.

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