

## Retrospective Study

**Indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 expression prediction for adverse prognosis in colorectal cancer**

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**Abstract****AIM**

To evaluate indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 (IDO1/COX2) expression as an independent

prognostic biomarker for colorectal cancer (CRC) patients.

## METHODS

We retrospectively studied the medical records of 95 patients who received surgical resection from August 2008 to January 2010. All patients were randomly assigned to adjuvant treatment with or without celecoxib groups after surgery. We performed standard immunohistochemistry to assess the expression levels of IDO1/COX2 and evaluated the correlation of IDO1/COX2 with clinicopathological factors and overall survival (OS) outcomes.

## RESULTS

The expression of nuclear IDO1 was significantly correlated with body mass index ( $P < 0.001$ ), and IDO1 expression displayed no association with sex, age, tumor differentiation, T stage, N stage, carcinoembryonic antigen, cancer antigen 19-9, CD3+ and CD8+ tumor infiltrating lymphocytes, and COX2. In univariate analysis, we found that nuclear IDO1 ( $P = 0.039$ ), nuclear/cytoplasmic IDO1 [hazard ratio (HR) = 2.044, 95% confidence interval (CI): 0.871-4.798,  $P = 0.039$ ], nuclear IDO1/COX2 (HR = 3.048, 95%CI: 0.868-10.7,  $P = 0.0049$ ) and cytoplasmic IDO1/COX2 (HR = 2.109, 95%CI: 0.976-4.558,  $P = 0.022$ ) all yielded significantly poor OS outcomes. Nuclear IDO1 ( $P = 0.041$ ), nuclear/cytoplasmic IDO1 (HR = 3.023, 95%CI: 0.585-15.61,  $P = 0.041$ ) and cytoplasmic IDO1/COX2 (HR = 2.740, 95%CI: 0.764-9.831,  $P = 0.038$ ) have significantly poor OS outcomes for the CRC celecoxib subgroup. In our multivariate Cox model, high coexpression of cytoplasmic IDO1/COX2 was found to be an independent predictor of poor outcome in CRC (HR = 2.218, 95%CI: 1.011-4.48,  $P = 0.047$ ) and celecoxib subgroup patients (HR = 3.210, 95%CI: 1.074-9.590,  $P = 0.037$ ).

## CONCLUSION

Our results showed that cytoplasmic IDO1/COX2 coexpression could be used as an independent poor predictor for OS in CRC.

**Key words:** Prognosis; Indoleamine-2,3-dioxygenase 1; Cyclooxygenase 2; Colorectal cancer

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**Core tip:** It was reported that indoleamine-2,3-dioxygenase 1 (IDO1) is an inhibitory factor that suppresses the T cell response to tumors. In this study, we evaluated IDO1/cyclooxygenase 2 (COX2) expression as an independent prognostic biomarker for colorectal cancer (CRC) patients. In our multivariate Cox model, high coexpression of cytoplasmic IDO1/COX2 was found to be an independent predictor of poor outcome in CRC patients and celecoxib subgroup patients. Our results showed that cytoplasmic IDO1/COX2 coexpression could be used as an independent predictor for poor overall survival in CRC.

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## INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. Nearly one million cases of CRC are diagnosed worldwide each year<sup>[1,2]</sup>. Because of genetic mutations and environmental factors, CRC development is a very complex process and is determined by multistage factors<sup>[3,4]</sup>. Currently, immunotherapy has become one of the most promising treatments for CRC<sup>[5]</sup>.

Recent studies have demonstrated that the tumor microenvironment plays a vital role in the progression of cancer development - *e.g.*, cancer cells, through expressing inhibitory proteins, such as PD-L1 and CTLA4, create an immunosuppressive microenvironment<sup>[6-8]</sup>. Clinical trials have shown that combining PD-1/PD-L1 with CTLA4 blockade therapy seems to be a better therapy than single blockade. However, this favorable outcome is achieved in only less than 40% of patients<sup>[9]</sup>. Other studies have confirmed that the tumor microenvironment has more inhibitory factors, including indoleamine-2,3-dioxygenase 1 (IDO1), and suppresses the T cell response to tumors. IDO1 belongs to a unique class of mammalian heme dioxygenase enzymes and is the first and rate-limiting enzyme in the degradation of the essential amino acid tryptophan, resulting in the accumulation of their metabolites such as kynurenine<sup>[9]</sup>. T cells sense low tryptophan and high kynurenine *via* mTORC and GCN2 signaling pathways to initiate an amino acid starvation response, resulting in T cell cycle arrest and cell death, and favoring the differentiation of regulatory T cells; as a result, the immune mediator is escapes in cancer<sup>[10]</sup>.

In humans, IDO1 is usually expressed only in placental endothelial cells and mature dendritic cells. Activating T lymphocytes could express interferon- $\gamma$  in the tumor microenvironment, resulting in inducing IDO1 expression in most tissues and cell types and inhibiting T cell responses to tumor cells<sup>[11]</sup>. Many human tumors still express IDO1 through PKC and PI3K signaling triggered by PGE2 in the absence of T cell infiltration. Constitutive expression of cyclooxygenase 2 (COX2) by MAPK signaling could induce PGE2 production<sup>[11]</sup>. Because many tumors harbor oncogenic mutations in these signaling pathways, they could express IDO1 constitutively in the absence of interferon- $\gamma$ . Therefore, IDO1 and COX2 are currently of great interest in cancer research as prognostic and therapeutic biomarkers of tissues and sera.

CRC has demonstrated high heterogeneity in recent years. Hence, biomarkers need to be identified and enabled to stratify the different subgroups. Similar to other tumors, such as endometrial carcinoma and liver and ovarian cancers, the IDO expression levels

are correlated with the overall survival (OS) of CRC patients<sup>[12-16]</sup>. One study showed that IDO1 expression at the invasive front was significantly associated with OS<sup>[17]</sup>. One report has hypothesized that the nuclear localization of IDO1 promotes the immunosuppression independence of enzyme activity<sup>[18]</sup>. In CRC, the level of COX2 expression was increased in up to 85 cases but not in the normal colonic epithelium. A selective COX2 inhibitor, celecoxib, could improve chemosensitivity when CRC cells are exposed to the combination with 5-FU and CPT-11<sup>[19]</sup> and could reduce hand-foot syndrome induced by capecitabine<sup>[20]</sup>. However, whether IDO1/COX2 coexpression is correlated with OS in CRC patients remains unknown.

In this study, we conducted a retrospective analysis for the potential prognostic importance of the correlation of IDO1 and COX2 in survival outcome prognosis, including their coexpression, cytoplasmic and nuclear localization of IDO1, and tumor-infiltrating lymphocytes (TILs).

## MATERIALS AND METHODS

### Patient characteristics

All tissues were collected from 95 patients who had undergone surgical resection from August 2008 to January 2010 at the Department of Colorectal Surgery of Sun Yat-sen University (Guangzhou, China). Patients were randomly assigned to adjuvant treatment with XELOX/capecitabine alone combined with or without celecoxib groups after surgery. All patients in the groups received celecoxib 200 mg/m<sup>2</sup> twice daily, given for 14 d (day 1 to day 14) of a 3-wk cycle for total of 6-8 cycles<sup>[20]</sup>. The eligibility criteria were as follows: (1) Stage II/III CRC eligible for adjuvant chemotherapy; (2) all tumor tissue pathological diagnoses confirmed to be CRC by a pathologist. The cases were selected consecutively based on the availability of resection tissues and follow-up data.

### Immunohistochemical staining

Formalin-fixed, paraffin-embedded tumor specimens were cut into 4- $\mu$ m sections. After baking at 60 °C for 2 h, the samples were deparaffinized in xylene and rehydrated in a series of graded ethanol. Next, the samples were incubated with 3% hydrogen peroxide for 10-15 min to block endogenous peroxidase activity. The sections were microwaved for antigen retrieval in 0.01 mol/L sodium citrate buffer (pH 6.0) for 30 min, and then were pre-incubated in 10% normal goat serum for 30 min to block nonspecific staining. The sections were then incubated with the primary rabbit anti-human IDO1 monoclonal antibody (working dilution, 1:100; Cell Signaling Technology, Danvers, MA, United States), rabbit antihuman COX2 monoclonal antibody (working dilution, 1:200; Beijing Golden Bridge Biotechnology, China), rabbit antihuman CD3 monoclonal (working dilution: 1:50; Beijing Golden Bridge Biotechnology) and mouse antihuman CD8 monoclonal (working dilution, 1:100; Beijing Golden Bridge Biotechnology) overnight

at 4 °C. Subsequently, the samples were incubated with secondary antibody (Dako, Glostrup, Denmark) at room temperature for 0.5 h.

All the stained slides were scored independently by two experienced pathologists who were blinded to the patients' identity and clinical status. H-scores of dominant staining intensity (0, 1+, 2+ and 3+) and the percentage of positive tumor cells (0 to 100%) of immunostaining were adopted for the expression data analysis. IDO1 expression was classified as high or low based on whether the H-score was above or below the score of 0.1. COX2 expression was considered high if the score was above 0.6 as the median cut-off. T cell infiltration of tumors was assessed by semiquantitative estimation of the density of CD3-positive/CD8-positive (CD3+/CD8+) cells and was scored as follows: 1+: No or sporadic CD3+/CD8+ cells; 2+: Moderate numbers of CD3+/CD8+ cells; 3+: Abundant occurrence of CD3+/CD8+ cells; and 4+: Highly abundant occurrence of CD3+/CD8+ cells<sup>[21]</sup>.

### Follow-up

The last date of follow-up was October 2017. All patients (51 males and 44 females) were followed up every 3 mo in the first 2 years and every 6 mo thereafter. History and physical examination should be given every 3 to 6 mo for 2 years, and then every 6 mo for a total of 5 years. A carcinoembryonic antigen (CEA) test and abdominal and pelvic ultrasound test were recommended at baseline and every 3 to 6 mo for 2 years, then every 6 mo for a total of 5 years. Colonoscopy is recommended at approximately 1 year after resection. Repeat colonoscopy is typically recommended at 3 years, and every 5 years thereafter, unless follow-up colonoscopy indicates advanced adenoma, in which case colonoscopy should be repeated in 1 year. Chest, abdominal and pelvic CT scans were recommended annually for up to 5 years. During the follow-up, 33 patients (34.7%) died of cancer-related causes. Sixty-two patients (65.3%) were still alive at the time of the last follow-up report.

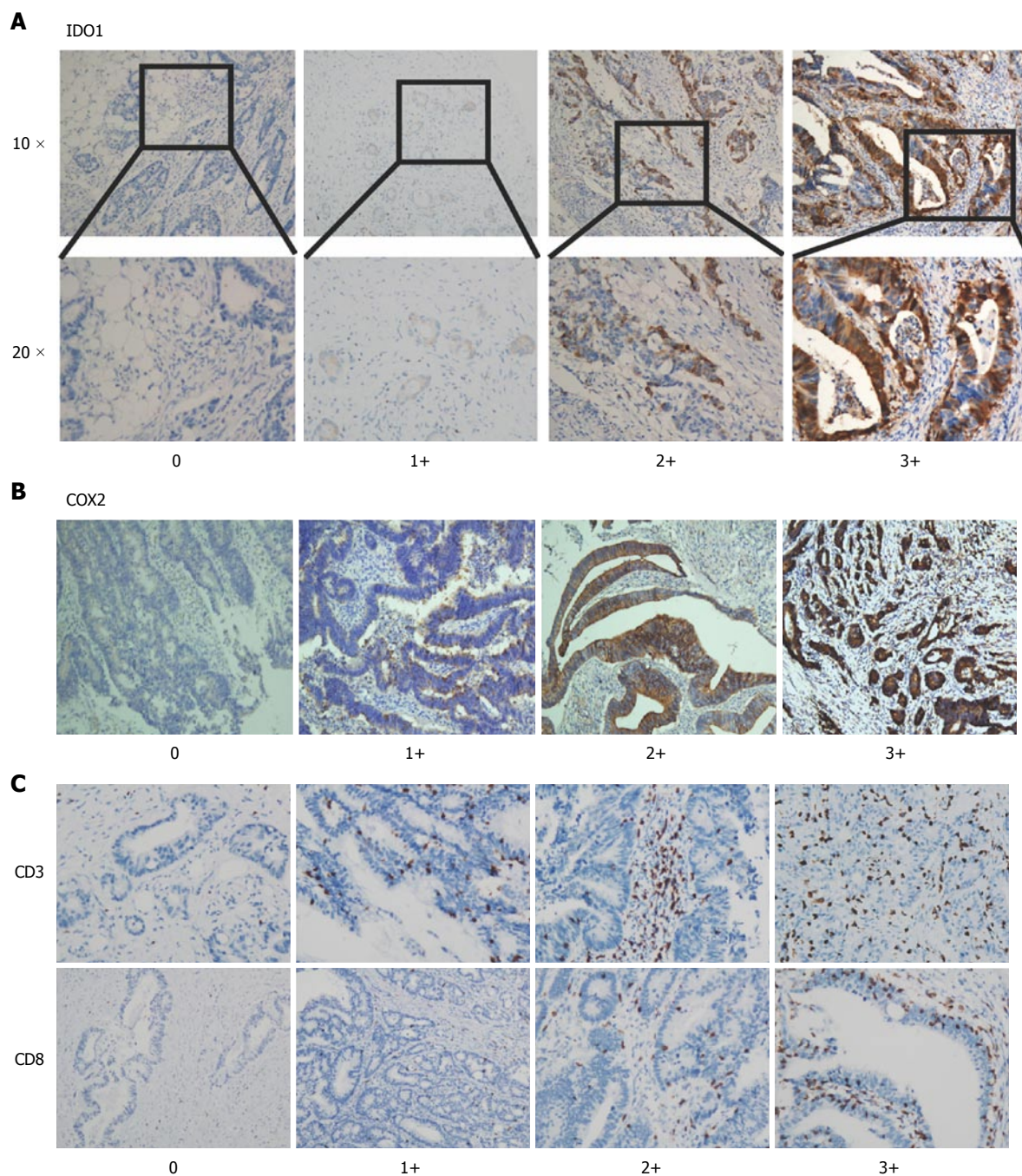
### Statistical analysis

The SPSS software package (version 23.0; IBM Corp, Armonk, NY, United States) and GraphPad Prism (version 7.0; GraphPad Software Inc, La Jolla, CA, United States) were used for statistical analysis. OS was defined as the time from the diagnosis of CRC to death of the patient or last date of follow-up. Chi-square test was used to assess the correlation of the IDO1 status with clinicopathologic characteristics. Survival curves were generated using the Kaplan-Meier method, and differences between curves were assessed by the log-rank test. The Cox multivariate proportional hazards regression model was used to determine the independent risk factors that influence OS. *P*-values < 0.05 were considered to be statistically significant.

## RESULTS

### IDO1 and COX2 expression in CRC

To elucidate the biological significance of IDO1/COX2 in



**Figure 1** Indoleamine-2,3-dioxygenase 1, cyclooxygenase 2, CD3 and CD8 expression in colorectal cancer. A: Examples of the tumoral staining intensity (0, 1+, 2+ and 3+) of IDO1 in immunohistochemistry analysis; B: Examples of the tumoral staining intensity (0, 1+, 2+ and 3+) of COX2 in immunohistochemistry analysis; C: Representative examples of tumors with intraepithelial CD3 and CD8 scores (1+, 2+, 3+ and 4+). CRC: Colorectal cancer; COX2: Cyclooxygenase 2; IDO1: Indoleamine-2,3-dioxygenase 1.

CRC, especially in the CRC celecoxib subgroup, we used immunohistochemical staining to test the expression of IDO1 and COX2 in the selected 95 CRC specimens. The results showed that IDO1 expression is primarily localized in the cytoplasm within the nucleus of tumor cells (Figure 1).

**Association of cytoplasmic and nuclear IDO1 expression with clinicopathological parameters in CRC patients**

To gain insight into the role of the localization of IDO1 protein in CRC, we correlated cytoplasmic and nuclear

IDO1 expression in the study cohort of 95 CRC patients with certain clinical and pathological factors. The expression of nuclear IDO1 was significantly correlated with body mass index (BMI) ( $P < 0.001$ ); however, cytoplasmic IDO1 showed no relationship with BMI ( $P = 0.16$ ). We observed no relationship between cytoplasmic and nuclear IDO1 expression and clinical factors such as sex, age, cancer (colon and rectum), tumor differentiation, T stage, N stage, CEA, cancer antigen (CA)19-9, CD3+ and CD8+ TILs, COX2, and celecoxib treatment (Tables 1 and 2).

**Table 1 Correlation of cytoplasmic indoleamine-2,3-dioxygenase 1 expression with colorectal cancer clinicopathologic parameters**

| Characteristic        | Total | Low IDO1  | High IDO1 | P-value |
|-----------------------|-------|-----------|-----------|---------|
| Sex                   |       |           |           | 0.074   |
| Male                  | 52    | 22 (42.3) | 30 (57.7) |         |
| Female                | 43    | 27 (62.8) | 16 (37.2) |         |
| Age in yr             |       |           |           | 0.65    |
| ≥ 60                  | 30    | 17 (56.7) | 13 (43.3) |         |
| < 60                  | 65    | 32 (49.2) | 33 (50.8) |         |
| Cancers               |       |           |           | 0.93    |
| Colon                 | 46    | 24 (52.2) | 22 (47.8) |         |
| Rectum                | 49    | 25 (51.0) | 24 (49.0) |         |
| BMI                   |       |           |           | 0.16    |
| ≥ 25                  | 20    | 7 (35.0)  | 13 (65.0) |         |
| < 25                  | 75    | 42 (56.0) | 33 (44.0) |         |
| Tumor differentiation |       |           |           | 0.47    |
| Moderate and poor     | 78    | 42 (54.5) | 35 (45.5) |         |
| Well                  | 17    | 7 (41.2)  | 10 (58.8) |         |
| Colon cancer stage    |       |           |           | 0.52    |
| 3                     | 20    | 12 (60.0) | 8 (40.0)  |         |
| 2                     | 26    | 12 (46.2) | 14 (53.8) |         |
| T stage               |       |           |           | 0.69    |
| 4                     | 29    | 14 (48.3) | 15 (51.7) |         |
| 2/3                   | 17    | 10 (58.8) | 7 (41.2)  |         |
| N stage               |       |           |           | 0.96    |
| 1/2                   | 20    | 11 (55.0) | 9 (45.0)  |         |
| 0                     | 26    | 13 (50.0) | 13 (50.0) |         |
| Rectum cancer stage   |       |           |           | 0.67    |
| 3                     | 24    | 11(45.8)  | 13 (54.2) |         |
| 2                     | 25    | 14 (46.0) | 11 (44.0) |         |
| T stage               |       |           |           | 0.68    |
| 4                     | 22    | 12 (55.6) | 10 (45.4) |         |
| 2/3                   | 27    | 12 (44.4) | 15 (55.6) |         |
| N stage               |       |           |           | 0.88    |
| 1/2                   | 24    | 12 (50.0) | 12 (50.0) |         |
| 0                     | 25    | 13 (52.0) | 12 (48.0) |         |
| CEA in ng/mL          |       |           |           | 0.45    |
| > 5                   | 42    | 24 (57.1) | 18 (42.9) |         |
| ≤ 5                   | 53    | 25 (47.2) | 28 (42.8) |         |
| CA19-9 in U/mL        |       |           |           | 0.22    |
| > 37                  | 17    | 6 (35.3)  | 11 (64.7) |         |
| ≤ 37                  | 78    | 43 (55.1) | 35 (44.9) |         |
| CD3 TILs              |       |           |           | 0.27    |
| High                  | 36    | 22 (61.1) | 14 (38.9) |         |
| Low                   | 59    | 28 (47.5) | 31 (42.5) |         |
| CD8 TILs              |       |           |           | 0.96    |
| High                  | 22    | 12 (54.5) | 10 (45.5) |         |
| Low                   | 73    | 38 (52.5) | 35 (47.5) |         |
| COX2                  |       |           |           | 0.92    |
| High                  | 48    | 26 (54.2) | 22 (45.8) |         |
| Low                   | 47    | 24 (51.1) | 23 (48.9) |         |
| Treatment group       |       |           |           | 0.58    |
| Celecoxib             | 44    | 25 (56.8) | 19 (43.2) |         |
| Non-celecoxib         | 51    | 25 (49.0) | 26 (51.0) |         |

Data are presented as *n* or *n* (%). BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; COX2: Cyclooxygenase 2; CRC: Colorectal cancer; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor-infiltrating lymphocytes.

**Correlation of IDO1/COX2 protein expression with poor prognosis in CRC**

We analyzed the correlation between IDO1 and traditional clinicopathologic parameters with patients' outcomes by univariate analysis. We also performed analyses to determine whether IDO1 and COX2 expression and localization represent potential independent

**Table 2 Correlation of nuclear indoleamine-2,3-dioxygenase 1 expression with colorectal cancer clinicopathologic parameters**

| Characteristic        | Total | Low IDO1  | High IDO1 | P-value |
|-----------------------|-------|-----------|-----------|---------|
| Sex                   |       |           |           | 0.70    |
| Male                  | 51    | 39 (76.5) | 12 (33.5) |         |
| Female                | 44    | 36 (81.8) | 8 (19.2)  |         |
| Age in yr             |       |           |           | 0.92    |
| > 60                  | 30    | 24 (80.0) | 6 (20.0)  |         |
| ≤ 60                  | 65    | 51 (78.5) | 14 (21.5) |         |
| Cancers               |       |           |           | 0.52    |
| Colon                 | 46    | 38 (82.6) | 18 (17.4) |         |
| Rectum                | 49    | 37 (75.5) | 12 (24.5) |         |
| BMI                   |       |           |           | < 0.001 |
| > 25                  | 20    | 9 (45.0)  | 11 (55.0) |         |
| ≤ 25                  | 75    | 66 (88.0) | 9 (12.0)  |         |
| Tumor differentiation |       |           |           | 0.87    |
| Moderate and poor     | 78    | 60 (76.9) | 18 (23.1) |         |
| Well                  | 17    | 14 (82.3) | 3 (17.7)  |         |
| Colon cancer stage    |       |           |           | 0.98    |
| 3                     | 20    | 17 (85.0) | 3 (15.0)  |         |
| 2                     | 26    | 21 (80.8) | 5 (19.2)  |         |
| T stage               |       |           |           | 0.71    |
| 4                     | 29    | 23 (19.3) | 6 (20.7)  |         |
| 2/3                   | 17    | 15 (88.2) | 2 (11.8)  |         |
| N stage               |       |           |           | 0.98    |
| 1/2                   | 20    | 16 (80.0) | 4 (20.0)  |         |
| 0                     | 26    | 22 (84.6) | 4 (15.4)  |         |
| Rectum cancer stage   |       |           |           | 0.44    |
| 3                     | 24    | 17 (70.8) | 7 (29.2)  |         |
| 2                     | 25    | 21 (84.0) | 4 (16.0)  |         |
| T stage               |       |           |           | 0.94    |
| 4                     | 22    | 16 (72.7) | 6 (27.3)  |         |
| 2/3                   | 27    | 21 (77.8) | 6 (22.2)  |         |
| N stage               |       |           |           | 0.28    |
| 1/2                   | 24    | 16 (66.7) | 8 (33.3)  |         |
| 0                     | 25    | 21(84.0)  | 4 (16.0)  |         |
| CEA in ng/mL          |       |           |           | 0.37    |
| > 5                   | 42    | 35 (83.3) | 7 (16.7)  |         |
| ≤ 5                   | 53    | 39 (73.6) | 14 (26.4) |         |
| CA19-9 in U/mL        |       |           |           | 0.78    |
| > 37                  | 81    | 41 (50.6) | 40 (49.4) |         |
| ≤ 37                  | 17    | 8 (47.1)  | 9 (52.9)  |         |
| CD3 TILs              |       |           |           | 0.96    |
| High                  | 36    | 28 (77.8) | 8 (22.2)  |         |
| Low                   | 59    | 47 (79.7) | 12 (20.3) |         |
| CD8 TILs              |       |           |           | 0.26    |
| High                  | 22    | 15 (68.2) | 7 (31.8)  |         |
| Low                   | 73    | 60 (82.2) | 13 (17.8) |         |
| COX2                  |       |           |           | 0.84    |
| High                  | 48    | 38 (79.2) | 10 (20.8) |         |
| Low                   | 47    | 37 (78.7) | 10 (21.3) |         |
| Treatment group       |       |           |           | 0.16    |
| Celecoxib             | 44    | 38 (86.4) | 6 (13.6)  |         |
| Non-celecoxib         | 51    | 37 (72.5) | 14 (27.5) |         |

Data are presented as *n* or *n* (%). BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; COX2: Cyclooxygenase 2; CRC: Colorectal cancer; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor-infiltrating lymphocytes.

predictors for the OS outcome in CRC patients. We observed that cytoplasmic IDO1 and COX2 expression could not predict OS outcomes in our univariate analysis (cytoplasmic IDO1: *P* = 0.10; COX2: *P* = 0.51). However, nuclear IDO1 (*P* = 0.039), nuclear/cytoplasmic IDO1 (hazard ratio (HR) = 2.044, 95% confidence interval (CI): 0.871-4.798, *P* = 0.039),

**Table 3** Univariate analysis of the correlation of clinicopathological parameters with overall survival in patients with colorectal carcinoma

|   | HR    | 95%CI       | P value |
|---|-------|-------------|---------|
| Sex, male <i>vs</i> female                              | 0.750 | 0.399-1.411 | 0.37    |
| Age in yr, ≤ 60 <i>vs</i> > 60                          | 0.899 | 0.472-1.714 | 0.74    |
| Cancer, colon <i>vs</i> rectum                          | 1.279 | 0.712-2.296 | 0.41    |
| BMI, > 25 <i>vs</i> ≤ 25                                | 1.579 | 0.697-3.579 | 0.21    |
| Tumor differentiation, moderate and poor <i>vs</i> well | 2.798 | 1.373-5.702 | 0.039   |
| Stage, 3 <i>vs</i> 2                                    | 1.003 | 0.534-1.882 | 0.99    |
| T stage, T4 <i>vs</i> T2/3                              | 1.418 | 0.755-2.664 | 0.27    |
| N stage, N1/2 <i>vs</i> N0                              | 1.005 | 0.536-1.887 | 0.99    |
| CEA in ng/mL, > 5 <i>vs</i> ≤ 5                         | 2.137 | 1.141-4.004 | 0.025   |
| CA19-9 in U/mL, > 37 <i>vs</i> ≤ 37                     | 1.262 | 0.547-2.911 | 0.56    |
| CD3 TILs, high <i>vs</i> low                            | 1.195 | 0.649-2.198 | 0.55    |
| CD8 TILs, high <i>vs</i> low                            | 2.096 | 0.975-4.504 | 0.018   |
| Nuclear IDO1, high <i>vs</i> low                        | 2.044 | 0.871-4.798 | 0.039   |
| Cytoplasmic IDO1, high <i>vs</i> low                    | 1.690 | 0.901-3.173 | 0.10    |
| Nuclear and cytoplasmic IDO1, high <i>vs</i> low        | 2.044 | 0.871-4.798 | 0.039   |
| COX2, high <i>vs</i> low                                | 1.235 | 0.659-2.314 | 0.51    |
| Nuclear IDO1/COX2, IV <i>vs</i> I / II / III            | 3.048 | 0.868-10.7  | 0.0049  |
| Cytoplasmic IDO1/COX2, IV <i>vs</i> I / II / III        | 2.109 | 0.976-4.558 | 0.022   |
| Treatment group, celecoxib <i>vs</i> non-celecoxib      | 0.943 | 0.489-1.826 | 0.86    |

BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; CI: Confidence interval; COX2: Cyclooxygenase 2; CRC: Colorectal carcinoma; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor infiltrating lymphocytes.

nuclear IDO1/COX2 (HR = 3.048, 95%CI: 0.868-10.7,  $P = 0.0049$ ), cytoplasmic IDO1/COX2 (HR = 2.109, 95%CI: 0.976-4.558,  $P = 0.022$ ), tumor differentiation (HR = 2.798, 95%CI: 1.373-5.702,  $P = 0.039$ ), CEA (HR = 2.137, 95%CI: 1.141-4.004,  $P = 0.025$ ), and CD8 TILs (HR = 2.096, 95%CI: 0.975-4.504,  $P = 0.018$ ) (Table 3) yielded significantly poor OS outcomes in CRC patients (Figure 2B-G, Supplementary Figure 1E) but not with other clinicopathologic parameters such as sex, age, BMI, T stage, N stage, CA19-9 and CD3+ TILs, including whether celecoxib was used or not (Figure 2A, 2C, 2H, Supplementary Figure 1A-D, 1F-J).

We also performed multivariate Cox modeling to analyze whether IDO1/COX2 represent potential independent predictors for the OS outcome in CRC patients. Combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with HR = 2.218 (95%CI: 1.011-4.48,  $P = 0.047$ ) in the IDO1<sup>High</sup>/COX2<sup>High</sup> group, and tumor differentiation was significantly correlated with OS (HR = 3.473, 95%CI: 1.201-10.046,  $P = 0.022$ ) (Table 4) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression. Our results revealed that cytoplasmic IDO1/COX2 coexpression and tumor differentiation were independent predictors for poor OS in CRC.

#### Correlation of IDO1/COX2 protein expression with poor prognosis in the CRC celecoxib subgroup

We also performed analyses to determine whether IDO1 and COX2 expression and localization represent potential independent predictors for OS outcome in CRC patients. We observed that cytoplasmic IDO1 and COX2 expression could not predict OS outcomes in univariate analysis (cytoplasmic IDO1:  $P = 0.31$ ; COX2:  $P = 0.25$ ). However, nuclear IDO1 ( $P = 0.041$ ), nuclear/

cytoplasmic IDO1 (HR = 3.023, 95%CI: 0.585-15.61,  $P = 0.041$ ), cytoplasmic IDO1/COX2 (HR = 2.740, 95%CI: 0.764-9.831,  $P = 0.038$ ) (Table 5), tumor differentiation (HR = 7.396, 95%CI: 2.749-19.90,  $P = 0.021$ ) and CD8 TILs (HR = 2.821, 95%CI: 0.774-10.29,  $P = 0.026$ ) have significantly poor OS outcomes for the CRC celecoxib subgroup (Figure 3B, 3D, 3F, 3H and 3I) but not with other clinicopathologic parameters such as sex, age, BMI, T stage, N stage, CEA, CA19-9 and CD3+ TILs (Figure 3A, 3C, 3E and 3G, Supplementary Figure 2A-I).

We further performed the multivariate Cox modeling to analyze whether IDO1/COX2 represents potential independent predictors for OS outcome in the CRC celecoxib subgroup. Combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with HR = 3.210 (95%CI: 1.074-9.590,  $P = 0.037$ ) in the IDO1<sup>High</sup>/COX2<sup>High</sup> group, and tumor differentiation was significantly correlated with OS (HR = 11.962, 95%CI: 1.526-23.787,  $P = 0.018$ ) (Table 6) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression. Our results revealed that cytoplasmic IDO1/COX2 coexpression and tumor differentiation were independent poor predictors of OS in the CRC celecoxib subgroup.

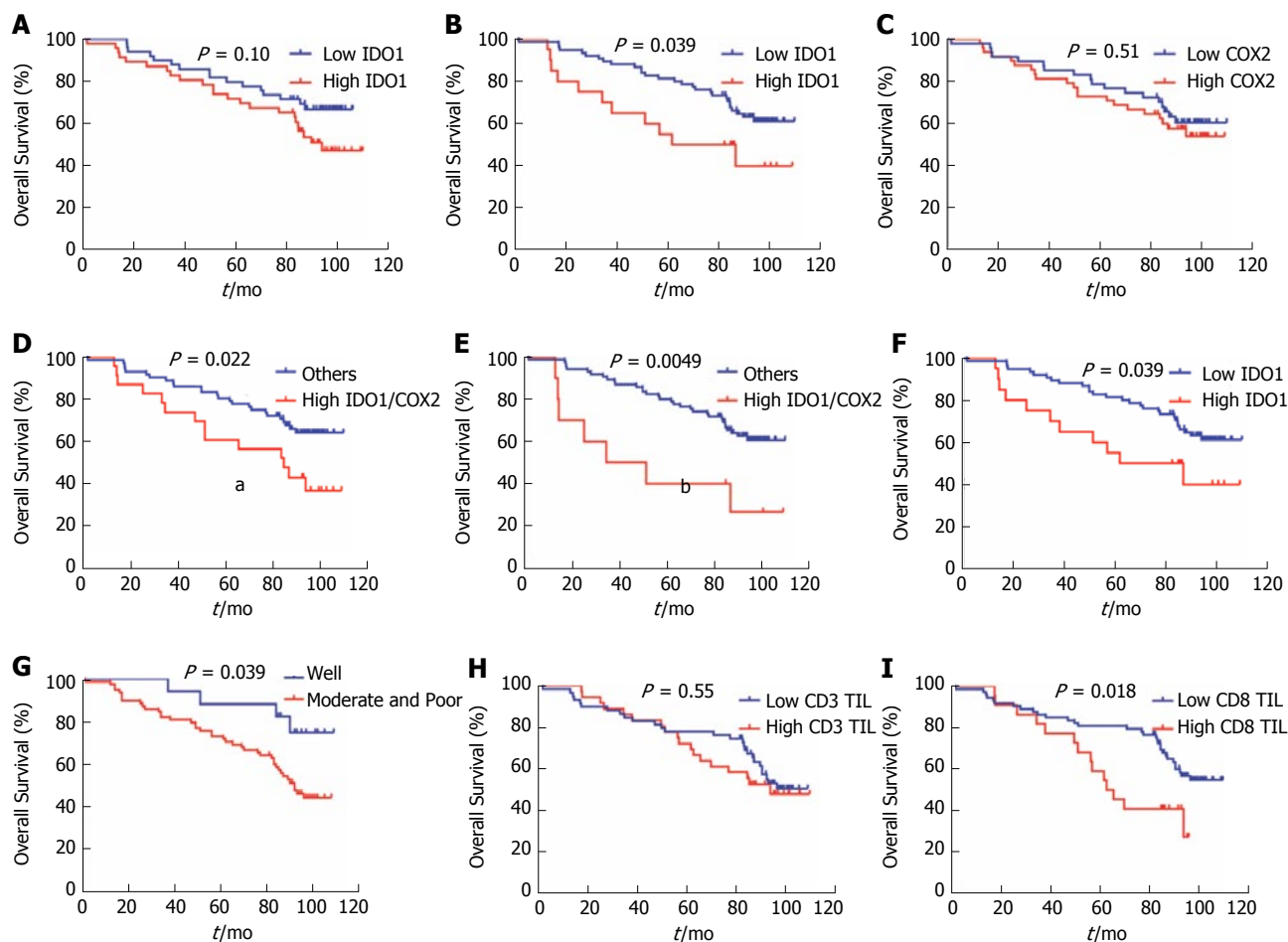
## DISCUSSION

Current immunotherapy has been achieving very effective and promising results, especially for stage IV disease. However, more than 50% of these patients who need more new therapies will progress with resistance to immunotherapy<sup>[22]</sup>. IDO1 is associated with T cell apoptosis through depleting tryptophan in the tumor microenvironment. Therefore, IDO1 inhibitors have emerged as new options for cancer therapy. However, a

**Table 4** Multivariate analysis of the correlation of indoleamine-2,3-dioxygenase 1 with overall survival in patients with Colorectal cancer

|   | HR    | 95%CI        | P-value |
|---|-------|--------------|---------|
| Cytoplasmic IDO1 and COX2, IV <i>vs</i> I / II / III    | 2.218 | 1.011-4.48   | 0.047   |
| Tumor differentiation, poor and moderate <i>vs</i> well | 3.473 | 1.201-10.046 | 0.022   |

CI: Confidence interval; COX2: Cyclooxygenase 2; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1.



**Figure 2** Correlation of Indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 protein expression with a poor prognosis in colorectal cancer. A-C: Correlation between nuclear or cytoplasmic IDO1 and COX2 expression with CRC patient OS. Survival curves were generated using the Kaplan-Meier method, and differences between survival curves were estimated by the log-rank test. Nuclear IDO1 showed a statistically significant correlation with OS; D-E: Correlation between the different expression levels of nuclear and cytoplasmic IDO1/COX2 and OS in CRC patients. Group I: IDO1<sup>Low</sup>COX2<sup>Low</sup>; Group II: IDO1<sup>High</sup>COX2<sup>Low</sup>; Group III: IDO1<sup>Low</sup>COX2<sup>High</sup>; Group IV: IDO1<sup>High</sup>COX2<sup>High</sup>. The association of the four groups (IV *vs* I / II / III) with OS was significant ( $P < 0.05$ ); F: Combined analysis of nuclear and cytoplasmic IDO1 and its correlation with OS in CRC. The association of nuclear and cytoplasmic IDO1 expression with OS was significant ( $P < 0.05$ ); G: Correlation between tumor differentiation and OS in CRC. The association of tumor differentiation (moderate and poor *vs* well) with OS was significant ( $P < 0.05$ ); H-I: Correlation between CD3 TILs and CD8 TILs and OS in CRC; H: CD3 TILs ( $P > 0.05$ ); I: CD8 TILs ( $P < 0.05$ ). CRC: Colorectal cancer; COX2: Cyclooxygenase 2; IDO1: Indoleamine-2,3-dioxygenase 1; OS: Overall survival; TILs: Tumor-infiltrating lymphocytes.

recent study suggested the alternative hypothesis that nuclear IDO1 promotes immunosuppression instead of enzyme activity<sup>[18]</sup>. In previous studies, high IDO expression in CRC has been found to be associated with the presence of metastatic disease and outcome and a reduction in CD3-positive TILs, revealing the important role in therapeutic blockade for this disease<sup>[12,17]</sup>. In up to 85% of CRC patients, COX2 is highly expressed but not in normal colonic epithelium. Celecoxib is a COX2 inhibitor used in the treatment regimen for CRC; previous studies have demonstrated celecoxib in combination

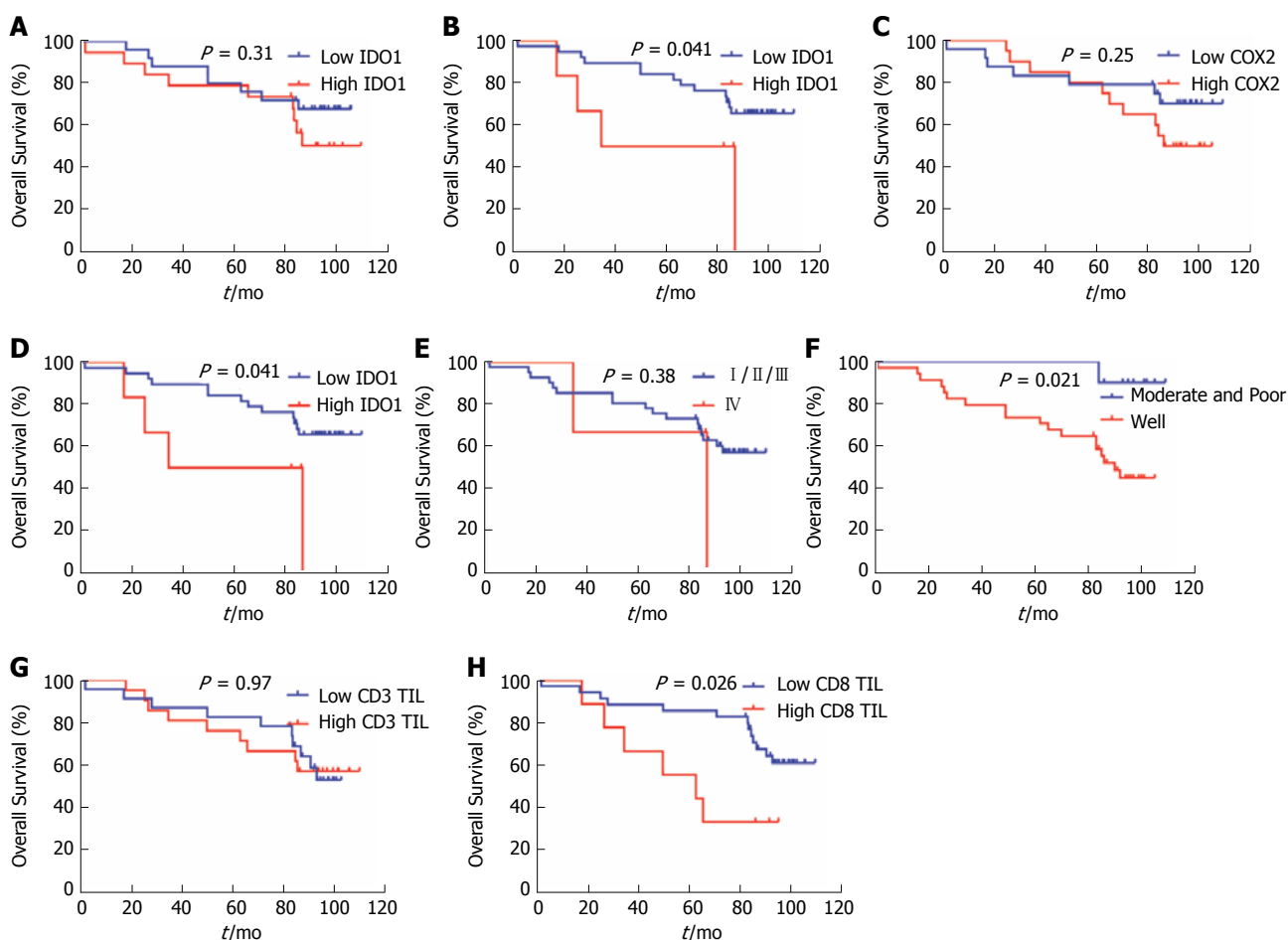
with chemotherapy to overcome resistance in therapy-refractory cancer cells *in vitro* and *in vivo*<sup>[19]</sup>. However, clinical studies have not been clarified to show the role of celecoxib in CRC patients and its potential prognostic importance.

In the present study, we evaluated CRC patients treated with or without celecoxib. We found no significant relationship with IDO1 or COX2 expression and OS in patients treated with or without celecoxib. However, our discovery revealed that cytoplasmic IDO1 and COX2 were correlated with OS in patients treated with or

**Table 5 Univariate analysis of the correlation of clinicopathological parameters with overall survival in CRC celecoxib subgroup**

|   | HR    | 95%CI       | P value |
|---|-------|-------------|---------|
| Sex, male <i>vs</i> female                              | 0.854 | 0.329-2.219 | 0.74    |
| Age in yr, ≤ 60 <i>vs</i> > 60                          | 1.249 | 0.432-3.609 | 0.70    |
| Cancer, colon <i>vs</i> rectum                          | 1.034 | 0.420-2.543 | 0.94    |
| BMI, > 25 <i>vs</i> ≤ 25                                | 1.328 | 0.351-5.020 | 0.71    |
| Tumor differentiation, moderate and poor <i>vs</i> well | 7.396 | 2.749-19.90 | 0.021   |
| Stage, 3 <i>vs</i> 2                                    | 1.075 | 0.415-2.782 | 0.88    |
| T stage, T4 <i>vs</i> T2/3                              | 1.389 | 0.537-3.596 | 0.50    |
| N stage, N1/2 <i>vs</i> N0                              | 1.075 | 0.415-2.782 | 0.88    |
| CEA in ng/mL, > 5 <i>vs</i> ≤ 5                         | 1.934 | 0.743-5.033 | 0.21    |
| CA19-9 in m/L, > 37 <i>vs</i> ≤ 37                      | 1.551 | 0.431-5.575 | 0.43    |
| CD3 TILs, high <i>vs</i> low                            | 1.02  | 0.414-2.510 | 0.97    |
| CD8 TILs, high <i>vs</i> low                            | 2.821 | 0.774-10.29 | 0.026   |
| Nuclear IDO1, high <i>vs</i> low                        | 3.023 | 0.585-15.61 | 0.041   |
| Cytoplasmic IDO1, high <i>vs</i> low                    | 1.623 | 0.617-4.267 | 0.31    |
| Nuclear and cytoplasmic IDO1, high <i>vs</i> low        | 3.023 | 0.585-15.61 | 0.041   |
| COX2, high <i>vs</i> low                                | 1.746 | 0.672-4.541 | 0.25    |
| Nuclear IDO1/COX2, IV <i>vs</i> I / II / III            | 1.885 | 0.279-12.76 | 0.38    |
| Cytoplasmic IDO1/COX2, IV <i>vs</i> I / II / III        | 2.740 | 0.764-9.831 | 0.038   |

BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; CI: Confidence interval; COX2: Cyclooxygenase 2; CRC: Colorectal carcinoma; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor infiltrating lymphocytes.



**Figure 3 Correlation of indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 protein expression with a poor prognosis in the colorectal cancer celecoxib subgroup.** A-D: Correlation between nuclear or cytoplasmic IDO1 and COX2 expression with OS in the CRC celecoxib subgroup. Survival curves were generated using the Kaplan-Meier method, and differences between survival curves were estimated by the log-rank test. Nuclear IDO1 and nuclear and cytoplasmic IDO1 showed a statistically significant correlation with OS; E: Correlation between different expression levels of nuclear and cytoplasmic IDO1/COX2 with the OS of the CRC celecoxib subgroup. Group I: IDO1<sup>Low</sup>COX2<sup>Low</sup>, Group II: IDO1<sup>High</sup>COX2<sup>Low</sup>, Group III: IDO1<sup>Low</sup>COX2<sup>High</sup>, Group IV: IDO1<sup>High</sup>COX2<sup>High</sup>. The association of four groups (IV *vs* I / II / III) with OS is not significant ( $P > 0.05$ ); F: Correlation between tumor differentiation and OS in CRC. The association of tumor differentiation (moderate and poor *vs* well) with OS is significant ( $P < 0.05$ ); G-H: Correlation between CD3 TILs and CD8 TILs with CRC OS; G: CD3 TILs ( $P > 0.05$ ); H: CD8 TILs ( $P < 0.05$ ). CRC: Colorectal cancer; COX2: Cyclooxygenase 2; IDO1: Indoleamine-2,3-dioxygenase 1; OS: Overall survival; TILs: Tumor-infiltrating lymphocytes.



**Table 6** Multivariate analysis of the correlation of indoleamine-2,3-dioxygenase 1 with overall survival in Colorectal cancer celecoxib subgroup

|   | HR     | 95%CI        | P-value |
|---|--------|--------------|---------|
| Cytoplasmic IDO1 and COX2, IV <i>vs</i> I / II / III    | 3.210  | 1.074-9.590  | 0.037   |
| Tumor differentiation, poor and moderate <i>vs</i> well | 11.962 | 1.526-23.787 | 0.018   |

CI: Confidence interval; COX2: Cyclooxygenase 2; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1.

without celecoxib. Additionally, our data further found that nuclear IDO1 and COX2 were not correlated with OS in patients of either group. However, one recent study showed that nuclear IDO1 plays a more important role in CRC instead of enzyme activity. From our data, nuclear IDO1 could not be an independent prognostic factor for CRC patients. Some other unknown factors in the nucleus might combine to nuclear IDO1, possibly influencing the OS of CRC patients. These patients in our study have not been treated with IDO1 inhibitors. Therefore, whether nuclear expression affects IDO1 inhibitors is unclear.

Constitutive IDO1 expression is dependent on an autocrine loop of PGE2 production through activating the PI3K and PKC pathways and subsequent activation of IDO1 transcription by factors such as ETV4. PGE2 production mediates the expression of COX2. However, in our study, we found that IDO1 or COX2 expression was not correlated with OS. Three explanations are possible. First, CRC patients were treated with celecoxib only for no more than 6 mo. COX2 might still influence the expression of IDO1, which would negatively regulate effector T cells. Second, another signaling pathway might activate IDO1 expression in CRC patients. Third, these patients were treated with celecoxib but not combined with IDO1 inhibitors.

There are some limitations in our current study. This study was a retrospective study, with its intrinsic associated limitations. Second, although our cohort size consists of well-annotated celecoxib groups, its number is still modest. Third, to minimize bias and immunohistochemistry methodological limitations, we have herein adopted rigorous standardized assay methods in our study. All immunohistochemistry scores were affirmed by two blinded, well-trained clinical pathologists working independently. Furthermore, a larger clinical sample cohort size would be valuable to validate our results, and more chemotherapy-resistant patients need to be considered.

The results of the current study demonstrate that the coexpression of cytoplasmic IDO1 and COX2 plays a key role in survival prognosis for CRC patients; IDO1 or COX2, nuclear IDO1 and COX2 alone may not serve as a feasible biomarker for prognostic prediction. Therefore, localization of IDO1 and COX2 may serve as a better biomarker to predict CRC patient OS.

Because of genetic mutations and environmental factors, CRC development is a very complex process and is determined by multistage factors. Currently, immunotherapy has become one of the most promising treatments for CRC. However, whether indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 (IDO1/COX2) coexpression is correlated with overall survival (OS) in CRC patients remains unknown.

### Research motivation

CRC has demonstrated high heterogeneity in recent years. Recent studies have demonstrated that IDO1 can suppress the T cell response to tumors. A selective COX2 inhibitor, celecoxib, could improve chemosensitivity when CRC cells are exposed to the combination of 5-FU and CPT-11 and could reduce hand-foot syndrome induced by capecitabine. In this study, we conducted a retrospective analysis for the potential prognostic importance of the correlation of IDO1 and COX2 in survival outcome prognosis, including their coexpression, cytoplasmic and nuclear localization of IDO1, and tumor-infiltrating lymphocytes.

### Research objectives

This study aimed to clarify the potential significance of IDO1/COX2 as a prognostic biomarker in CRC *in vitro*.

### Research methods

Immunohistochemical staining of IDO1 and COX2 was performed in a clinical cohort consisting of 96 CRC cases. Expression of IDO1 and COX2 was correlated with clinicopathological indicators and the clinical outcome of CRC patients.

### Research results

In the CRC group, combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with hazard ratio (HR) = 2.218 (95% confidence interval (CI): 1.011-4.48,  $P = 0.047$ ) in the IDO1<sup>High</sup>/COX2<sup>High</sup> group, and tumor differentiation was significantly correlated with OS (HR = 3.473, 95%CI: 1.201-10.046,  $P = 0.022$ ) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression. Our results revealed that cytoplasmic IDO1/COX2 coexpression and tumor differentiation were independent predictors for poor OS in CRC.

In the CRC celecoxib subgroup, combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with HR = 3.210 (95%CI: 1.074-9.590,  $P = 0.037$ ) in the IDO1<sup>High</sup>/COX2<sup>High</sup> group, and tumor differentiation was significantly correlated with OS (HR = 11.962, 95%CI: 1.526-23.787,  $P = 0.018$ ) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression.

### Research conclusions

The results of the current study demonstrate that the coexpression of cytoplasmic IDO1 and COX2 plays a key role in survival prognosis in CRC patients.

### Research perspectives

IDO1 could be a novel therapeutic target for human CRC, especially as a bio-target of immunotherapy.

## ARTICLE HIGHLIGHTS

### Research background

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide.

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