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# High SLC28A2 expression endows an inferior survival for rectal cancer patients managed by neoadjuvant CCRT

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ABSTRACT

For rectal cancer patients with stage T3–4 disease or positive lymph node, neoadjuvant concurrent chemoradiotherapy (CCRT) has become the standard treatment, but the clinical outcomes are still far from satisfactory. Accordingly, a more precise predictive tool such as genetic biomarkers is urgently required to optimize therapy decisions. Colorectal cancer (CRC) development has been considerably correlated with cellular metabolic process involving nucleotides, but the underlying molecular mechanisms remain unclear. In this study, we employed a transcriptome dataset comprising 46 rectal adenocarcinoma patients undergoing preoperative CCRT and focused on nucleobase-containing compound metabolic process (GO: 0055134) for data mining. We identified solute carrier family 28 member 2 (*SLC28A2*) as the most considerably upregulated gene among rectal cancer patients with CCRT resistance. Afterwards, there were a total of 172 rectal cancer tissue blocks procuring from our biobank, and the immunointensity of SLC28A2 was appraised utilizing immunohistochemical staining. Strong SLC28A2 immunointensity was significantly linked to female patients ($p = 0.032$), vascular invasion ($p = 0.021$), and post-CCRT tumor invasion and regional lymph node involvement ($p < 0.001$ and $p = 0.005$). Notably, patients with strong SLC28A2 immunointensity had no tumor downstaging ($p < 0.001$). Univariate analysis revealed that high SLC28A2 immunoexpression was considerably unfavorably linked to all three endpoints: local recurrence-free survival (LRFS), metastasis-free survival (MeFS), and disease-specific survival (DSS) (all $p \leq 0.0333$). Moreover, both high SLC28A2 immunoexpression and low tumor regression grade were independently unfavorable prognostic factors for all three endpoints (all $p \leq 0.013$) in the multivariate analysis. Utilizing function prediction analysis, *SLC28A2* upregulation was more likely to be linked with stem cell homeostasis in rectal cancer. In brief, we demonstrated that high SLC28A2 immunoexpression is substantially linked to an

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advanced stage, poor response to CCRT, and worse patient survival. Consequently, SLC28A2 expression can be a valuable predictive and prognostic marker for rectal cancer patients and be an encouraging therapeutic target for those with CCRT resistance.

## 1. Introduction

Colorectal cancer (CRC) constitutes about 10% of all new cancer cases and cancer deaths and can be considered as a marker of socio-economic development [1]. In general, CRCs comprise both colon cancer and rectal cancer. Especially, incidence rates of rectal cancer in Eastern Asia rank among the highest in 2020 [1]. At present, total mesorectal excision (TME) is thought as the regular surgical intervention for rectal cancer. But for locally advanced rectal cancer (LARC) patients with stage T3–4 disease or positive lymph node, fluoropyrimidine-based neoadjuvant concurrent chemoradiotherapy (CCRT) is introduced before surgical resection to reduce local recurrence and allow tumor downstaging [2]. Despite the fact that many patients benefit from local control of preoperative CCRT, approximately one-third of patients relapses with metastasis [3] and patient survival has not significantly improved [4]. Accordingly, potential predictive biomarkers are urgently required to optimize therapy decisions.

The rectal delivery of peptide/oligonucleotide drugs has been indicated to have greater systemic bioavailability than oral administration, especially with the optimization of dosage form [5]. This implies that the rectum, connecting the sigmoid colon to the anus, dose not just adsorb water and electrolytes and store stool. Interestingly, it has also been reported that the transcriptional regulation of aberrantly activated Wnt-mediated CRC development is considerably associated with cellular metabolic process involving nucleotides [6]. The nitrogenous nucleobases found in nucleotides are classified as purines, including adenine or guanine, or pyrimidines, such as cytosine, thymine, or uracil. Uric acid is produced during the breakdown of purine found in certain food and drinks. Intriguingly, the risk of gout is positively correlated with the intake of purine-rich red meat and alcohol, which correspond to two of risk factors for CRC. Moreover, gout has been linked to the development of several cancers, including CRC [7]. However, the underlying molecular mechanism of how purine promotes rectal cancer formation remains elusive.

Solute carrier family 28 member 2 (SLC28A2), also called concentrative nucleoside transporter 2 (CNT2), is found mainly in the gastrointestinal tract and kidney [8]. The *SLC28A2* gene, which is mapped to chromosome 15q21.1 in humans, encodes a sodium/nucleoside symporter preferentially located at the apical side of polarized epithelia, thereby mediating the unidirectional uptake of sodium ions coupled with nucleosides [9]. There are three members of the concentrative nucleoside transporter family: SLC28A1(CNT1), SLC28A2(CNT2), and SLC28A3(CNT3), which differ in their substrate selectivity, apart from uridine, which could be transported by all members. SLC28A1 carries pyrimidines and SLC28A2 transports purines, while SLC28A3 mediates the uptake of both pyrimidines and purines. In addition to natural substrates, their ability to transport nucleoside analogs used to treat several diseases has been considered as biomarkers of drug responsiveness [10]. SLC28A2, a sodium-dependent and purine-selective transporter, has been reported to translocate antiviral purine analogs. Additionally, it has also been suggested that deficiency in SLC28A2 contributes to 5–fluorouracil (5-FU), an anticancer uracil analog, resistance in pancreatic cancer [11]. However, in terms of intestinal epithelial cell polarity, the apical side confronts the intestinal lumen, while the basolateral side faces the blood [12]. Although SLC28A2 is favorably located at the apical side of polarized epithelia, in this study, 5-FU-based chemotherapy was given by continuous infusion into the blood circulation. Consequently, whether SLC28A2 expression affects CCRT effectiveness through its ability to transport nucleoside analogs or other unknown mechanisms remain an open question. Intriguingly, according to the human disease database MalaCards (https://www.malacards.org/search/results/SLC28A2), the *SLC28A2* gene is more likely to be associated with hyperuricemia, which may affect CRC formation. Accordingly, this study aimed at correlating SLC28A2 expression with patient survival and elucidating the role of SLC28A2 in rectal cancer development.

## 2. Patients and methods

### 2.1. Transcriptome profiling of rectal cancer

With the purpose of linking potential genes to CCRT effectiveness, a published dataset (GSE35452) of rectal cancer tissue blocks ($n = 46$) was employed for transcriptomic profiling. In this dataset, during colonoscopic screening before getting CCRT, biopsy specimens were collected. The raw microarray data (CEL files) from Human Genome U133 Plus 2.0 Array were computerized with the statistical software Nexus Expression 3.0 to determine the gene expression levels using all probe sets without any filtering/mapping method. As determined by the response to CCRT, the samples were split into “nonresponders” and “responders” groups, and the comparison of two groups was carried out under supervision. We identified differentially expressed genes in relation to nucleobase-containing compound metabolic process (GO: 0055134) and further selected those with a $p$-value under 0.005 and log2 ratio $> 0.5$ for further appraisal.

### 2.2. Patient recruitment

Approved by the Ethics Committee of Chi Mei Medical Center (IRB10302014), this research was undertaken using formalin-fixed paraffin-embedded (FFPE) rectal cancer tissue blocks ($n = 172$) from our biobank. The data regarding clinical and pathological findings and treatment outcomes were retrospectively reviewed and collected. The original clinical staging was defined by colonoscopy, and patients without distant metastasis as stated by chest X-ray and/or abdominopelvic computed tomography (CT) were included. Before proctectomy, all patients got a regimen of 45–50 Gy in twenty-five fractions over five weeks with continuous infusional 5-fluorouracil (5-FU)-based treatment concurrently. Before or after chemoradiotherapy, for patients with tumoral status at least T3 or nodal status at least N1, adjuvant chemotherapy was given and the most common adjuvant chemotherapy agents included FOLFOX, CapOX, and 5-FU for at least 4 months. All patients presented free circumferential resection margin following sphincter-saving low anterior resection. As a rule, lateral node dissection was performed when metastatic involvement was suspected, and none of our patients received lateral node dissection. Our rectal cancer patients usually visited the doctor and received follow-up screening every 3–6 months for 5 years. The surveillance included a carcinoembryonic antigen (CEA) blood test every 3 months and a colonoscopy and an abdominal CT or magnetic resonance imaging (MRI) scan every year.

### 2.3. Histopathological appraisal and immunohistochemical scoring

Without the clinical profiles of the patients, two skilled pathologists (Wan-Shan Li and Tzu-Ju Chen) surveyed all tumor samples to acquire more objective appraisal. Before and after chemoradiotherapy, the tumor and node stages were defined as reported by the seventh edition of the American Joint Committee on Cancer (AJCC) cancer staging system. The tumor regression grading system as reported by Dworak et al. [13] was utilized to forecast CCRT effectiveness in rectal cancer

patients. Immunohistochemical staining was carried out following protocols as mentioned by our prior research [14], and tissue slides were incubated with SLC28A2 primary antibody (NBP2–38763, 1:100) (Novus Biologicals, Littleton, CO, USA). The immunointensity of SLC28A2 staining was labeled as 0 (absent), 1 + (weak), 2 + (moderate), 3 + (strong), or 4 + (intense). Stained tumor cells with intensity identical to or beyond 3 + were regarded to have high SLC28A2 expression.

### 2.4. Statistical analysis

All data were statistically analyzed by utilizing Statistical Product and Service Solutions (SPSS) software version 22.0. The correlations between the clinicopathological characteristics and the expression levels of SLC28A2 were appraised using Pearson's chi-squared test. The Kaplan–Meier method was utilized to plot survival curves, and the log-rank test was employed to statistically compare two groups measured from surgery to the date of cancer death (DSS), first local recurrence (LRFS), or first metastasis (MeFS). Multivariate Cox proportional hazards regression analysis using parameters that have prognostic utility at the univariate level was utilized to identify independent prognostic variables. A two-tailed test with a $p$-value under 0.05 was considered as statistical significance.

## 3. Results

### 3.1. SLC28A2 is the most considerably upregulated gene associated with nucleobase-containing compound metabolic process in CCRT-resistant rectal cancer patients

To link potential genes with the efficacy of neoadjuvant CCRT, a published dataset (GSE35452) of rectal cancer tissue blocks ($n = 46$) was employed for transcriptomic profiling. There were twenty-two patients (47.8 %) grouping as nonresponders and twenty-four patients (52.2 %) labeling as responders, which were utilized to compare and find predictive genetic biomarkers. Paying attention to nucleobase-containing compound metabolic process (GO: 0055134), we identified 2 probes covering 2 transcripts: *SLC28A2* and guanine deaminase (*GDA*), related to CCRT resistance (Table 1 and Fig. 1). Between these two genes, the mRNA expression level of *SLC28A2* was prominently higher (log2 ratio = 1.3578, $p = 0.0014$) and observed specifically in the intestine. Accordingly, we were curious about the expression levels and clinical significance of SLC28A2 in our rectal cancer cohort.

### 3.2. Clinicopathological features of our patient cohort in rectal cancer

We recruited 172 rectal adenocarcinoma patients getting preoperative CCRT, and their clinicopathological characteristics are recorded in Table 2. Most cases were male ($n = 108$, 62.8 %) and fewer than seventy ($n = 106$, 61.6 %). At the time of original clinical diagnosis, the tumoral status of 91 patients (52.9 %) was advanced (cT3–T4), and the lymph node status of 47 patients (27.3 %) was positive (cN1–N2). Following CCRT, positive lymph node (ypN1–N2) was examined in 49 patients (28.5 %), and 86 patients (50 %) had an invasion depth over the muscularis propria (ypT3–T4). Moreover, vascular invasion and perineural invasion were examined in 15 cases (8.7 %) and 5 cases (2.9 %), correspondingly. Furthermore, to forecast CCRT effectiveness in rectal cancer patients, the tumor regression grade was used. The scores of the Dworak system revealed that 37 cases (21.5 %) had little or no regression (grade 0–1), while 17 cases (9.9 %) had a total regression (grade 4). Additionally, tumor downstaging was observed in 22 patients (12.8 %).

### 3.3. SLC28A2 immunointensity and its relationship to clinicopathological variables

We carried out immunohistochemical staining to explore the immunointensity and clinical relevance of SLC28A2 in our rectal cancer cohort. As displayed in Fig. 2**A–C**, the immunointensity of SLC28A2 staining was significantly stronger in CCRT-resistant rectal cancer tissue blocks. Strong SLC28A2 immunointensity was significantly linked to female patients ($p = 0.032$), vascular invasion ($p = 0.021$), and post-CCRT tumor invasion and positive lymph node ($p < 0.001$ and $p = 0.005$). Notably, patients with strong SLC28A2 immunointensity had no tumor downstaging ($p < 0.001$).

### 3.4. The prognostic involvement of SLC28A2 immunoexpression in rectal cancer

As listed in Table 3, 31 patients (18 %) died owing to rectal cancer. Besides, the development of local recurrence and distant metastasis were found in 27 patients (15.7 %) and 31 patients (18 %), correspondingly. Subsequently, we performed univariate and multivariate analyses to appraise the prognostic markers of local recurrence-free survival (LRFS), metastasis-free survival (MeFS), and disease-specific survival (DSS). At the univariate level, high SLC28A2 immunoexpression (Fig. 3**A–C**), advanced post-CCRT tumoral status, and low tumor regression grade were considerably unfavorably linked to all three endpoints (all $p \leq 0.0333$). Additionally, presence of vascular invasion was substantially correlated with inferior DSS and LRFS (both $p \leq 0.0184$), and pre-CCRT lymph node metastasis was an unfavorable prognostic marker only for LRFS ($p = 0.007$). In the multivariate analysis, both high SLC28A2 immunoexpression and low tumor regression grade were independently unfavorable prognostic factors for all three endpoints (all $p \leq 0.013$) (Table 4).

### 3.5. SLC28A2 upregulation is more likely to be linked with stem cell homeostasis in rectal cancer

A gene coexpression analysis was carried out to link *SLC28A2* with unrevealed biological functions in rectal cancer. We reviewed the top two hundred differentially expressed transcripts presenting positive correlations (Supplementary Table 1) or negative correlations (Supplementary Table 2) with *SLC28A2* employing the colorectal adenocarcinoma dataset (TCGA, $n = 594$). Subsequently, these genes were utilized to predict *SLC28A2* biological functions applying the PANTHER classification system. Regarding biological processes, the most prominent term positively correlated with *SLC28A2* was intestinal stem cell

**Table 1**
Summary of differentially expressed genes associated with nucleobase-containing compound metabolic process (GO: 0055134) in CCRT-resistant rectal adenocarcinoma.

| Probe | Comparison log ratio | Comparison $p$-Value | Gene symbol | Gene name | Biological process | Molecular function |
|---|---|---|---|---|---|---|
| 207249_s_at | 1.3578 | 0.0014 | ***SLC28A2*** | solute carrier family 28 (sodium-coupled nucleoside transporter); member 2 | nucleobase; nucleoside; nucleotide and nucleic acid metabolic process, purine nucleoside transport, transport | nucleoside binding, nucleoside:sodium symporter activity, purine nucleoside transmembrane transporter activity |
| 224209_s_at | 0.7893 | < 0.0001 | *GDA* | guanine deaminase | nervous system development, nucleobase; nucleoside; nucleotide and nucleic acid metabolic process | guanine deaminase activity, hydrolase activity, zinc ion binding |

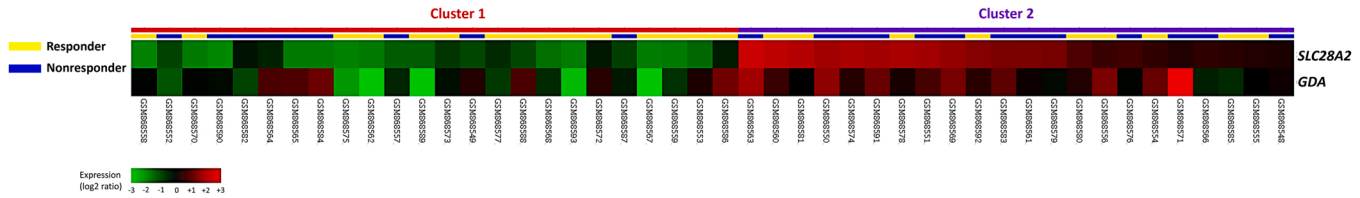


**Fig. 1. Transcriptome analysis of genes associated with nucleobase-containing compound metabolic process and the response to CCRT.** Green color represents the downregulated genes, and red color indicates the upregulated genes. We recognized *SLC28A2* as the most considerably upregulated gene correlated with nucleobase-containing compound metabolic process (GO: 0055134) among rectal cancer patients with CCRT resistance.

**Table 2**
Associations between SLC28A2 expression and clinicopathological factors in 172 rectal cancer patients managed by neoadjuvant CCRT.

| Parameter | | No. of case | SLC28A2 Expression | | *p*-Value |
|---|---|---|---|---|---|
| | | | Low Exp. (0–2) | High Exp. (3–4) | |
| **Gender** | Male | 108 | 76 | 32 | **0.032*** |
| | Female | 64 | 34 | 30 | |
| **Age** | < 70 | 106 | 66 | 40 | 0.625 |
| | ≧ 70 | 66 | 44 | 22 | |
| **Pre-Tx tumor status (Pre-T)** | T1–T2 | 81 | 54 | 27 | 0.527 |
| | T3–T4 | 91 | 56 | 35 | |
| **Pre-Tx nodal status (Pre-N)** | N0 | 125 | 82 | 43 | 0.480 |
| | N1–N2 | 47 | 28 | 19 | |
| **Post-Tx tumor status (Post-T)** | T1–T2 | 86 | 67 | 19 | **< 0.001*** |
| | T3–T4 | 86 | 43 | 43 | |
| **Post-Tx nodal status (Post-N)** | N0 | 123 | 87 | 36 | **0.005*** |
| | N1–N2 | 49 | 23 | 26 | |
| **Vascular invasion** | Absent | 157 | 105 | 52 | **0.021*** |
| | Present | 15 | 5 | 10 | |
| **Perineural invasion** | Absent | 167 | 109 | 58 | 0.057 |
| | Present | 5 | 1 | 4 | |
| **Tumor regression grade** | Grade 0–1 | 37 | 21 | 16 | 0.368 |
| | Grade 2–3 | 118 | 76 | 42 | |
| | Grade 4 | 17 | 13 | 4 | |
| **Tumor downstaging** | No | 150 | 88 | 62 | **< 0.001*** |
| | Yes | 22 | 22 | 0 | |

Tx, treatment; *, statistically significant.

homeostasis (GO: 0036335, fold enrichment: 76.47) (Fig. 4**A**). As to molecular functions, we identified that asparaginase activity (GO: 0004067, fold enrichment: > 100) was the most distinguished term positively connected to *SLC28A2* (Fig. 4**B**). Since cancer stem cells have been linked to therapy resistance in CRC [15], we speculate that CCRT resistance in rectal cancer may at least in part attribute to *SLC28A2* upregulation.

## 4. Discussion

Neoadjuvant CCRT is delivered before surgery to shrink a tumor or prevent cancer from spreading, which makes surgery less invasive and more effective. For patients with locally advanced rectal cancer, neoadjuvant CCRT has become the standard treatment, but the clinical outcomes are still far from satisfactory. Additionally, CCRT efficacy in rectal cancer patients is still determined by clinical assessment, which is lack of a more precise predictive tool such as genetic biomarkers. As the lower digestive system dominates cellular metabolism and absorption and CRC development has been considerably correlated with cellular metabolic process involving nucleotides [6], we employed a transcriptome dataset and focused on nucleobase-containing compound metabolic process (GO: 0055134) for data mining. We then identified the *SLC28A2* gene and demonstrated that high SLC28A2 immunoexpression is considerably linked to poor response to CCRT and inferior clinical outcomes in our patient cohort of rectal cancer. Accordingly, SLC28A2 expression can be a valuable predictive and prognostic biomarker for selecting patients who can benefit from neoadjuvant CCRT.

Disease associated with *SLC28A2* is more likely to be hyperuricemia. Intriguingly, the other gene *GDA* identified in this study can encode guanine deaminase, which converts guanine into xanthine, providing an indirect source of uric acid. One study indicated that gout is linked to the development of several cancers, including CRC [7], whereas a recent report showed that patients with gout have no significant correlation with increased risk of CRC incidence in Taiwan [16]. In the latter study, they speculated that the similar risks of CRC occurrence observed in patients with or without gout may attribute to the use of urate-lowering drug allopurinol, a xanthine oxidase inhibitor, which had been suggested to reduce the risk of CRC occurrence. However, we cannot rule out the possibility that allopurinol treatment may reduce the risk of CRC incidence through its anti-inflammatory effect.

It is widely accepted that excessive consumption of purine-rich red meat and alcohol may increase the risk of early CRC onset. In addition to dietary purines, some gut microbiota such as *E. coli*, another CRC risk factor, can trigger a substantial release of adenosine triphosphate (ATP) from host intestinal cells [17]. This ATP is then converted into adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine extracellularly by CD39 and CD73 expressed on the intestinal epithelium [18]. Furthermore, colon cancer within a hypoxic niche has been indicated to cause more adenosine production via stepwise CD39 and CD73 reactions [19]. In the tumor microenvironment (TME) of CRC, the accumulation of the immunosuppressive metabolite adenosine has been suggested to be implicated in tumor immune escape [18]. Nevertheless, in terms of cancer therapy, most studies targeted extracellular adenosine without appraisal of intracellular adenosine metabolism and function. It has been suggested that adenosine can stimulate the growth of colorectal cancer cells [20], but the underlying molecular mechanism remained unclear. As a sodium-dependent and purine-selective transporter, SLC28A2 can transport adenosine and guanosine in the large intestine. Upon absorbed, the purines can be utilized by intestinal cells or be metabolized into uric acid. Intracellular adenosine can also be interconverted into ATP. A recent study has indicated that intracellular ATP can drive cell motility and cytoprotection [21]. Collectively, in view of the redundant sources, including diet, *E. coli*, and hypoxia, controlling adenosine levels, targeting a purine-selective transporter such as SLC28A2 may provide a more specific way for rectal cancer therapies. In addition, we speculate that rectal cancer development is more likely to be affected by adenosine but not its end product uric acid.

It has been suggested that the gastrointestinal tract may maintain energy homeostasis partly via nutrient sensing and subsequent signal transduction to the brain and other tissues [22]. In fact, SLC28A2 has been considered as a putative transceptor, combining transporter and receptor functions, and to give rise to nucleoside sensing and signal transduction [9]. Adenosine transported by SLC28A2 is able to trigger

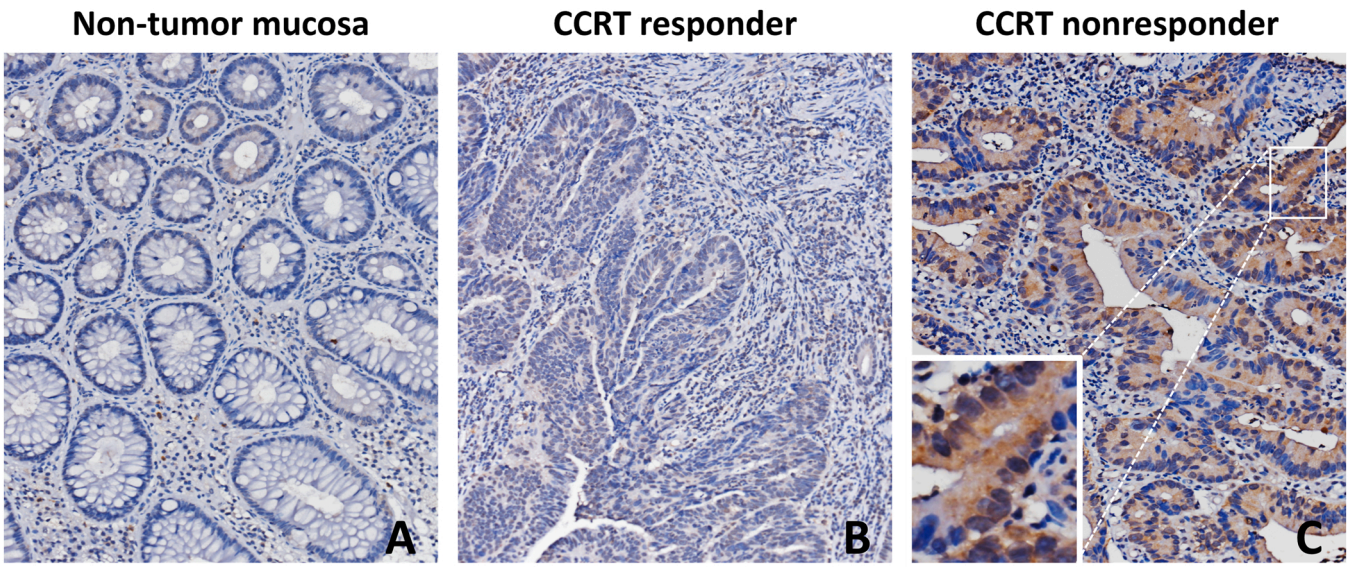


**Fig. 2. Immunohistochemical staining of SLC28A2.** (A) Non-neoplastic rectal mucosa showed no SLC28A2 immunoexpression. Rectal cancer samples exhibited (B) low SLC28A2 immunoexpression in patients responding to CCRT and (C) high SLC28A2 immunoexpression in patients with CCRT resistance. The staining showed predominantly cytoplasmic staining.

**Table 3**
Univariate log-rank analysis for important clinicopathological variables and SLC28A2 expression.

| Parameter | | No. of case | DSS | | LRFS | | MeFS | |
|---|---|---|---|---|---|---|---|---|
| | | | No. of event | *p*-Value | No. of event | *p*-Value | No. of event | *p*-Value |
| **Gender** | Male | 108 | 20 | 0.9026 | 7 | 0.2250 | 17 | 0.3520 |
| | Female | 64 | 11 | | 20 | | 14 | |
| **Age** | < 70 | 106 | 19 | 0.8540 | 18 | 0.6615 | 20 | 0.7427 |
| | ≧ 70 | 66 | 12 | | 9 | | 11 | |
| **Pre-Tx tumor status (Pre-T)** | T1–T2 | 81 | 10 | 0.0776 | 10 | 0.2261 | 11 | 0.1745 |
| | T3–T4 | 91 | 21 | | 17 | | 20 | |
| **Pre-Tx nodal status (Pre-N)** | N0 | 125 | 19 | 0.0711 | 15 | **0.0070*** | 19 | 0.0973 |
| | N1–N2 | 47 | 21 | | 12 | | 12 | |
| **Post-Tx tumor status (Post-T)** | T1–T2 | 86 | 7 | **0.0006*** | 7 | **0.0040*** | 8 | **0.0033*** |
| | T3–T4 | 86 | 24 | | 20 | | 23 | |
| **Post-Tx nodal status (Post-N)** | N0 | 123 | 21 | 0.5998 | 16 | 0.1320 | 20 | 0.4634 |
| | N1–N2 | 49 | 10 | | 11 | | 11 | |
| **Vascular invasion** | Absent | 157 | 25 | **0.0184*** | 21 | **0.0028*** | 27 | 0.4470 |
| | Present | 15 | 6 | | 6 | | 4 | |
| **Perineural invasion** | Absent | 167 | 29 | 0.2559 | 25 | 0.0940 | 30 | 0.9083 |
| | Present | 5 | 2 | | 2 | | 1 | |
| **Tumor regression grade** | Grade 0–1 | 37 | 13 | **0.0038*** | 10 | **0.0090*** | 14 | **0.0006*** |
| | Grade 2–3 | 118 | 17 | | 17 | | 16 | |
| | Grade 4 | 17 | 1 | | 0 | | 1 | |
| **SLC28A2 expression** | Low Exp. | 110 | 9 | **< 0.0001*** | 13 | **0.0333*** | 9 | **< 0.0001*** |
| | High Exp. | 62 | 22 | | 14 | | 22 | |

DSS, disease-specific survival; LRFS, local recurrence-free survival; MeFS, metastasis-free survival; *, statistically significant.

AMP-activated protein kinase (AMPK) activation in intestinal epithelial cells [23]. Moreover, intracellular AMP converted from adenosine can also activate the AMPK signaling [21], which implies that SLC28A2 function is dependent on energy metabolism. Metabolic stress may ascribe to nutrient depletion or nutrient excess. Generally, under nutrient deficiency, AMPK responds to increased AMP/ATP ratio for energy production, and AMPKα1 has been suggested to confer colorectal cancer cell survival under glucose limitation [24]. Nonetheless, we speculate that CRC cell survival may also be due to excessive purine intake and subsequent adenosine/SLC28A2-mediated AMPK activation. In addition, AMPK has also been indicated to sustain colorectal cancer stem cell activity [25], which at least in part provides a link between *SLC28A2* and intestinal stem cell homeostasis in our function prediction analysis (Fig. 4**A**).

With the ability to transport antiviral or antitumor purine analogs, SLC28A2 could be used as drugs and regarded as biomarkers of drug responsiveness [10,26]. In addition to purine nucleosides, SLC28A2 also transports uridine and its deficiency has been indicated to cause 5-FU, an anticancer uracil analog, resistance in pancreatic cancer [11]. Nevertheless, the role of SLC28A2 expression in 5-FU CCRT effectiveness of rectal cancer remains poorly understood. Interestingly, we found that high SLC28A2 expression is associated with 5-FU CCRT resistance in rectal cancer, which suggests that SLC28A2 may link unknown factors instead of its ability to transport nucleoside analogs to CCRT resistance. To link *SLC28A2* with unrevealed biological functions in rectal cancer, we performed bioinformatic analysis and noticed that *SLC28A2* overexpression is more likely to correlate with stem cell homeostasis (Fig. 4**A**). Considering that cancer stem cells have been connected to both 5-FU and radiotherapy resistance in CRC [27,28], we conjecture that CCRT resistance in rectal cancer may at least in part be ascribed to *SLC28A2* overexpression. However, more experiments are needed to validate this hypothesis.

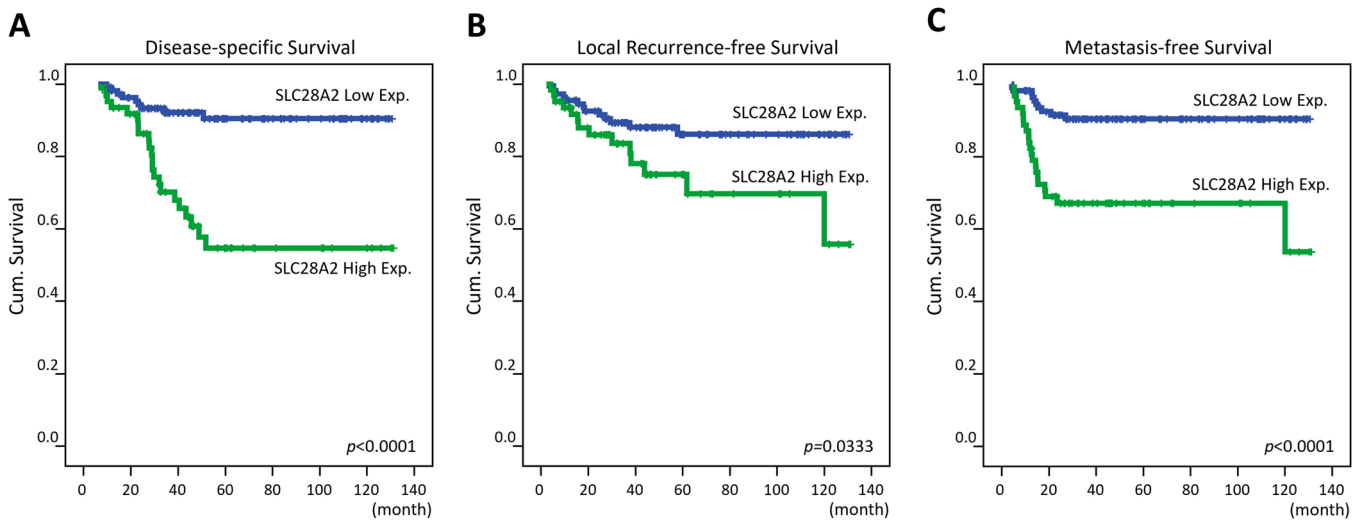


**Fig. 3. Kaplan–Meier survival analysis.** The results of Kaplan–Meier method with a log-rank test showed that high SLC28A2 immunoexpression was substantially connected to inferior (A) disease-specific survival (DSS), (B) local recurrence-free survival (LRFS), and (C) metastasis-free survival (MeFS).

**Table 4**
Multivariate analysis.

| Parameter | DSS | | | LRFS | | | MeFS | | |
|---|---|---|---|---|---|---|---|---|---|
| | H.R. | 95% CI | *p*-Value | H.R. | 95% CI | *p*-Value | H.R. | 95% CI | *p*-Value |
| **Tumor regression grade** | **2.450** | **1.215–4.950** | **0.012*** | **2.132** | **1.020–4.785** | **0.013*** | **2.702** | **1.353–5.405** | **0.005*** |
| **SLC28A2 expression** | **3.873** | **1.707–8.790** | **0.001*** | **2.145** | **0.899–12.414** | **< 0.001*** | **3.581** | **1.648–7.781** | **0.001*** |
| **Vascular invasion** | 1.683 | 0.648–4.374 | 0.285 | 1.363 | 0.500–3.714 | 0.545 | – | – | – |
| **Post-Tx tumor status (Post-T)** | 1.815 | 0.728–4.524 | 0.201 | 1.833 | 0.744–4.517 | 0.188 | 1.552 | 0.657–3.666 | 0.316 |
| **Pre-Tx nodal status (Pre-N)** | – | – | – | 1.313 | 0.599–2.877 | 0.496 | – | – | – |

DSS, disease-specific survival; LRFS, local recurrence-free survival; MeFS, metastasis-free survival; *, statistically significant.

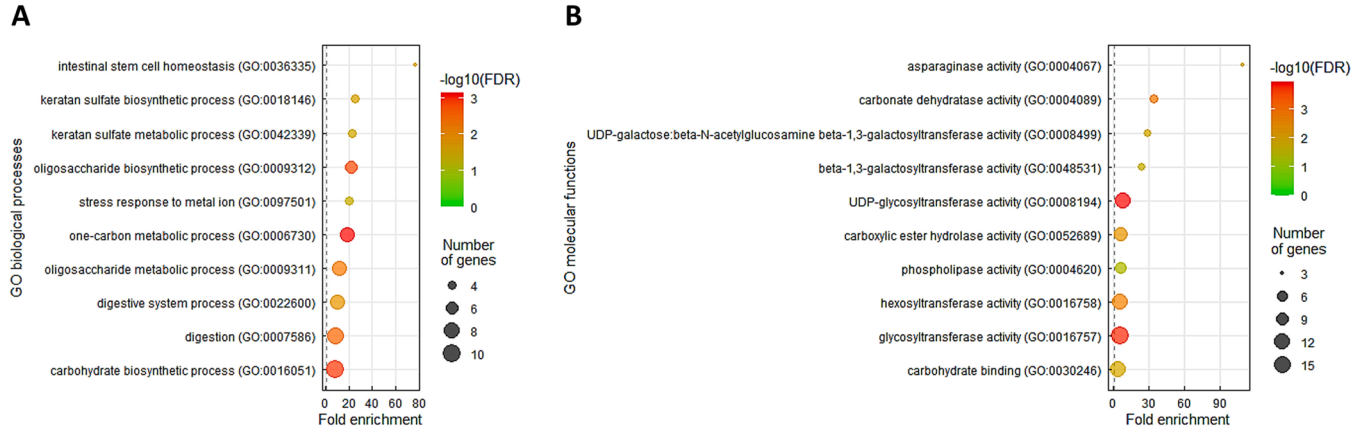


**Fig. 4. The Gene Ontology (GO) terms enriched in *SLC28A2* upregulation.** The genes that were coexpressed with *SLC28A2* in the colorectal adenocarcinoma dataset (TCGA, $n = 594$) were reviewed employing the cBioPortal online platform. The top two hundred transcripts co-upregulated with *SLC28A2* were further analyzed applying the PANTHER classification system based on (A) biological processes or (B) molecular functions and ranked by fold enrichment.

Intriguingly, our function prediction analysis also revealed that asparaginase activity was the most notable term correlated with *SLC28A2* upregulation (Fig. 4**B**). Asparagine plays a critical role for leukemic cell survival, but some leukemic cells cannot synthesize asparagine *de novo* from glutamine and aspartate owing to the absent of the enzyme asparagine synthase. This provides a therapeutic vulnerability in leukemia. Accordingly, with the ability to hydrolyze asparagine, asparaginase has been used for leukemia treatment for over three decades [29]. However, asparaginase has been shown to have little efficacy in *APC*- or β-catenin-mutant [downstream of glycogen synthase kinase 3 alpha (GSK3α)] colorectal cancer, because GSK3α-dependent protein degradation provides a catabolic asparagine source and causes asparaginase resistance [30]. Since approximately 85 % of CRCs have *APC* or β-catenin mutations, especially in left-sided colon and rectum [31], *SLC28A2* upregulation with high asparaginase activity may not be toxic to most rectal cancers.

## 5. Conclusion

We demonstrated that high SLC28A2 immunoexpression is

substantially linked to an advanced stage, poor response to CCRT, and worse patient survival in our rectal cancer cohort. An enhanced understanding of the molecular characterization of rectal cancer may provide a link between *SLC28A2* and its biological functions. Collectively, SLC28A2 expression can be a valuable predictive and prognostic marker for rectal cancer patients and be an encouraging therapeutic target for those with CCRT resistance.

## CRediT authorship contribution statement

**Conceptualization**: C.-L. Chou and H.-Y. Lai; **Methodology:** H.-P. Chen, C.-I. Chen, K.-W. Liu, T.-J. Chen, Y.-F. Tian, Y.-H. Kuo, W.-S. Li, H.-H. Tsai, L.-C. Wu, C.-F. Yeh, and C.-F. Li; **Investigation**: H.-P. Chen, C.-I. Chen, K.-W. Liu, T.-J. Chen, Y.-F. Tian, Y.-H. Kuo, W.-S. Li, H.-H. Tsai, L.-C. Wu, C.-F. Yeh, and C.-F. Li; **Formal analysis**: H.-P. Chen, C.-I. Chen, K.-W. Liu, T.-J. Chen, Y.-F. Tian, Y.-H. Kuo, W.-S. Li, H.-H. Tsai, L.-C. Wu, C.-F. Yeh, and C.-F. Li; **Resources**: L.-C. Wu, C.-F. Yeh, and C.-F. Li; **Validation**: H.-P. Chen, C.-I. Chen, K.-W. Liu, T.-J. Chen, Y.-F. Tian, Y.-H. Kuo, W.-S. Li, and H.-H. Tsai; **Visualization**: H.-P. Chen, C.-I. Chen, K.-W. Liu, T.-J. Chen, Y.-F. Tian, Y.-H. Kuo, W.-S. Li, and H.-H. Tsai; **Writing – original draft**: C.-L. Chou and H.-Y. Lai; **Writing – review & editing**: H.-Y. Lai; **Funding acquisition**: C.-L. Chou; **Supervision:** C.-L. Chou and H.-Y. Lai. All authors contributed to the article and approved the submitted version.

## Ethics approval and consent to participate

This study and its use of tumor samples that were deidentified from the biobank was approved by the Ethics Committee and Institutional Review Board of Chi Mei Medical Center (10302014) and followed the ethical guidelines of the Helsinki Declaration and the regulations of our government.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Availability of data and materials

The dataset analyzed in the current study (GSE35452) is available in a published transcriptome dataset from the Gene Expression Omnibus (GEO) database (National Center for Biotechnology Information, Bethesda, MD, USA).

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2022.154158.

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