

Increased *ENT2* expression and its association with altered purine metabolism in cell lines derived from different stages of colorectal cancer

SAFAA M. NAES^{1,2}, SHARANIZA AB-RAHIM¹, MUSALMAH MAZLAN¹,
NURUL AZMIR AMIR HASHIM¹ and AMIRAH ABDUL RAHMAN¹

¹Department of Biochemistry and Molecular Medicine; ²Institute of Medical and Molecular Biotechnology,
Faculty of Medicine, Universiti Teknologi MARA, Cawangan Selangor, Kampus Sungai Buloh, Jalan Hospital
Sungai Buloh, Selangor 47000, Malaysia

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Abstract. Colorectal cancer (CRC) is one of the most prevalent malignant cancer types worldwide. Although the purine metabolism pathway is vital for cancer cell survival, little is known about the role of equilibrative nucleoside transporter 2 (*ENT2*) in CRC development and its association with purine metabolites. The aim of the present study was to evaluate the levels of hypoxanthine phosphoribosyl transferase (HPRT), hypoxanthine and uric acid (UA), as well as xanthine oxidase (XO) activity, and investigate their association with *ENT2* expression levels in a normal human colon cell line and CRC cell lines derived from different stages of CRC. These analyses were performed using the normal colon CCD-841CoN cell line and a panel of human CRC cell lines comprising SW480, HCT15 and HCT116, which represent Dukes' B, C and D stages, respectively. Reverse transcription-quantitative PCR was performed to determine the level of *ENT2* mRNA expression. In cells of all CRC stages, the levels of HPRT and hypoxanthine were significantly higher ($P < 0.05$), while XO activity and UA levels were significantly decreased ($P < 0.05$), compared with those in the CCD-841CoN cell line. *ENT2* expression was found to be elevated in cells derived from all

stages of CRC. The Dukes' D stage cell line had higher levels of HPRT and hypoxanthine, although its *ENT2* level was not significantly lower than that of the Dukes' B and C stage cell lines. Increased levels of HPRT and hypoxanthine in various stages of CRC may indicate an increase in the activity of the salvage pathway. The increased expression of *ENT2* implies the importance of the *ENT2* protein in facilitating hypoxanthine transport, which is required for enhanced DNA synthesis via hypoxanthine recycling. In conclusion, *ENT2* may have potential as a target in the development of CRC therapeutics.

Introduction

Globally, colorectal cancer (CRC) is the third most common malignant cancer in terms of incidence and the second in terms of mortality (1). In 2018, ~10% of all new cases of cancer and cancer-associated mortalities were recorded as being due to CRC (2). The Dukes' staging system classifies CRC into four groups known as Dukes' A, B, C and D, which represent stages I, II, III and IV, respectively (3). CRC develops through a multistep process, including morphological, histological and genetic alterations that accumulate over time (4). Furthermore, metabolic reprogramming allows cancer cells to maintain redox homeostasis while increasing ATP production to meet their high energy demand (5).

Recently, purine metabolism has become a topic of interest in CRC research, and alteration of the purine metabolism pathway, including the modification of enzyme activities, has been shown to be associated with disease progression (6). Purine metabolites supply cells with vital energy and cofactors to enhance cell survival and proliferation (7). Purine nucleosides (purine metabolites), such as adenosine, guanine and inosine, function as signaling molecules (8) and precursors to ATP and GTP nucleotides during DNA and RNA synthesis (7). An insufficient supply of nucleotides during DNA replication may reduce the rate of cell replication, eventually triggering DNA damage and genomic instability (9).

Purine levels are maintained by the *de novo* and salvage biosynthetic pathways, whereby under normal physiological conditions, degraded bases are recycled via a salvage pathway

Correspondence to: Dr Amirah Abdul Rahman, Department of Biochemistry and Molecular Medicine, Faculty of Medicine, Universiti Teknologi MARA, Cawangan Selangor, Kampus Sungai Buloh, Jalan Hospital, Sungai Buloh, Selangor 47000, Malaysia
E-mail: amirahar@uitm.edu.my

Abbreviations: CRC, colorectal cancer; ELISA, enzyme-linked immunosorbent assay; ENT, equilibrative nucleoside transporter; HPRT, hypoxanthine phosphoribosyl transferase; IMP, inosine monophosphate; NT, nucleoside transporter; RT, reverse transcription; qPCR, quantitative PCR; ROS, reactive oxygen species; UA, uric acid; XO, xanthine oxidase

Key words: CRC, *ENT2*, hypoxanthine, hypoxanthine phosphoribosyl transferase, XO

to sustain the cellular purine pool (10). Moreover, existing bases in the extracellular matrix can be transported into the cells to generate nucleotides (7). Conversely, high purine levels are required in the environment of cancer cells, which necessitates upregulation of the *de novo* biosynthetic pathway (7). Previous research has indicated that the treatment of cancer cells with purine anti-metabolites blocks DNA synthesis in the cells, thereby inhibiting their growth (11,12).

Hydrophilic purine nucleosides require specific membrane transport proteins known as nucleoside transporters (NTs) to transport them across plasma membranes. NTs are classified according to their transport mechanisms as Na⁺-dependent concentrative transporters, which are encoded by the solute carrier family 28 (*SLC28*) gene, and equilibrative NTs (ENTs), which are encoded by the *SLC29* gene (13). The ENTs are bidirectional transporter proteins that facilitate the movement of nucleosides across the cell membrane driven solely via the concentration gradient between nucleoside permeants inside and outside the cell (14). The ENT2 protein is able to transport nucleosides and a wide range of purine and pyrimidine nucleobases. Although the exact function of ENT2 is unclear, its capacity and affinity for the translocation of hypoxanthine has been reported to be higher than that of ENT1 (15,16).

Hypoxanthine is a purine substrate in the salvage pathway for nucleotide synthesis, as well as the first metabolite in the purine catabolism process (17). Consequently, any interruption in the expression or activity of *ENT2* could be deleterious to the cells, as the intracellular accumulation of reactive oxygen species (ROS), a by-product of the purine catabolism pathway, might disrupt the redox balance and ultimately kill the cancer cells (18). Furthermore, ENT2 and other NTs play crucial roles in the clearance from the body of certain anticancer nucleoside analogs, such as 5-fluorouracil, which is widely used as a chemotherapeutic drug for the treatment of gastrointestinal cancer, particularly CRC (19), and antiviral drugs, such as remdesivir, which is used as a treatment for COVID-19 (20). The current study therefore aimed to compare the levels of hypoxanthine phosphoribosyl transferase (HPRT), hypoxanthine/xanthine and uric acid (UA), as well as the activity of xanthine oxidase (XO) in CRC cell lines and a normal colorectal cell line. It may be noted that enzyme activity measurements are typically used to estimate the concentration of enzyme present in a sample under certain conditions (21). In addition, the levels of *ENT2* mRNA expression were evaluated and compared in cells derived from CRC of different stages.

Materials and methods

Cell culture. All cell lines were purchased from the American Type Culture Collection (ATCC). A panel of human CRC cell lines was used, where each cell line represented a different CRC stage, as well as a normal colon epithelial cell line. CCD-841CoN (cat. no. CRL-1790) was used as the normal colon epithelial cell line, and SW480 (cat. no. CCL-228; Dukes' B), HCT15 (cat. no. CCL-225; Dukes' C) and HCT116 (cat. no. CCL-247; Dukes' D) were used as the CRC cell lines. These CRC stages were classified according to the tumor stage following Dukes' classification criteria by the ATCC and previous studies (22,23). All cells were cultured in Dulbecco's modified Eagle's medium (high glucose; Nacalai Tesque, Inc.)

supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (MilliporeSigma). The cells were incubated at 37°C in a humidified incubator (BINDER GmbH) with 5% CO₂.

Cell lysate preparation. Following cell quantification, 1x10⁶ cells were resuspended in 100 µl appropriate buffer as provided with the kit used for each assay. The cells were then homogenized using an Ultrasonic Vibra-Cell™ homogenizer (Sonics & Materials, Inc.) with pulses of 5 sec on and 25 sec off for 10 cycles. The tube containing the cell lysate was homogenized in ice to reduce the heat produced by the ultrasonic treatment. Tubes containing cell lysate and debris were then centrifuged at 24,104 x g for 10 min at 4°C. The supernatants were transferred to new tubes and kept at -20°C until required for further use.

HPRT enzyme-linked immunosorbent assay (ELISA) assay. The HPRT level in the cells was measured using a sandwich ELISA kit for HPRT (cat. no. SEA717Hu; Cloud-Clone Corp.) according to the manufacturer's instructions. The cells were lysed as aforementioned. The detection method is based on the colorimetric reaction of horseradish peroxidase following the addition of a tetramethylbenzidine substrate. The absorbance was measured immediately at 450 nm using a microplate reader (BMG Labtech GmbH).

XO assay. The activity of XO was quantified spectrophotometrically using a Xanthine Oxidase Activity Colorimetric/Fluorimetric Assay Kit (cat. no. K710; BioVision, Inc.; Abcam). In this assay, XO in the sample oxidizes xanthine to hydrogen peroxide (H₂O₂), which reacts stoichiometrically with OxiRed Probe to generate a color that can be detected at 570 nm. Briefly, 44 µl assay buffer, 2 µl enzyme mix, 2 µl substrate mix and 2 µl OxiRed probe were mixed and added to the designated wells. The reactions were initiated by the addition of 50 µl prepared standard or cell lysate into each well. Absorbance (A) measurements A1 and A2 were taken after 20 min incubation in the dark at room temperature using a microplate reader (BMG Labtech GmbH) at a wavelength of 570 nm. A standard curve was plotted for several dilutions of H₂O₂ standard and the volume was adjusted to 50 µl/well using distilled water. The XO activity of each sample was calculated using the following equation: XO activity (mU/ml)=(B x dilution factor)/(T2-T1) x V, in which B is the amount of H₂O₂ generated by XO according to the standard curve (nmol), T1 is the time of the first reading (A1, min), T2 is the time of the second reading (A2, min) and V is the pre-treated sample volume added to the reaction well (ml).

Hypoxanthine/xanthine assay. The hypoxanthine/xanthine level in all cell lines was detected using a Xanthine/Hypoxanthine Colorimetric/Fluorimetric Assay Kit (cat. no. K685; BioVision, Inc.; Abcam) according to the manufacturer's instructions. In brief, 1x10⁶ cells were incubated on ice with 100 µl ice-cold xanthine assay buffer for 10 min. After centrifugation at 24,104 x g for 5 min, the supernatant was collected and incubated with the reaction mix for 30 min at room temperature. A standard curve was plotted using several dilutions of xanthine standard and the volume was adjusted

Table I. Primer sequences used for quantitative PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
<i>ENT2</i>	CCACTCTCTCACC GAAGCCTAA	GCAGGAAGAACAGCACCAACA
<i>GAPDH</i>	GCATCCTGGGCTACACTGAG	TCCTCTTGTGCTCTTGCTGG
<i>HPRT1</i>	GAGTCCTATTGACATCGCCAGT	TCCGCCCAAAGGGAAGTAT

ENT2, equilibrative nucleoside transporter 2; *HPRT*, hypoxanthine phosphoribosyl transferase.

to 50 μ l/well with xanthine assay buffer. The absorbance was measured at a wavelength of 570 nm.

UA assay. UA quantification was performed using a Uric Acid Colorimetric/Fluorometric Assay Kit (cat. no. K608; BioVision, Inc. Abcam) according to the manufacturer's instructions. Briefly, 46 μ l UA assay buffer, 2 μ l UA enzyme mix and 2 μ l UA probe were mixed and added to the designated wells on the plate. The reactions were initiated by the addition of 50 μ l prepared UA standard or cell lysate to each well. The plates were then incubated for 30 min at 37°C and protected from light. A standard curve was plotted using several dilutions of UA standard and the volume was adjusted to 50 μ l/well with UA assay buffer. The absorbance was measured at a wavelength of 570 nm.

RNA isolation and cDNA synthesis. Total RNA from all cell lines was extracted using a NucleoSpin RNA mini kit for RNA purification (cat. no. 740955; Macherey-Nagel, GmbH & Co. KG) according to the manufacturer's instructions. The total RNA concentration and the A230/208 and A260/280 nm ratios of the initial RNA were measured to assess the RNA quality using a SpectraMax® QuickDrop™ UV-Vis Spectrophotometer (Molecular Devices, LLC). RNA integrity was assessed via a gel electrophoresis assay. Next, 200 ng total RNA was used for reverse transcription (RT) using a SensiFAST™ cDNA Synthesis kit (cat. no. BIO-650531 Bioline; Meridian Bioscience, Inc.) according to the manufacturer's instructions.

Quantitative PCR (qPCR) of *ENT2*. Following RT, qPCR was performed using a CFX96™ Touch Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). The reactions were carried out in triplicate using a SensiFAST™ SYBR® & Fluorescein Kit (cat. no. BIO96005; Bioline; Meridian Bioscience, Inc.) following the manufacturer's instructions. The sequences of the oligonucleotide primers that were used for qPCR amplification are listed in Table I. Each reaction was performed in a final volume of 10 μ l and the primer concentration was 400 nM. Thermocycling was conducted with an initial start cycle at 95°C for 2 min, followed by 39 cycles at 95°C for 5 sec and 60°C for 30 sec. To confirm product specificity, melting curve analysis was performed after each amplification. As normalization against a single reference gene may not be considered adequate (24), the mRNA level of *ENT2* was normalized by the geometric averaging of two reference genes, *GAPDH* and *HPRT*, to obtain the most reliable results in the gene transcription analysis. The stability and reliability of *GAPDH* and *HPRT1* as reference genes in the CRC and normal colon cell

lines were determined via measurement of their quantification cycle (Cq) value and calculating the coefficient of variation using CFX96 software (Bio-Rad Laboratories, Inc.). The fold-change in *ENT2* expression of the human CRC cell lines relative to the normal colon cell line was determined using the $2^{-\Delta\Delta Cq}$ method (25). The geometric average (GeoMean) of the relative quantities $[(E_{REF})^{\Delta Cq_{REF}}]$ of the two reference genes was utilized to determine the expression of the gene of interest (GOI) as shown in the following equation: Relative gene expression = $[(E_{GOI})^{\Delta Cq_{GOI}}] / \text{GeoMean}[(E_{REF})^{\Delta Cq_{REF}}]$.

Statistical analysis. All data were statistically analyzed using IBM SPSS Statistical Software version 27 (IBM Corp.). The results are expressed as the mean \pm SD obtained from three independent experiments where each assay was performed in triplicate. The significant differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons tests. Statistical evaluation of *ENT2* gene expression was performed using CFX96 software followed by one-way ANOVA with Tukey's multiple comparisons tests. The correlations between *ENT2* expression and the different stages of CRC were determined by Spearman's correlation test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Determination of *HPRT* levels. *HPRT* levels in the normal colon and CRC cell lines were determined by the binding of *HPRT* to an antibody in an ELISA experiment. When compared with normal colon cells, the levels of *HPRT* in the SW480 ($P = 0.0001$), HCT15 ($P = 0.013$) and HCT116 ($P < 0.0001$) cell lines were significantly higher. *HPRT* levels in the cell lines representing Dukes' stages B and D CRC were significantly higher compared with that in the cell line representing Dukes' stage C ($P = 0.0079$ and $P = 0.0003$, respectively; Fig. 1).

Determination of *XO* activity. The *XO* enzymatic activity in the CRC cell lines representing the different stages of CRC were all significantly lower (all $P < 0.0001$) than those in the normal colon cells (Fig. 2).

Determination of hypoxanthine/xanthine level. The hypoxanthine/xanthine levels of the Dukes' B, C and D stage CRC cell lines were significantly higher compared with those in the normal colon cells ($P = 0.007$, $P = 0.004$ and $P < 0.0001$, respectively; Fig. 3). The hypoxanthine/xanthine levels ranged from 2.86 to 5.08 μ M in the CRC cell lines compared with 0.92 μ M

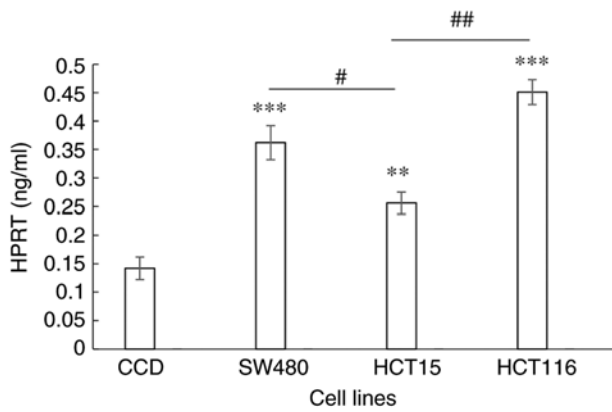


Figure 1. HPRT level in a normal colon epithelial cell line and the colorectal cancer SW480, HCT15 and HCT116 cell lines, which are derived from Dukes' B, C and D stage cancer, respectively. Data are presented as the mean \pm SD (n=9). Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc tests. ** $P \leq 0.001$, *** $P \leq 0.0001$ vs. CCD; # $P \leq 0.05$, ## $P \leq 0.01$. HPRT, hypoxanthine phosphoribosyl transferase; CCD, CCD-841CoN normal colon epithelial cells.

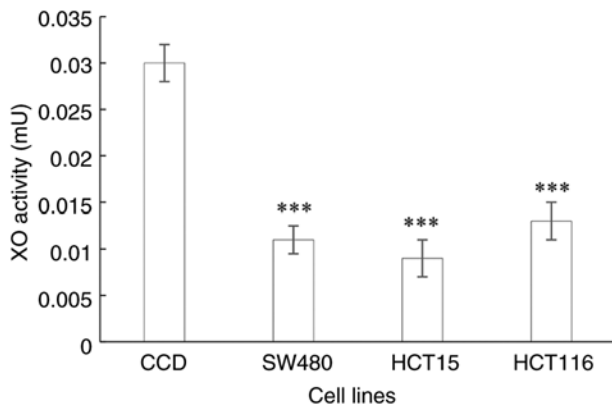


Figure 2. XO activity in a normal colon epithelial cell line and the colorectal cancer SW480, HCT15 and HCT116 cell lines, which are derived from Dukes' B, C and D stage cancer, respectively. Data are presented as the mean \pm SD (n=9). Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc test. *** $P \leq 0.0001$ vs. CCD. XO, xanthine oxidase; CCD, CCD-841CoN normal colon epithelial cells.

in the normal colon cells. The hypoxanthine/xanthine levels were observed to increase as the CRC stage advanced, with 3.1-, 4.2- and 5.5-fold increases in Dukes' B, C and D stages, respectively, compared with the normal colon control. The hypoxanthine/xanthine level was significantly higher in the Dukes' D CRC cell line than that in the Dukes' B cell line ($P=0.003$).

Determination of UA level. The end product of purine catabolism is UA. When compared with that in the normal colon cells, the UA level in the HCT15 and HCT116 cell lines was significantly downregulated ($P < 0.0001$ and $P = 0.0002$, respectively; Fig. 4). Furthermore, UA levels were significantly decreased in the CRC cell lines representing Dukes' C and D stages compared with those in the Dukes' B CRC cell line ($P < 0.0001$ and $P = 0.0008$, respectively).

***ENT2* gene expression.** *ENT2* was significantly upregulated in all CRC cell lines when compared with that in the normal

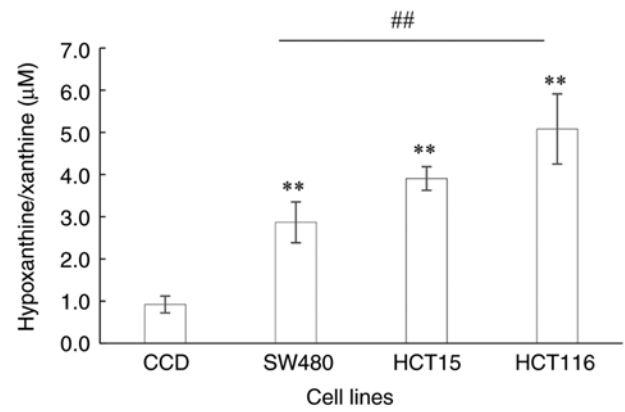


Figure 3. Level of hypoxanthine/xanthine in a normal colon epithelial cell line and the colorectal cancer SW480, HCT15 and HCT116 cell lines, which are derived from Dukes' B, C and D stage cancer, respectively. Data are presented as the mean \pm SD (n=9). Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc test. ** $P \leq 0.001$, *** $P \leq 0.001$. CCD, CCD-841CoN normal colon epithelial cells.

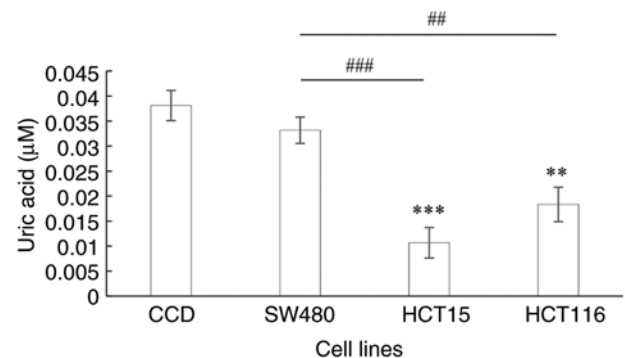


Figure 4. Uric acid level in a normal colon epithelial cell line and the colorectal cancer SW480, HCT15 and HCT116 cell lines, which are derived from Dukes' B, C and D stage cancer, respectively. Data are presented as the mean \pm SD (n=9). Statistical analysis was performed using one-way analysis of variance followed by Tukey's post hoc test. ** $P \leq 0.001$, *** $P \leq 0.0001$ vs. CCD; ## $P \leq 0.001$, ### $P \leq 0.0001$ between CRC stages. CCD, CCD-841CoN normal colon epithelial cells.

colon cells ($P < 0.0001$; Fig. 5). The *ENT2* expression level was found to be 186-, 471- and 123-fold higher in cells from the Dukes' B, C and D stages of CRC, respectively, compared with that in the normal colon cell line. The *ENT2* expression level was the highest in the Dukes' C stage CRC cells. Notably, *ENT2* expression was lower in the cells of Dukes' stage D compared with those of Dukes' stage B, however this was insignificant. However, Spearman's correlation analysis for *ENT2* expression and CRC stage did not reveal a significant correlation ($\rho = 0.475$; $P = 0.128$; data not shown).

Discussion

Purine metabolism has been observed to be one of the most affected pathways in CRC (26,27) and other cancer types, including breast cancer (28) and urothelial carcinoma (29). Moreover, alterations in its enzymatic pattern (30) and purine metabolism dysfunction have been found to be associated with cancer progression. For example, several cancer types have

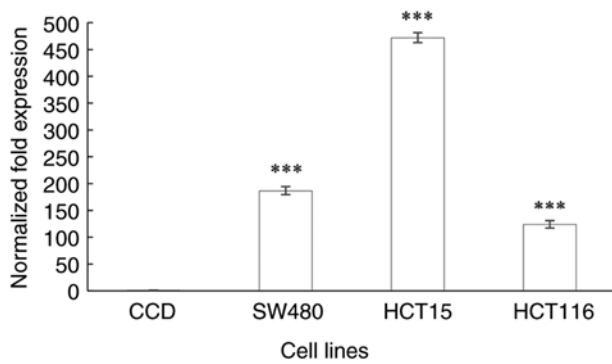


Figure 5. Differential expression of the equilibrative nucleoside transporter 2 gene (NM_001300868.1) in a normal colon epithelial cell line and the colorectal cancer SW480, HCT15 and HCT116 cell lines, which are derived from Dukes' B, C and D stage cancer, respectively. *GAPDH* and *HPRT1* were used as the reference genes. Data are presented as the mean \pm SD (n=9). Statistical differences were examined using one-way analysis of variance followed by Tukey's post hoc test. ***P \leq 0.0001 vs. CCD. CCD, CCD-841CoN normal colon epithelial cells.

been demonstrated to express a high level of HPRT, the key enzyme in the salvage pathway, thus promoting metabolic flux to produce purine mononucleotides, which are precursors in nucleic acid synthesis (31). While the hypoxanthine concentration gradient alone may not provide a compelling argument for the involvement of ENT2 in hypoxanthine transport, as hypoxanthine influx/efflux was not observed in the present study, the molecules and substrates involved in the intracellular purine metabolic pathway, including HPRT level, XO activity and UA level, were examined. The present study recorded a significant upregulation of HPRT and hypoxanthine/xanthine levels in different stages of CRC, notably in advanced stages, whereas the XO activities and UA levels in the CRC cell lines were markedly decreased compared with those in the normal colon cells.

The upregulation of HPRT in several solid cancer types, including breast, lung, colon and prostate cancer (32), the significantly higher HPRT activities in colorectal (33) and breast (34) cancer, and a notable increase in HPRT activity with the clinical progression of human colon carcinoma (35) have been documented, which support the findings of the current study. A metabolomic study reported elevated hypoxanthine levels in lymphoblastic leukemia and lung and prostate cancer (36), whereas under normal physiological conditions, hypoxanthine levels are very low (37). Similarly, another study recorded elevated hypoxanthine and decreased UA levels in CRC tissues (38). The data in the present study suggest that increased hypoxanthine levels are associated with CRC progression. However, further investigation into the association between hypoxanthine elevation and cancer progression *in vivo* is required to verify this.

Consistent with the findings of the present study, several investigations have reported diminished XO activity in cancer types such as colon (39), kidney and bladder (40) cancer. Low XO expression has been shown to be associated with more aggressive ovarian cancer (41), indicating that it plays a vital role in cancer progression and could be a risk factor (42). Furthermore, the reduction in XO activity may benefit cancer cells by lowering the generation of ROS,

which can trigger apoptosis and cell death. It has been demonstrated that reduced XO activity is advantageous to cancer cells, as it attenuates the detrimental effect of ROS generation on hypoxanthine degradation and catabolic pathway activation (43). Another parameter examined in the present study is UA levels. Previous studies have recorded elevated UA levels in advanced stages of rectal cancer (44) and head and neck carcinoma (45). Conversely, the current study recorded significantly decreased UA levels in the CRC cells of different stages compared with those in normal colon cells. The decrease in UA levels may be due to the low XO activity in all CRC stages evaluated.

The role of UA in tumorigenesis has not yet been determined. Several investigations have suggested that an elevated serum level of UA (hyperuricemia) is associated with an increased cancer risk due to its pro-inflammatory properties (46). It has also been suggested that UA plays an essential role as a potent antioxidant (31). Moreover, elevated UA levels might serve as a tumor defense, by protecting the tumor cells against free-radical oxidative damage by acting as antioxidant and by stimulating the immune system (47). Elevated UA levels have been shown to be associated with an increased survival time in patients with CRC (48). It is notable that the investigated metabolites and enzyme levels in the present study did not exhibit a strict association with Dukes' stage. This may be due to different mutational statuses in the different stages (49). Therefore, the rate of purine metabolism may not increase with higher staging. Advanced stages of cancer may have alternative cancer-protective/resistance mechanisms that are not limited to the purine metabolism pathway. Cells are particularly adaptable to intrinsic oxidative stress in the advanced stages of cancer, as they develop a strong ability to maintain the homeostatic balance between the generation and elimination of ROS (50-52).

The current study found that *ENT2* expression was significantly higher in cells from all stages of CRC than that in normal colon cells. Similarly, in previous studies, the level of *ENT2* was observed to be 2- to 5.5-fold higher in breast, kidney and prostate cancer cells than in corresponding normal cells (18,53), with the highest expression of *ENT2* detected in cancer derived from digestive organ tissues (54). *ENT2* has been reported to be highly expressed in colorectal biopsy samples (55), and to be highly expressed in four metastatic cell lines, namely LoVo, Colo205, SK-CO-1 and T84, and four primary CRC cell lines, namely HT29, Caco2, Colo320 and HCT116 (56). Furthermore, high levels of *ENT2* gene expression have been associated with the advanced stages of a variety of malignancies, including hepatocellular carcinoma, mantle cell lymphoma and ovarian carcinoma (57).

In the present study, *ENT2* expression was significantly higher in CRC cell lines that representing Dukes' B, C, and D compared with that in normal colon cells, but was not significantly lower in Dukes' D stage cell line (stage IV) than in the Dukes' B and C stage cell lines (stage II and III). This suggests that the expression of *ENT2* is not associated with CRC stage. This may imply that cancer cells, particularly in advanced stages, regulate *ENT2* expression for protection and survival. However, the mechanism of action of ENT2, specifically on nucleoside permeant influx/efflux, requires further exploration via *in vivo* studies. ENT2 is a bidirectional

transporter that has the ability to translocate a wide range of nucleosides into and out of cells; it has been documented that ENT2 is the main transporter responsible for cellular hypoxanthine efflux (58). In the current study, the low expression of *ENT2* in Dukes' D stage cells corresponded to the highest level of hypoxanthine, an endogenous substrate of *ENT2* (31). Increased *ENT2* expression may allow cancer cells to escape from the damaging effects of ROS on hypoxanthine degradation, while lowered *ENT2* expression may limit hypoxanthine efflux, in a process whereby hypoxanthine recycling supplies nucleotides to cancer cells to meet the demand for rapid cell proliferation.

During salvage purine metabolism, hypoxanthine is recycled to inosine monophosphate (IMP) by HPRT or synthesized through the deamination of adenosine by adenosine deaminase to produce inosine, which is converted to hypoxanthine by purine nucleoside phosphorylase (33). Subsequently, hypoxanthine is oxidized to produce xanthine via the purine catabolism pathway and then converted to UA via XO. During the catabolism pathway, ROS are produced as a by-product (42).

Studies have shown that intrinsic oxidative stress, such as ROS-induced stress, can effectively kill tumor cells via the activation of apoptotic processes (59–62). It has been observed that when ROS accumulation reaches a specific threshold that is incompatible with cell survival, cytotoxic effects are exerted, which induce apoptosis and slow cancer growth (63). ROS generation mediated by XO or NADH dehydrogenase has been linked with mitochondrial malfunction, which is considered to be the first indication of apoptotic intrinsic pathway activation (64). Excess hypoxanthine in cancer cells is then transported out of the cancer cells via the ENT2 transporter (58) to maintain low to average levels of ROS, or recycled to generate new nucleotides for DNA synthesis in order to meet the demand for rapid cell proliferation. Cancer cells may alter their metabolic regulation processes, in order to avoid the harmful effects of ROS and allow the cells to adapt and thrive in a hostile environment (38). These findings may explain the contradictory data observed for hypoxanthine levels in the serum of patients with CRC (6) and in CRC cells (65), particularly in advanced stages (27).

The upregulated HPRT and hypoxanthine levels reported in the CRC cells in the present study may imply that the activity of the salvage pathway is increased to meet the demand for rapid cell proliferation via the generation of nucleotides for DNA synthesis by hypoxanthine recycling, as previously proposed (31). Furthermore, the activity of the catabolism pathway may be reduced, as suggested by the decreased XO and UA levels, in order to preserve the antioxidant balance and avoid ROS-induced damage, which would lead to apoptosis (Fig. 6). Changes in the levels of enzymes and metabolites associated with the purine salvage and catabolism pathways may have diagnostic and prognostic importance for CRC progression. Furthermore, ENT2 may play a crucial role in the regulation of metabolite transport to enhance cancer cell survival and proliferation.

Since hypoxanthine influx/efflux was not observed in the present study, the role of ENT2 in hypoxanthine transport and the hypoxanthine concentration gradient has not been clearly demonstrated. However, various molecules and substrates

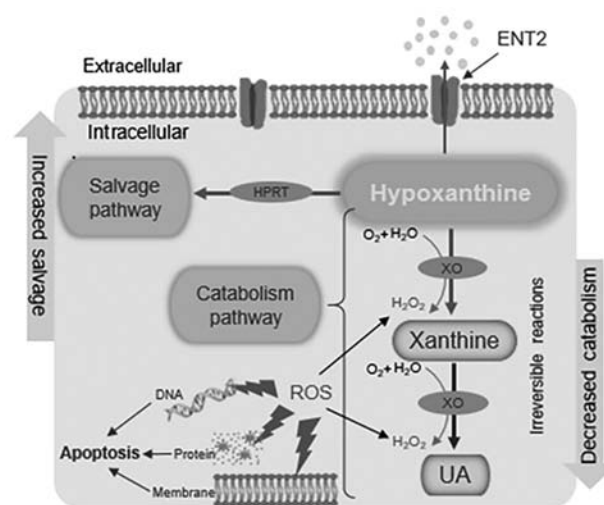


Figure 6. Schematic diagram of purine salvage and catabolism pathways in CRC cells. Modification of purine metabolism in CRC cells affects two processes: The activity of the salvage pathway is increased to meet the demands of rapid cell proliferation by the generation of new nucleotides for DNA synthesis via hypoxanthine recycling, and the activity of the catabolism pathway is decreased in order to avoid the detrimental effects of ROS. CRC, colorectal cancer; ROS, reactive oxygen species; ENT2, equilibrative nucleoside transporter 2; HPRT, hypoxanthine phosphoribosyl transferase; UA, uric acid; XO, xanthine oxidase.

involved in the purine metabolic pathway were examined, including XO activity, and HPRT and UA levels, which act intracellularly.

Increased *ENT2* expression in cells of higher Dukes' stages suggests that hypoxanthine may be being transported out of the cells as a form of defense, resulting in fewer substrates being available for the purine metabolism pathway. Evidence for this is provided by the inverse correlation of hypoxanthine levels with XO activity and UA levels. In this case, the degradation pathway is unfavorable to cells, and extracellular transport avoids the generation of high levels of ROS, which are toxic to cancer cells, and the antioxidant UA.

Notably, the level of HPRT was found to be upregulated in the CRC cell lines of the present study, which could imply that the hypoxanthine available inside the cells is converted to IMP via the salvage pathway to produce the nucleosides GMP and AMP for conversion to the nucleotides required for DNA synthesis and cell growth. However, further investigation is required for a definitive conclusion on the latter process. An *in vivo* experiment, which would give tangible support for the findings, is lacking and is a limitation of the present study; therefore, it could be the subject of future research.

In conclusion, CRC cells may utilize ENT2 in dual roles for protection and survival, that is, for avoiding the detrimental effects of ROS accumulation via purine catabolic metabolism, as demonstrated by decreased XO enzyme and UA production, and for utilizing hypoxanthine to meet the energy demands of rapid cell proliferation via the purine salvage pathway, as indicated by increased HPRT levels. Accordingly, the purine metabolism pathway appears to be an important metabolic pathway in the progression of CRC. However, the mechanism of action, in particular, whether additional pathways are affected by aberrant *ENT2* expression, requires further investigation.

In the current study, HPRT, XO, hypoxanthine and UA levels, and *ENT2* expression, were detected in a panel of CRC cell lines. However, further investigations such as measuring hypoxanthine influx/efflux and its association with low and high staging, assessment of protein expression levels by western blotting, performing *ENT2* gene silencing and detecting the ROS level in different stages of colorectal carcinogenesis are recommended to determine the relevance and role of *ENT2*, and its association with purine metabolism in colorectal carcinogenesis. However, although the metabolomic analysis of cell lines has been extensively used in disease research due to certain advantages, including lower costs, ease of control and simpler data interpretation than the analysis of *in vivo* models using human tissues and bio-fluids (66), *in vivo* experiments are necessary to provide concrete support to the findings and a solid conclusion. Despite these limitations, the findings from the present study suggest that targeting *ENT2* could potentially be a therapeutic strategy for the enhancement of CRC treatment efficacy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SMN drafted the manuscript. SMN and NAAH conducted the experiments, and analyzed and interpreted the data. SAR, MM and AAR designed the study, discussed experimental approaches and outcomes, and critically revised the manuscript. MM and AAR obtained funding. AAR, SAR and MM confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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