MicroRNA-24 alleviates colorectal cancer progression via a rs28382740 single nucleotide polymorphism in the long noncoding region of X-linked inhibitor of apoptosis protein

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Abstract. Colorectal cancer (CRC) is one of the most prevalent malignant diseases worldwide. Recurrence is associated with the poor survival of patients with CRC. Targeted therapy and precision medicine for recurrent CRC may improve the clinical outcome. Therefore, finding biomarkers that can detect CRC early, assess its prognosis and survival, and predict its treatment response is key to improving the clinical prognosis. The aim of this study was to assess CRC recurrence by analyzing molecular differences using postoperative specimens. Whole-exome sequencing was first used to evaluate the molecular differences in CRC tissues from patients with recurrent disease, and the results were then verified with tissue array methods. The regulation of single nucleotide polymorphisms (SNPs) in long noncoding regions of interest was analyzed in the presence of target microRNAs (miRs) using luciferase assays. The results demonstrated that in patients with recurrent CRC, the G allele was mainly detected at the rs28382740 SNP in the 3'-untranslated region of the X-linked inhibitor of apoptosis (XIAP)-encoding gene. From the tissue arrays, 60% (3/5) of patients with the G allele of the rs28382740 SNP were diagnosed with CRC recurrence, whilst only 10% (1/10) of patients without the G allele had recurrent CRC (P=0.077). Furthermore, XIAP levels were high in non-CRC (50%; 2/4) and CRC (75%; 3/4) tissues of patients with recurrent disease and CRC (54.5%; 6/11) tissues of patients without recurrent disease. However, but only 9.1% (1/11) of non-CRC tissues of nonrecurrent patients had significantly high XIAP expression levels (P=0.022). Using a luciferase assay, it was demonstrated that miR-24s (miR-24-1-5p and miR-24-2-5p) targeting the rs28382740 SNP reduced XIAP levels in CRC cells with rs28382740 SNP genotype G. These results indicate that apoptosis-related proteins, such as XIAP, may be therapeutic targets or biomarkers for tumor development. The data from the present study support an inhibitory effect of miR-24s on

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Abbreviations: CRC, colorectal cancer; IAP, inhibitor of the apoptosis protein; XIAP, X-linked IAP; SNP, single nucleotide polymorphism; miR, microRNA; FFPE, formalin-fixed paraffin-embedded; IRB, Institutional Review Board; 3'-UTR, 3'-untranslated region; H&E, hematoxylin and eosin; IHC, immunohistochemistry; β-gal, β-galactosidase; OR, odds ratio; CI, confidence interval

Key words: recurrent CRC, XIAP, rs28382740 SNP, miR-24

XIAP expression. However, this inhibitory potency depends on the rs28382740 SNP genotype and may alleviate CRC progression by regulating the expression of XIAP.

Introduction

Colorectal cancer (CRC) accounts for about one in 10 cancer cases and deaths worldwide (1). Recurrence, which contributes to its poor prognosis by increasing 5-year CRC-related mortality to 33.6%, is frequently observed in most patients undergoing curative treatment or resection (2,3). Clinically, the delayed diagnosis and treatment failure of CRC remain the main reasons for the poor prognosis. Therefore, appropriate biomarkers that can detect CRC early, assess its prognosis and survival, and predict its treatment response are key to improving its clinical prognosis (4). Furthermore, an understanding of the postoperative follow-up program for CRC recurrence is important (5). For instance, using a liquid biopsy to monitor or evaluate recurrent CRC has been frequently reported (6). In addition, mutational status in formalin-fixed paraffin-embedded (FFPE) blocks showing high concordance may indicate the current reality or possible future prognosis of patients with CRC (7,8).

Several biomarkers modulating apoptosis have been described for their prognostic value for CRC recurrence. These biomarkers may provide new insights into monitoring CRC recurrence and therapeutic targets (9-12). Increased expression of an inhibitor of the apoptosis protein (IAP) family is involved in colon tumorigenesis (13). In contrast, the downregulation of IAPs by modulating molecules in the tumor microenvironment, such as tumor necrosis factor-a, may repress tumor growth (14). Therefore, certain apoptosis-related proteins, including X-linked IAP (XIAP), cIAP1, cIAP2 and survivin, may have potential as biomarkers for tumor development, including in CRC (13-15). Dysregulation of these antiapoptotic molecules has been reported to promote tumorigenesis in humans (16). The most important of these is XIAP, which is an emerging therapeutic target for different human cancers (17-20). Notably, inhibition of XIAP expression has been reported to control the proliferation and invasion of CRC cells, especially in metastatic CRC (21,22). Therefore, identifying potent XIAP antagonists [such as drugs, antisense oligonucleotides or microRNAs (miRs)] may have clinical implications for cancers that overexpress XIAP (23-25).

Dysregulation of small noncoding RNAs, such as miRs, contributes to the pathogenesis of all types of cancer (26). Exosomal miRs in serum or plasma are potential biomarkers for the diagnosis and prognosis of CRC (27,28). Furthermore, certain miRs may be potential candidate targets for treating CRC (29) and provide an attractive antitumor approach to cancer therapy (30). These miRs may act as tumor suppressors (31).

The aim of the present study was to evaluate the molecular differences in recurrent CRC via whole-exome sequencing (WES) and assess the role of XIAP. Luciferase expression was used to assess the regulation of single nucleotide polymorphisms (SNPs) in the long noncoding region of XIAP with target miRs. It is hoped that the information obtained may lead to improved and new treatment strategies to improve the prognosis of CRC.

Materials and methods

CRC sample acquisition. Delinked FFPE tissue samples from 9 patients with CRC [nonrecurrent, n=4 and recurrent cases, n=5; American Joint Committee on Cancer (AJCC) stage II; median (range) time to recurrence, 4.1 (1.9-7.0) months; Table I] were obtained from the Department of Pathology at Taipei Veterans General Hospital (Taipei, Taiwan). The protocol for the present study was reviewed and approved by the Institutional Review Board (IRB) of Taipei Veterans General Hospital (approval no. 2017-07-030AC). In addition, paired archived FFPE samples (non-CRC and CRC tissues) from 15 patients (AJCC stage II, n=7 and AJCC stage III, n=8; Table II) for fabrication into tissue arrays were acquired from the Cathay General Hospital Biobank (Taipei, Taiwan), which approved their use (approval no. HBKEC-20200928-1). The IRB of the Cathay General Hospital exempted the obtaining of informed consent for tissue procurement through the Cathay General Hospital Biobank after an anonymous unlinked process (approval no. CGH-P108136). The time period of Taipei Veterans General Hospital tissue samples collection was between February 2009 and December 2015, whilst the period for the Cathay General Hospital was between January 2000 and July 2020. The histological diagnosis of CRC in the present study was made by certified anatomical pathologists at Taipei Veterans General Hospital or Cathay General Hospital. The present study was performed in accordance with the Declaration of Helsinki, and all clinical characteristics of patients, including sex, onset age, primary or recurrent, initial AJCC stage and follow-up information, were obtained retrospectively.

DNA purification from FFPE colonic tissues. Genomic DNA from FFPE colonic tissues was extracted and purified using the High Pure FFPET DNA Isolation Kit (cat. no. 06650767001, Roche Diagnostics). Briefly, the fixed paraffin-embedded colonic tissue sections (10-mm thick) were immersed in xylene to remove the extra paraffin and rehydrated with 100% alcohol at room temperature. After RNase treatment, the genomic DNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and the quality and integrity of the genomic DNA was verified using the Qubit™ dsDNA Quantification Assay Kit (cat. no. Q32851; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

Library construction and sequencing by WES. To generate standard exome capture libraries, the Agilent SureSelect XT Reagent kit (cat. no. G9611A; Agilent Technologies, Inc.) for the Illumina Multiplexed Paired-End sequencing library was used with the SureSelect XT Clinical Research exome V2 (cat. no. 5190-9492; Agilent Technologies, Inc.) probe set. A total of 1 mg genomic DNA was used to construct a library with the Agilent SureSelect XT Reagent kit. The amplification adapter-ligated sample was purified using Agencourt AMPure XP beads (cat. no. A63882; Beckman Coulter, Inc.) and analyzed on a TapeStation 4200 D1000 screentape (Agilent Technologies, Inc.). A 750 ng-genomic DNA library was prepared for hybridization with the capture baits, and the sample was hybridized for 24 h at 65˚C, captured with

Table I. Clinical characteristics of non-recurrent (n=4) and recurrent (n=5) patients for next-generation sequencing analysis.

Characteristic	No recurrence	Recurrence
Sex, n (%)		
Female	1 (25.0)	0 (0.0)
Male	3 (75.0)	5 (100.0)
Median onset age (range), years	57.3 (45.3-74.1)	74.8 (62.8-81.9)
Median time to recurrence (range), months	-	4.1 (1.9-7.0)
Stage, n (%)		
T3N0M0	4 (100.0)	5 (100.0)
AJCC II	4 (100.0)	5 (100.0)

T, tumor; N, node; M, metastasis; AJCC, American Joint Committee on Cancer.

Table II. Clinical characteristics of non-recurrent (n=11) and recurrent (n=4) patients for the tissue array.

Characteristic	No recurrence	Recurrence
Sex, n (%)		
Female	6 (54.5)	1 (25.0)
Male	5 (45.5)	3 (75.0)
Median age of onset (range), years	71.3 (34.6-84.5)	61.0 (48.5-78.0)
Median time to recurrence (range), months	-	102.0 (7.3-174.1)
T stage, n (%)		
T3	10 (90)	4 (100.0)
T4	1 (9.1)	0 (0.0)
N stage, n (%)		
N0	6 (54.5)	1 (25.0)
N1	2 (18.1)	3 (75.0)
N2	3 (27.3)	0 (0.0)
AJCC stage, n (%)		
II	6 (54.5)	1 (25.0)
III	5 (45.5)	3 (75.0)

T, tumor; N, node; M, metastasis; AJCC, American Joint Committee on Cancer.

Dynabeads™ MyOne™ Streptavidin T1 (cat. no. 65602; Thermo Fisher Scientific, Inc.), and purified using Agencourt AMPure XP beads. The Agilent protocol was used to add index tags by posthybridization amplification. Finally, all samples were sequenced on an Illumina NovaSeq 6000 platform (cat. no. 20012850; Illumina, Inc.) using the 150PE protocol. The sequence reads are deposited in the National Center for Biotechnology Information (NCBI) under the accession number PRJNA-1063437.

Variant analysis of sequencing data. The qualified read data were then processed through genomic alignment against the Ensembl database (version 86; https://ftp.ensembl.org/pub/release-86) and using the Burrows-Wheeler Aligner (version 0.7.17; https://github.com/lh3/bwa) to obtain basic sequence information (32,33). The Genome Analysis Toolkit (version 3.7.0) was used to analyze variants (34,35), and the Variant Effect Predictor (version 86; https://github.com/Ensembl/ensembl-vep/releases?page=7) predicted the effects SNPs on proteins (36,37). Paired-end reads were quality-checked using FastQC (version 0.32; https://github.com/s-andrews/FastQC/releases) and trimmed using Trimmomatic (version 0.11.7; https://github.com/usadellab/Trimmomatic/releases) (38). Finally, the association between the sequencing data and nonrecurrent and recurrent CRC was assessed.

CRC cell lines and their rs28382740 SNP genotypes. In the present study, four CRC cells classed as AJCC stage II, LS 123 [cat. no. CCL-255; American Type Culture Collection (ATCC)], HCT 116 (cat. no. CCL-247; ATCC), LS 174T (cat. no. CL-188; ATCC) and SW480 (cat. no. CCL-228; ATCC), and two CRC cells classed as AJCC stage III, SW620 (cat. no. CCL-227; ATCC) and LoVo (cat. no. CCL-229; ATCC), were cultured according to the protocol of the ATCC. Briefly, all cells were cultured to 80% confluence with specific culture medium [LS 123 and LS 174T: Minimum Essential Medium (cat. no. 41500034; Thermo Fisher Scientific, Inc.); HCT 116 and LoVo: Dulbecco's Modified Eagle's Medium (cat. no. 12800017; Thermo Fisher Scientific, Inc.); SW480 and SW620: Leibovitz's L-15 Medium (cat. no. 41300039; Thermo Fisher Scientific, Inc.)] in the presence of 10% fetal bovine serum (cat. no. A06806-35; NQBB International biological Corp.) and 1X antibiotic-antimycotic solution (100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B; cat. no. 15240062; Thermo Fisher Scientific, Inc.) at 37˚C and 5% CO_2, except that SW480 and SW620 cells were cultured in the absence of CO_2. A 170-bp fragment containing the rs28382740 SNP in the 3'-untranslated region (3'-UTR) of XIAP from these cell lines was PCR-amplified in the presence of 0.25 units of AmpliTaq Gold 360 DNA Polymerase (cat. no. 4398833; Thermo Fisher Scientific. Inc.), 2.0 mM $MgCl_2$, 50 ng of genomic DNA and 1 μM primer pair (Table III) in a 10-μl reaction mixture. The PCR program was as follows: Denaturation at 95˚C for 10 min, a 45-cycle program (95˚C, 30 sec; 60˚C, 30 sec; 72˚C, 30 sec), and a final extension at 72˚C for 7 min. Sequences of the rs28382740 SNP were then determined by Sanger sequencing.

Hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC) and interpretation. Tissues were immersed in 4% formaldehyde for 1 day at room temperature, processed in Tissue-Tek VIP 5 (Sakura Finetek USA, Inc.) for dehydration, and embedded in Paraplast Plus (cat. no. 39602004; Leica Biosystems, Inc.) to form a donor block. Tissue arrays were then constructed by removing a core of tissue from a donor block and transferring this core to a predetermined position on a recipient block. Sections of 5 mm were cut from the blocks of tissue arrays, stained with H&E and immunostained using an avidin-biotin-immunoperoxidase method. Briefly,

Table III. Primers for the amplification, sequencing and cloning of DNA fragments containing rs28382740 single nucleotide polymorphism of X-linked inhibitor of the apoptosis protein.

Use of primer	Direction	Sequence (5'-3')	Product length, bp
Sequencing of DNA fragments within the 3'-UTR	Forward	TGACAAGTGTCCCATGTGCT	170
	Reverse	TTGGTAGCAAATGCTAATGGAA	
Cloning 3'-UTR fragment	Forward-1[a]	*ACTAGT*TCTAACTCTATAGTAGGCATG	604
	Forward-2[b]	*ACTAGT*TCTAACTCTATGGTAGGCATG	
	Reverse	*GTTTAAAC*ATCATTAAATACGCTTTCAA	

[a]Used to amplify genotype A; [b]Used to amplify genotype G. The rs28382740 SNP is indicated by underlined fonts; SpeI (ACTAGT) and PmeI (GTTTAAAC) are indicated by italics and underlined fonts. 3'-UTR, 3'-untranslated region.

H&E staining was performed using a Tissue-Tek DRS™ 2000 Automated Slide Stainer (Sakura Finetek USA, Inc.) following a general and serial protocol at room temperature: Deparaffinization (two consecutive xylene changes of 5 min each, followed by a 7 min change), rehydration [a sequential alcohol changes (100% alcohol, 60 sec; 100% alcohol, 90 sec; 95% alcohol, 60 sec; 75% alcohol, 60 sec) and running water, 3 min] and staining with hematoxylin for 5 min, followed by dipping the slides 5 times in 1% acid alcohol (1% HCl in 70% alcohol). Before mounting the tissue sections, the sections were rinsed, stained with eosin for 3 min at room temperature, dehydrated with graded alcohol, and washed in xylene.

IHC assays were performed on a BenchMark GX IHC/ISH slide automated system (Ventana Medical Systems, Inc.). The automated IHC program included deparaffinization with EZ Prep solution (cat. no. 950-102; Ventana Medical Systems, Inc.) at 75˚C for 8 min, antigen retrieval with Cell Conditioning 1 solution (cat. no. 950-124; Ventana Medical Systems, Inc.) at 95˚C for 64 min, and incubation with primary anti-XIAP antibody (dilution 1:100; cat. no. SC-55550; Santa Cruz Biotechnology, Inc.) at 37˚C for 1 h. Finally, positive signals were developed following an incubation with secondary antibody and chromogen (OptiView DAB IHC Detection Kit; cat. no. 760-700; Roche Diagnostics). The Reaction Buffer (cat. no. 950-300; Ventana Medical Systems, Inc.) was used for all wash steps at room temperature. All sections were further processed by counterstaining with hematoxylin II (cat. no. 790-2208; Ventana Medical Systems, Inc.) for 8 min at room temperature and Bluing Reagent (cat. no. 760-2037; Ventana Medical Systems, Inc.) for 4 min at room temperature, and then visualized using light microscopy (Olympus BX41 Microscope; Olympus Corp.).

Sections were evaluated at a high magnification by a research pathologist blinded to tissue type to determine the proportion of cells expressing XIAP, and the tissue sections were scored using a semiquantitative method based on a four-layer system: Score 0 for negative expression; score 1 for weakly positive expression; score 2 for positive expression; and score 3 for strongly positive expression (39).

Knockdown of XIAP in SW480 cells and cell proliferation assessment. For XIAP knockdown in SW480 cells, the plasmid (pLKO_005) carrying a non-targeting control lentivirus-mediated small hairpin (sh)RNA (cat. no. TRCN0000231719; shLuc; 5'-GCGGTTGCCAAG AGGTTCCAT-3') or a specific lentivirus-mediated shRNA targeting XIAP (shXIAP; cat. no. TRCN0000231579; 5'-ACA CGTACTTGTGCGAATTAT-3') was purchased from the National RNAi Core Facility of Academia Sinica, Taiwan. The first-generation lentiviral vectors were used to package lentiviruses. Infection of each lentivirus into SW480 cells and selection of stable clones with shLuc (shLuc-SW480) or with shXIAP (shXIAP-SW480) by puromycin and efficacy validation of XIAP knockdown were performed according to a previous protocol (TRC protocol: Lentivirus infection V3) (40). After washing cells with PBS, bright-field images of live cells were taken using an ECHO Revolve microscope (ECHO RVL-100-M; BICO Group AB). To determine cell proliferation, images were analyzed using QuPath (version 0.3.0; http://qupath.github.io) and Adobe Photoshop 2022 (version 23.0.1; Adobe Systems, Inc.) to obtain the daily coverage area of cells (41,42).

Cloning of 3'-UTR regions of XIAP into a pMIR-REPORT vector and luciferase assay. The pMIR-REPORT™ miRNA Expression Reporter Vector System (cat. no. AM5795; Thermo Fisher Scientific. Inc.) was used to analyze the potential target of miR-24 in the 3'-UTR of XIAP, including the significant rs28382740 SNP. Fragments of 608 bp in length with different genotypes at the rs28382740 SNP within this long noncoding region were amplified from genomic DNA of HCT116 (type A on the rs28382740 SNP) in the presence of 1.25 units of GoTaq DNA Polymerase (cat. no. M3001; Promega Corp.), 2.5 mM $MgCl_2$, 50 ng of genomic DNA and 0.8 μM appropriate primer pair (Table III) in a 10-μl reaction mixture using the following PCR program: Denaturation at 95˚C for 10 min, a 45-cycle program (95˚C, 30 sec; 60˚C, 30 sec; 72˚C, 50 sec) and a final extension at 72˚C for 7 min. Amplified fragments were cloned into the SpeI and PmeI restriction sites of the pMIR-REPORT vector with T4 DNA ligase (cat. no. T4L0500; Bioman Scientific Co., Ltd.) for 16 h at 4˚C. Subsequently, changes in the luciferase activity of two pMIR-REPORT vectors (pMIR-REPORT-A and pMIR-REPORT-G) in HCT116 cells were assessed by cotransfection of pCMV-MIR vectors (cat. no. PCMVMIR; OriGene Technologies, Inc.) with miR-24-1 precursor (cat. no. SC400296; OriGene Technologies, Inc.) or miR-24-2

Table IV. rs28382740 single nucleotide polymorphism in patients with colorectal cancer (n=15).

		Prognosis		
rs28382740 SNP allele	Total patients with CRC, n	No recurrence	Recurrence	P-value
G allele	5	2 (40.0)	3 (60.0)	0.077
A allele	10	9 (90.0)	1 (10.0)	

Values are expressed as n (%). SNP, single nucleotide polymorphism; CRC, colorectal cancer.

precursor (cat. no. SC400297; OriGene Technologies, Inc.), and a pMIR-REPORT β-galactosidase (β-gal) control plasmid (cat. no. AM5795; Thermo Fisher Scientific. Inc.). Briefly, $7.5x10^3$ HCT116 cells/well were seeded on to a 96-well plate and co-transfected with 60 ng pMIR-REPORT vector, 80 ng empty vector (pCMV-MIR) and 10 ng pMIR-REPORT β-gal control plasmid the next day or when cells reached 30-50% confluency, using 450 nl jetPRIME® reagent (cat. no. 101000046; Polyplus-transfection SA), according to the manufacturer's instructions. Following an initial 24-h cultivation after transfection, the medium was replaced with fresh complete medium, and culture continued for an additional 24 h before detecting luciferase activity using the Luc-Screen Extended-Glow Luciferase Reporter Gene Assay System (cat. no. T1035; Thermo Fisher Scientific, Inc.) and galactosidase with the Galacto-Light Plus™ β-Galactosidase Reporter Gene Assay System (cat. no. T1007; Invitrogen™; Thermo Fisher Scientific, Inc.). Finally, the luciferase activity [measured in relative light units (RLUs)] was normalized to the activity of β-gal as RLU/β-gal. Transfection efficiency was calculated as the number of pCMV-MIR green fluorescent protein-positive cells as a % of the number of cells with Hoechst 33342-stained nuclei (cat. no. 910-3015; ChemoMetec A/S). The ECHO Revolve microscope (RVL-100-M; BICO Group AB) was also used to capture fluorescent images of transfected cells, and positive cells were quantified using QuPath (version 0.3.0; http://qupath.github.io) (41).

Statistical analysis. The frequencies of the G allele in the rs28382740 SNP of the XIAP 3'-UTR and CRC recurrence in the 15 patients with CRC were compared using Fisher's exact test using SPSS Statistics for Windows (version 20; IBM Corp.). Risk analysis was estimated by calculating the odds ratio (OR) and the 95% confidence interval (CI). The relative XIAP expressions of two groups (shLuc-SW480 and shXIAP-SW480) were compared using the unpaired Student's t-test, and the relative luciferase activity and cell proliferation of different groups were compared using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Significant SNPs in patients with recurrent CRC. For long follow-up times, archived FFPE specimens and appropriate samples were available to assess genes involved in recurrent CRC. Therefore, patients with CRC were recruited who were followed ≥5 years to confirm their recurrence status. Using WES, 27 SNPs with common variant sequences and distribution in 21 genes were revealed in the group of patients with recurrent CRC (Table SI). Due to the significant molecular functions of XIAP in cell proliferation, apoptosis, invasion and metastasis, this apoptosis-related protein was further studied for CRC progression. As presented in Table IV, the most genotype of the rs28382740 SNP in the XIAP 3'-UTR was type A in nonrecurrent cases and type G in recurrent cases. In addition, three early-stage CRC cell lines (LS123, HCT116 and LS174T) demonstrated genotype A, and two CRC cell lines with recurrence potential (SW480 cell line and its metastasis-derived SW620 cell line) were sequenced as genotype G in the rs28382740 SNP.

The expression of XIAP in the shXIAP-SW480 cells was significantly reduced in comparison with that in shLuc-SW480 cells (Fig. 1A). Furthermore, the proliferation of shXIAP-SW480 cells was notably reduced compared with that of shLuc-SW480 cells (Fig. 1B), and the relative quantitative results, which represented the level of proliferation by cell coverage, also demonstrated that shXIAP-SW480 cells had significantly lower levels of proliferation than that of shLuc-SW480 cells from day 2 ($P<0.001$ for Day 2 and $P<0.0001$ for Day 3; Fig. 1C).

XIAP levels and genotypes of patients with CRC in the tissue array. Among the 15 patients with CRC whose tissues were assembled into the tissue array, 4 were diagnosed with recurrent CRC (median time to recurrence, 102.0 months; range, 7.3-174.1 months), whilst 11 were not demonstrated to have any signs of recurrence during the follow-up period (median follow-up time, 64.4 months; range, 26.9-124.9 months). To assess the clinical relevance of XIAP in these patients with CRC, paired colon tissues (non-CRC and CRC cores) were evaluated for XIAP levels using IHC. The results demonstrated that markedly higher levels of XIAP were detected in the CRC tissue with a more aggressive phenotype, whereas the corresponding non-CRC tissue showed negative immunostaining (Fig. 2A). The XIAP levels were then semi-quantitatively scored based on the intensity and % of epithelial cells in colon tissues. Fig. 2B presents representative images of the four scores (scores 0-3). Briefly, tissues with an intensity score ^{3}2 were classified into the high XIAP-expressed group, and tissues with an intensity score <2 were considered the low XIAP-expressed group. In the paired tissues of 4 recurrent patients, 50% (2/4) of non-CRC tissues and 75% (3/4) of CRC tissues expressed high XIAP levels. In comparison, among the 11 nonrecurrent patients, 54.5% (6/11) of the CRC tissues

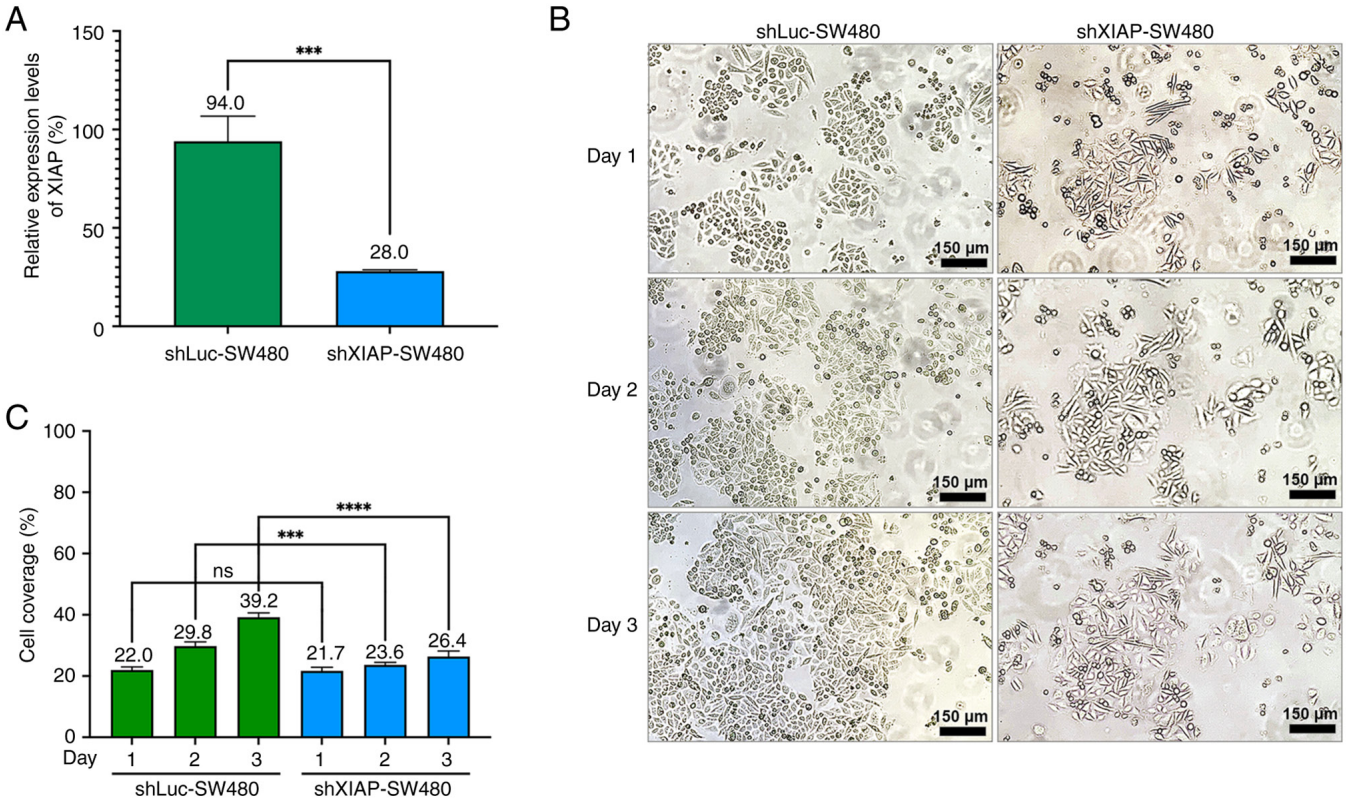

Figure 1. Assessment of the effect of XIAP on SW480 cell proliferation. (A) Knockdown efficiency of shXIAP in SW480 cells. (B) Proliferation of shLuc-SW480 and shXIAP-SW480 cells over 3 days in bright field. (C) Changes in the cell coverage of shLuc-SW480 and shXIAP-SW480 cells over 3 days of culture. To determine cell proliferation, cell images were taken using an ECHO Revolve microscope daily over the 3-day incubation period and identified by using QuPath (version 0.3.0). Finally, Adobe Photoshop 2022 (version 23.0.1) was used to calculate the % coverage area of cells. ***P<0.001; ****P<0.0001. XIAP, X-linked inhibitor of apoptosis; sh, small hairpin; ns, no significance.

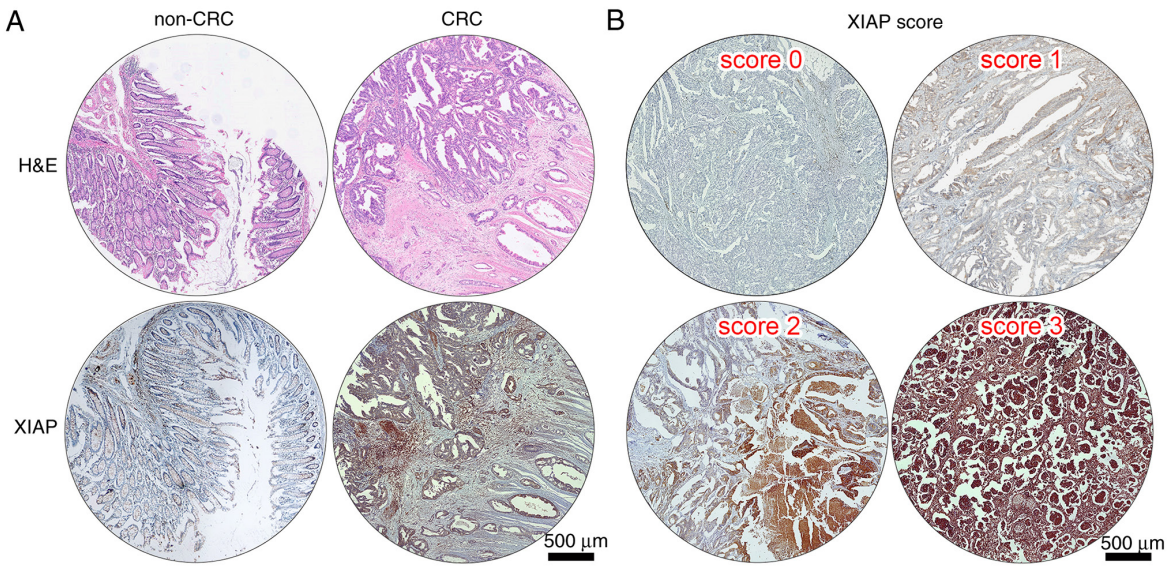

Figure 2. Immunohistochemical analyses of XIAP in CRC tissues. (A) Higher levels of XIAP in CRC tissues. (B) Scores of XIAP-positive signals. Scores were determined by a pathologist based on a four-layer system: Score 0 for negative expression; score 1 for weakly positive expression; score 2 for positive expression; and score 3 for strongly positive expression. XIAP expression was detected from the paired CRC tissue array (scale bars, 50 μm). CRC, colorectal cancer; H&E, hematoxylin and eosin; XIAP, X-linked inhibitor of apoptosis.

had high XIAP levels, but only 9.1% (1/11) of non-CRC tissues had high XIAP levels. However, this difference in patients with no recurrence was not statistically significant (P=0.063; Table V). Further observation of the subcellular localization of the XIAP protein revealed markedly increased nuclear XIAP intensity in patients with CRC recurrence (Fig. 3). In addition, the genotypes of the rs28382740 SNP in the XIAP 3'-UTR of patients with CRC in the tissue array were analyzed (the

Table V. Expression level of X-linked inhibitor of the apoptosis protein in non-recurrent (n=11) and recurrent (n=4) patients with colorectal cancer.

A, No recurrence

	Expression level		
Sample type	High	Low	P-value
Non-CRC tissue	1 (9.1)	10 (90.9)	0.063
CRC tissue	6 (54.5)	5 (45.5)	

B, Recurrence

	Expression level		
Sample type	High	Low	P-value
Non-CRC tissue	2 (50.0)	2 (50.0)	1.000
CRC tissue	3 (75.0)	1 (25.0)	

Values are expressed as n (%). CRC, colorectal cancer.

A/G heterotype of the rs28382740 SNP; Fig. 4). The frequency of G-allele carriers was 14.3% (1/7) in patients with AJCC stage II and 50.0% (4/8) in patients with AJCC stage III. Among the G-allele carriers, 60% (3/5) were diagnosed with CRC recurrence, whilst only 10% (1/10) of patients without a G allele had recurrent CRC (P=0.077; Table IV). Furthermore, the G allele at the rs28382740 SNP notably increased the risk of recurrent CRC at AJCC stages II and III, with an OR of 13.5 (95% CI, 0.88-207.62). Conversely, according to the refSNP cluster ID number (e.g. rs28382740) (43), the Asian population has a markedly higher proportion of the G allele in the rs28382740 SNP (36.6-48.0%) than the European and African populations (15.7-19.2%; Table VI).

rs28382740 SNP in miR binding sites. SNPs in miR binding sites are known to be potential cancer biomarkers with clinical significance (44). The rs28382740 SNP in the XIAP 3'-UTR was demonstrated to have putative binding sites for two different miR-24s (miR24-1-5p and miR24-2-5p) (45). Therefore, the clinical significance of this miR binding to the rs28382740 SNP in CRC was assessed. The fragment of the XIAP 3'-UTR containing any genotype of the rs28382740 SNP was cloned and inserted into separate luciferase reporters (pMIR-REPORT-A and pMIR-REPORT-G) and transfected into HCT116 cells (Fig. 5A). Following an appropriate antibiotic selection, green fluorescence was expressed in the transfected cells (Fig. S1), and the transfection efficiencies of pCMV-MIR-miR24-1 and pCMV-MIR-miR24-2 were 20.1 and 20.5%, respectively (Fig. 5B). The luciferase activity was determined following co-transfection with a different effector plasmid (pCMV-MIR-miR-24-1 or pCMV-MIR-miR24-2). Both miR-24-1-5p and miR-24-2-5p significantly downregulated the relative luciferase activity of HCT116 cells with different rs28382740 SNP genotypes compared with cells with the empty vector pCMV-MIR (Fig. 5C). Furthermore, in HCT116 cells with rs28382740 SNP genotype A, miR-24-1-5p significantly reduced the relative luciferase activity compared with miR-24-2-5p (Fig. 5C), whilst this reduction was undetectable in HCT116 cells with rs28382740 SNP genotype G (Fig. 5C).

Discussion

CRC is a common malignant disease of the gastrointestinal system, and recurrence results in poor clinical outcomes following surgery and postsurgical treatment (46). Despite established clinical strategies, including surgery, adjuvant chemotherapy and targeted therapy, the recurrence rate of CRC has not yet decreased (47). Currently, CRC recurrence is associated with multiple risk factors, such as molecular subtypes, clinical stages and epigenetic alterations (29,48,49). Targeted therapy and precision medicine for recurrent CRC may improve the clinical outcomes of these vulnerable patients. Therefore, whether the prognosis will improve when patients with CRC experience a significant reduction in cancer recurrence after surgery warrants exploration. Using postoperative specimens to assess recurrence is the most advantageous and convenient clinical strategy, and FFPE tissue, which contains most of the pathological information of a patient, is a feasible source to assess molecular and clinical follow-up data (50,51).

From archived tissue samples, the present study demonstrated that the XIAP gene with genotype G at the rs28382740 SNP was primarily detected in patients with recurrent CRC. A total of >50% of the non-CRC and CRC tissues of patients with recurrent CRC, and CRC tissues of patients with nonrecurrent CRC, expressed higher levels of XIAP. Nevertheless, only the non-CRC tissues of patients with nonrecurrent CRC expressed lower levels of XIAP. These results indicate that the genotypes and levels of XIAP may be associated with patient prognosis. Furthermore, the results of the present study were partially consistent with the conclusion of Xiang *et al* (39), who reported that the status of XIAP expression could be an independent prognostic marker in CRC. Moreover, it has been reported that XIAP is the strongest member of the family of inhibitors of apoptosis proteins (52). Therapeutic benefits in diseases such as cancers caused by inappropriate inhibition of cell death, may result from reinduction or triggering of apoptosis (53). Therefore, XIAP, as a potent inhibitor of cell death, may be involved in chemotherapy resistance and tumor aggressiveness in several cancers (45,52,54,55). High levels of XIAP may be a potential therapeutic target (18). For example, inhibition of XIAP has been reported to increase carboplatin sensitivity in ovarian cancer (56). In contrast, the present study demonstrated that patients with CRC and advanced-stage disease had increased XIAP expression in the nucleus. This finding was similar to that of Delbue *et al* (57) who revealed that an elevated expression of nuclear XIAP may be associated with drug resistance and poor prognosis in breast cancer. Taken together, the results of the present study imply that increases in XIAP expression, not only in whole cells or the nucleus, appear to be an adverse prognostic factor for clinical outcomes in many cancers (57,58). XIAP may be targeted in CRC with a poor prognosis, but its concrete role needs to be further explored.

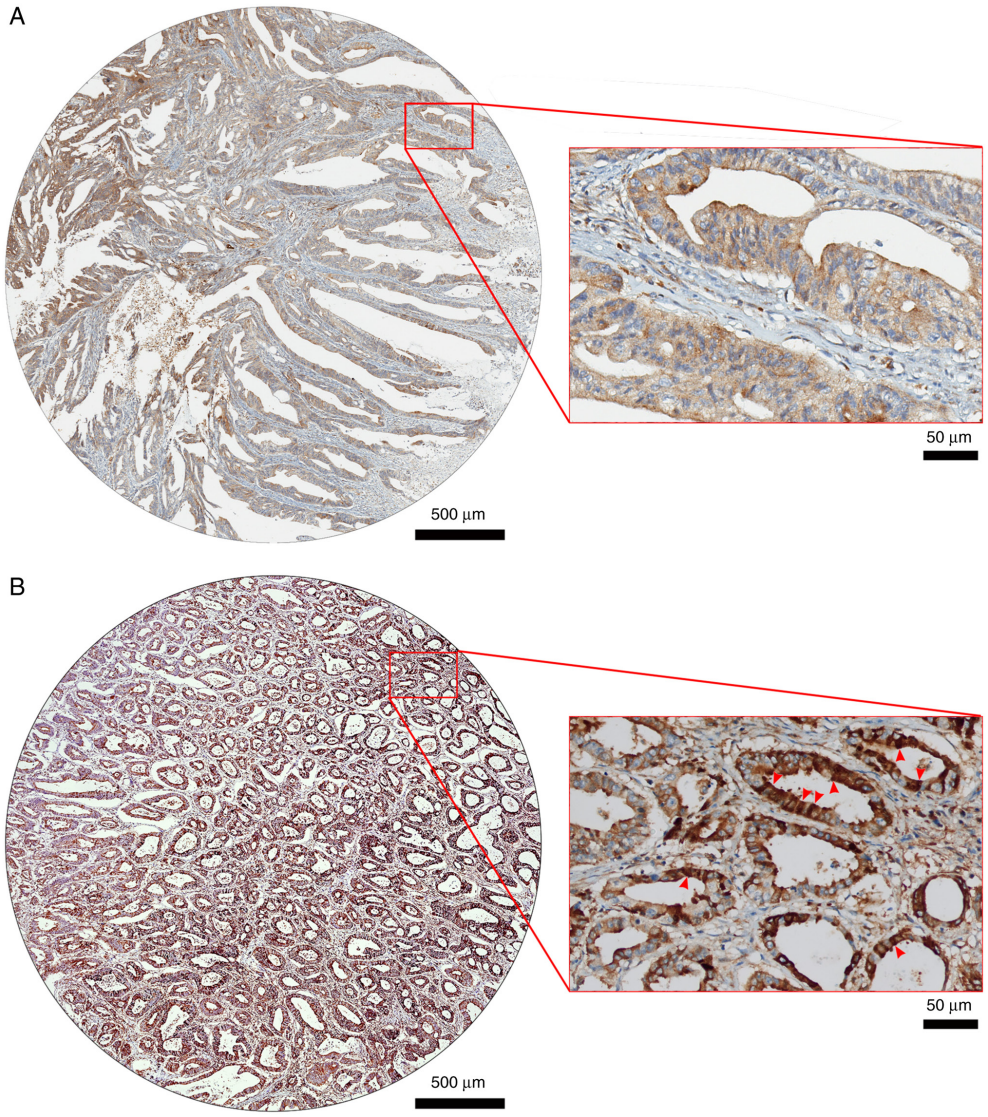

Figure 3. Nuclear XIAP intensity in CRC tissues. Images of primary CRC tissues from two male patients were captured. Tissue from patient with CRC with (A) no recurrence (age, 84.5 years; AJCC stage III) and (B) recurrence (age, 48.5 years; initially diagnosed with AJCC stage II; recurrence at age 63.0 years). The increased nuclear XIAP is indicated with red arrows (scale bars, 500 and 50 μm in magnified windows). XIAP, X-linked inhibitor of apoptosis; CRC, colorectal cancer; AJCC, American Joint Committee on Cancer.

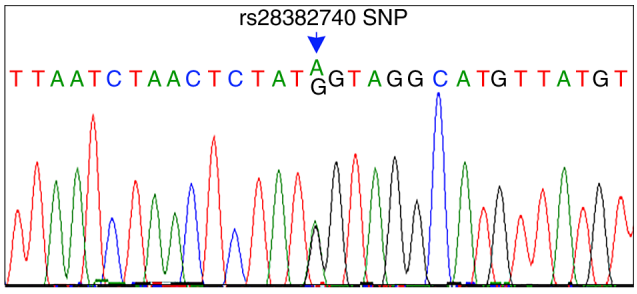

Figure 4. Heterotypic genotype of the rs28382740 SNP. The sequence was determined by direct sequencing of the target 3'-untranslated region of XIAP. SNP, single nucleotide polymorphism; XIAP, X-linked inhibitor of apoptosis.

XIAP has been known to inhibit the growth of several cancers through different pathways (22,57,59). This inhibition was also demonstrated by the results of the present study, which indicated that parental SW480 cells grew faster than XIAP-knockdown SW480 cells in CRC. The inhibition could be inferred to slow CRC cell growth by reducing XIAP expression. Due to the molecular significance of XIAP in the apoptosis and growth of cells, it has become a potential therapeutic target in tumors and inflammatory diseases (60,61).

As XIAP is critical for CRC progression and miRs are known to develop or mitigate cancer by modulating target expression, it is important to understand the interaction between XIAP and specific miRs. Prabhu *et al* (62) reported that changes in gene expression were caused by the interaction of miRs and SNPs, and this effect was related to the sequence of the miR-mRNA binding site within the target gene. For example, in CRC, high miR-503 and high miR-183 have been positively associated with tumor progression (63) and poor prognosis (28). Conversely, patients with CRC and high miR-23b in plasma have been reported to exhibit an improved survival rate (64). Taken together, miRs in CRC have been studied for years, but whether they are oncogenic or tumor suppressor miRs remains to be determined (65).

Table VI. Allele frequency of rs28382740 in different populations.

Population	Sample size	Allele frequency[a], %	
		A	G
European	20,114	80.8	19.2
African	3,394	84.3	15.7
African American	3,280	84.3	15.7
Latin American	610	81.8	18.2
Asian	168	58.3	41.7
East Asian	112	63.4	36.6
South Asian	98	52.0	48.0

[a]Frequency data were compiled from https://www.ncbi.nlm.nih.gov/snp/rs28382740.

Understanding the regulation of several miRs with clinical significance for CRC is warranted.

Taking XIAP as an example in several cancers, miR-215 and miR-122-5p have been reported to restrain XIAP levels to negatively regulate tumor growth for cancers in the gastrointestinal system (52,66). In addition, miR-214-3p and miR-618 have been reported to act as tumor suppressors against retinoblastoma (67) and degenerative thyroid cancer (68), respectively, by interacting with the XIAP 3'-UTR. The present study revealed the rs28382740 SNP at the XIAP 3'-UTR using WES, indicated it might overlap with the miR-24 binding region and speculated that XIAP was indeed one of the genes in the list that miR-24 could bind to through the algorithm of miRDB (http://www.mirdb.org/). This interaction may decrease the expression level of XIAP in CRC cells. Furthermore, other studies have reported that the interaction of miRs and their target SNPs produces intracellular effects with clinical importance (69,70). miR-24 has been widely studied in several human cancers with different roles (71). Further information on miR-24-1-5p and miR-24-2-5p was obtained from miRDB (Table SI). Among the top five high-scoring targets, there are three other CRC-related genes [Caldesmon 1 (CALD1), serine and arginine rich splicing factor 11 (SRSF11), and SRSF protein kinase 2 (SRPK2)] besides XIAP, which scored the third highest one (72-75). Inhibiting oncomiR or inducing tumor suppressors through miR-based therapies may be effective in treating cancer (76). Therefore, miR-24-1-5p and miR-24-2-5p may contribute to regulating their potential targets, especially those known to be possible therapeutic targets for CRC, including XIAP, CALD1 and two genes associated with serine- and arginine-rich splicing factor (SRSF11 and SRPK2) (74,75,77,78). Taken together, further research is needed to explore changes in these genes in association with the genotype (A or G) of rs28382740 SNP in CRC cells. As reviewed by Mukherjee *et al* (79), miR-24, along with its passenger strands, miR-24-1-5p and miR-24-2-5p, may be involved in the pathobiology of several diseases and have potential for their diagnosis and prognosis. Zhang *et al* (80) highlighted that upregulated miR-24-1-5p may provide preventive and therapeutic strategies for CRC through intracellular molecular mechanisms. These findings illustrate that miR-24 has molecular functions for disease prevention and treatment.

In the present study, the molecular regulation of miR-24 on the downregulation of XIAP expression was genotype-dependent. The results demonstrating that miR-24-1-5p reduced the luciferase activity of SW480 cells with rs28382740 SNP genotype A indicate that patients with CRC and rs28382740 SNP genotype A may have lower levels of XIAP expression in the presence of a certain level of miR-24-1-5p. These results from clinical specimens and CRC cell lines demonstrate that the expression levels and genotypes of XIAP in CRC cells may be associated with CRC prognosis. However, the high proportion of the genotype G allele at rs28382740 SNP in the Asian population must have clinical significance. This implies that it is also important to determine the rs28382740 SNP genotype in patients in the Asian population with AJCC stage II and III CRC as patients with CRC and rs28382740 SNP genotype G have higher levels of XIAP expression and are not suitable for receiving miR-24 (miR-24-1-5p and miR-24-2-5p). The findings of the present study may benefit the future development of molecular diagnosis and personalized therapies for recurrent CRC.

Genes in the IAP family are frequently expressed at elevated levels in tumor maintenance and progression (13). Targeting IAP proteins could be an option for antitumor therapeutic intervention (81). That is, preventing apoptosis may inhibit tumor growth and recurrence and lead to an improved prognosis (82). In other words, XIAP, as a key molecule for cell death, exerts its oncogenic potential by inhibiting apoptosis to promote cell proliferation and could be a molecular target for anti-CRC (44). Clinical trials of several miR replacement therapies may demonstrate their potential for cancer treatment (26). However, there are limitations to the present study that should be noted. First, although the present study used 24 clinical samples, including 15 paired CRC tissue samples for validation, the number of clinical samples assessed should be expanded to approximate clinical complexity more closely. Second, the multigene panel is frequently considered to be associated with CRC progression (83,84). Further study of multiple genes related or unrelated to XIAP is necessary to understand the molecular biology of CRC recurrence. Third, the targets of miRs are not unique genes, so further research should assess the molecular effects of other genes regulated by miR-24 (miR-24-1-5p or miR-24-2-5p) on CRC cells. Further investigations should clarify the relationship between XIAP and CRC recurrence.

In conclusion, the results of the present study indicate that miR-24-1-5p and miR-24-2-5p could directly target the rs28382740 SNP in the 3'-UTR of XIAP to exert an inhibitory effect on XIAP protein expression, especially for miR-24-1-5p. Understanding the impact of the rs28382740 SNP on CRC recurrence may be beneficial to reduce cancer recurrence and enhance treatment. The findings infer that miR-24-1-5p downregulates XIAP expression in CRC cells due to the rs28382740 SNP genotype, which may inhibit CRC tumor growth and thereby prevent CRC recurrence, especially in patients with AJCC stages II and III.

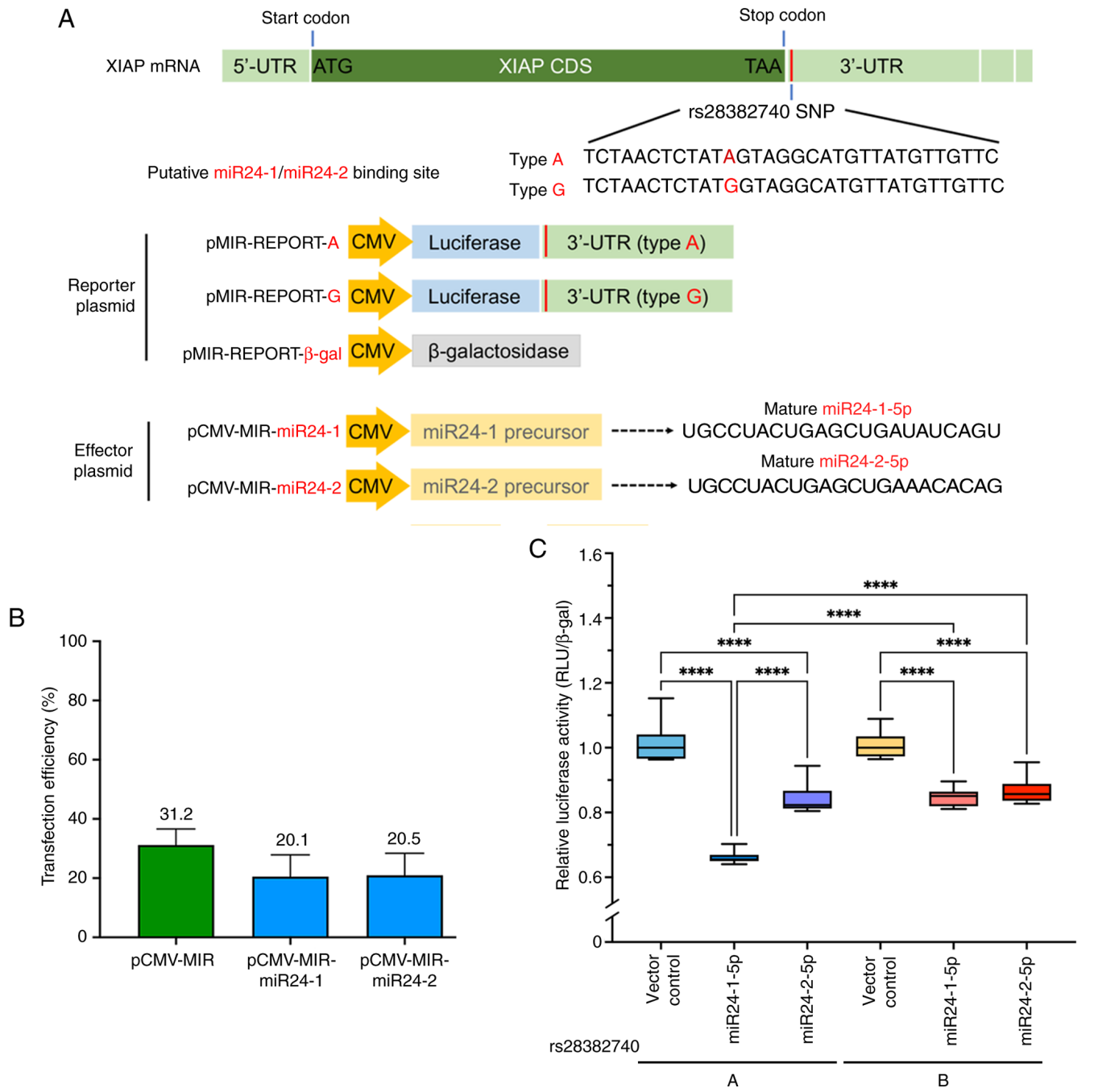

Figure 5. Determination of the association between the rs28382740 SNP in the XIAP 3'-UTR and miR-24s in XIAP expression in CRC cells. (A) Constructions of plasmids with different genotypes of the rs28382740 SNP and miR-24s. (B) Transfection efficiency of different pCMV-MIR vectors evaluated using the proportion of green fluorescent protein-positive cells among Hochest-positive cells. (C) Relative luciferase activity of HCT116 cells with different rs28382740 SNP and miR-24s. The reporter plasmids were pMIR-REPORT-A and pMIR-REPORT-G. Luciferase activity was assessed in the presence of pCMV-MIR-miR-24-1 or pCMV-MIR-miR24-2 compared with the empty vector pCMV-MIR. β-gal was used as an internal control was used to normalize the data. The data are presented as the mean ± standard deviation. ****P<0.0001. SNP, single nucleotide polymorphism; XIAP, X-linked inhibitor of apoptosis; UTR, untranslated region; CDS, coding sequence; miR/MIR, microRNA; CMV, cytomegalovirus; RLU, relative luciferase unit; β-gal, β-galactosidase.

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Availability of data and materials

The original sequencing data generated in the present study may be found in the National Center for Biotechnology Information database under accession number PRJNA1063437 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1063437). All other data generated in the present study may be requested from the corresponding author.

Authors' contributions

YFT and CCC conceived the project, oversaw the study and secured funding. YFT, SHY, CSH and CCC analyzed and interpreted the patient data. CJH and CYL performed the histological and pathological assessments. CJH, SHY and KYL collected and analyzed the data. CJH performed most of the experiments. KYL and CLL provided technical support in cloning and the research reagents. CLL obtained and interpretated cloning data, ensured accuracy of cell coverages, and carefully approved the relevant revision. YFT, CJH, CYL and CCC drafted the manuscript. YFT, CJH and CCC confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from the Ethical Committees of Taipei Veterans General Hospital (Taipei, Taiwan; approval no. 2017-07-030AC) and Cathay General Hospital Biobank (Taipei, Taiwan; approval no. HBKEC-20200928-1). The Institutional Review Board of Cathay General Hospital exempted the present study from obtaining informed consent for tissue procurement through the Biobank after an anonymous unlinked process (approval no. CGH-P108136). The study was performed in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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