# Expression of CYP4X1 in colorectal carcinoma is associated with metastasis and poor prognosis

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## Abstract

# Background

Colorectal cancer (CRC) is the third most common cancer and has the second highest mortality rate. The recurrence rate of colorectal cancer is 30–50%, and the survival rate of patients with recurrence and metastasis is very low. Prognostic biomarkers of colorectal cancer can be guidelines for the therapeutic management of the cancer patients. The cytochrome P450 (CYP) family is upregulated in various cancers and contributes to cancer cell proliferation, signaling and drug metabolism. In addition, the Cytochrome P450 family is related to various xenobiotics and endogenous compounds. Nevertheless, the role of *CYP4X1* in regulating the progression and growth of colorectal cancer remains unclear.

# Methods

To investigate the relationship between *CYP4X1* expression and colorectal cancer, *CYP4X1* expression was inhibited in colorectal cancer cells using siRNA and confirmed at mRNA and protein levels. WST-1, Transwell and colony formation assays were performed using *CYP4X1* downregulated cells. We performed immunohistochemistry for the *CYP4X1* expression of 243 colorectal cancer tissues and investigated the expression with the patient's clinical parameters.

# Results

In colorectal cancer, downregulated *CYP4X1* suppressed proliferation, migration, invasion, and colony formation. *CYP4X1* overexpression was found to be related to TNM stage, degree of tumor differentiation, invasion of the primary tumor, clinical stages, and lymph node metastasis. In addition, the high *CYP4X1* expression revealed a shorter survival period than those with low *CYP4X1* expression by Kaplan-Meier survival analysis.

# Conclusions

Upregulated *CYP4X1* may be an independent prognostic marker for CRC, and *CYP4X1* may be a therapeutic target for CRC patients. Epoxyeicosatrienoic acid (EET) derived from arachidonic acid has been implicated in carcinogenesis due to its CYP polymorphism. Whether *CYP4X1* regulates EET in CRC remains unknown. Therefore, the increased *CYP4X1* in EET requires further studies on the correlation between EGFR phosphorylation and PI3K/AKT and MAPK signaling.

## Background

Cancer is the most common cause of death worldwide, and colorectal cancer (CRC) is the third most common malignant tumor and the second most common cause of death [1]. In addition, the relapse rate

of CRC after resection is 30–50%, and half of the patients with CRC develop metastasis [2, 3]. In the United States, the 5-year relative survival rate of CRC patients ranges from 90% of patients with a diagnosis of confined disease to 14% of patients with distant metastasis [4]. Predicting relapse and metastasis is crucial for the efficient treatment of CRC patients. However, despite many studies, prognostic biomarkers that can predict the relapse and metastasis of CRC remain limited.

Cytochrome P450 (CYP4) enzymes are categorized into 18 families and 43 subfamilies [5]. The CYP4 enzyme family is a multigene family of inducible enzymes that play essential roles in the oxidative metabolism of various xenobiotics and endogenous compounds [6, 7]. In addition, CYP4 plays a significant role in tumorigenesis by metabolizing several carcinogens [8]. Compounds implicated in CRC etiology include polycyclic aromatic hydrocarbons, particularly heterocyclic amines, many of which demand metabolic activation by CYP4 before exhibiting their genotoxic effects [9, 10]. Abnormal CYP4 expression in cancer contributes to cell proliferation, signaling, and drug metabolism for treatment outcomes [11]. Approximately a quarter of the cytochrome P450 enzymes are considered orphans because their expression patterns, regulation, and functional information remain unknown [12–14].

Cytochrome P450 family 4, subfamily X, polypeptide 1 (*CYP4X1*) is a member of the cytochrome P450 superfamily. Human *CYP4X1* is classified as an orphan CYP owing to its unknown function. Human *CYP4X1* mainly occurs in the adult human aorta, organs, and skeletal muscle [15]. The metabolic capacity of *CYP4X1* is unknown; however, recent studies have confirmed that arachidonic acid derivatives are involved in many essential physiological processes [16]. The arachidonic acid derivative arachidonoyl ethanol amide (anandamide) is a natural endocannabinoid in human tissues that functions as a vital signaling mediator in immune, neuronal, and cardiovascular functions [16]. And *CYP4X1* is known to be expressed and play a role in neurovascular brain function [17]. According to recent research, *CYP4X1* plays an essential role in several cancers. *CYP4X1* shows an association with cancer activity [17–20], and *CYP4X1* overexpression has been reported in breast cancer [19], gastric cancer [20], brain cancer [17]. Therefore, human *CYP4X1* is a potential drug target for cancer therapy. However, the role of *CYP4X1* in CRC remains unknown. This study investigated, for the first time, the effects of *CYP4X1* expression in vitro, and correlated the *CYP4X1* expression with clinical parameters of cancer patients.

## Material and methods

# Cell lines and cell culture conditions

Human colorectal cancer cells (SW480, SW620, HCT116, and HT29) were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). All the cell lines were maintained in Roswell Park Memorial Institute 1640 (RPMI1640 medium, Hyclone, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Corning, NY, USA), 100 units penicillin and 100 ug/ml streptomycin (Corning) in an atmosphere of 5% $CO_2$ at 37°C. All human colorectal cancer cells were purchased within the past five years. Cell identity was confirmed via short tandem repeat (STR) profiling of KCLB.

# Transfection of small interfering RNA

The *CYP4X1* siRNA was used to down-regulate the expression of *CYP4X1* in human colorectal cancer cell lines, and pre-made siRNA was purchased from Bionner (Daejeon, Korea). siRNA transfection was performed according to the manufacturer's protocol. Four CRC cell lines were seeded on 6-well plates with RPMI1640 containing 10% FBS and cultured for 24 hours (cell confluent of 50–60%). Then the culture medium was discarded from the cells, 30 nM of small interfering RNA was mixed with Lipofectamine RNAiMAX (Thermo Fisher Scientific, MA, USA) in opti-MEM (Thermo Fisher Scientific), and the cells were transfected for 48 hours. *CYP4X1*-specific siRNA sequence target is NM_001320289.1, NM_001320290.1, NM_178033.1, and XM_017000973.1. The negative control consisted of CRC cells non-targeted with siRNA (AccuTarget™ Negative Control siRNA).

# RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from negative control cells and *CYP4X1*-inhibited cells using an RNA extraction kit (GENEALL, Seoul, Korea) following the manufacturer's instructions. The RNA purity and concentration were assessed via optical density measurements using a microvolume spectrophotometer (Pultton, CA, USA). Furthermore, 500ng RNA was reverse transcribed into cDNA using the HyperScript RT Master Mix Kit (GENEALL). RT-PCR was performed using the SYBR Green real-time PCR master mix kit (TOYOBO, Osaka, Japan). The following primers were used: *CYP4X1* sense primer 5 $TGAGCAG\forall CAGATC\mathbb{C}AAGT3$; *CYP4X1* antisense primer 5 $CAG\forall TGAG\mathbb{C}ATCA\mathbb{C}TC\forall T3$; GAPDH sense primer 5$GTCT\mathbb{C}TCTGACT\mathbb{C}\forall CAGCG3$; GAPDH antisense primer 5$A\mathbb{C}CTG\top GCTGTAG\mathbb{C}AA3$. The amplicons were loaded onto an 2% agarose and visualized using Neogreen (Neoscience, Daejeon, Korea). Then, amplicons were shown using a FluoroBox (Neoscience) nucleic acid gel imaging system, and mRNA expression levels were analyzed using Image J 1.53t software (National Institutes of Health). All analyses were performed in triplicate.

# Western blot assay

The total cellular proteins of negative control cells and *CYP4X1*-inhibited cells were lysed with PRO-PREP (iNtRON, Seongnam, Korea) and quantified using BCA kit (Thermo Fisher Scientific). 30 μg total protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred for 100 minutes onto polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) for 1 hour. Primary antibodies were added to the blocked membranes and incubated overnight at 4°C. The primary antibodies were used anti-*CYP4X1* rabbit polyclonal antibody (1:1,000; MyBioSource, Vancouver, Canada), anti-β-actin mouse monoclonal antibody (1:1,000; Santacruz, Santa Cruz, CA, USA). The next day, secondary antibodies were added and incubated for 2 hours. Membranes were visualized using reagents detected via enhanced chemiluminescence (ECL, Thermo Fisher Scientific) and the Molecular Imager ChemiDoc XRS + System (Bio-Rad Laboratories, CA, USA). The acquired images were analyzed using Image J 1.53t software (National Institutes of Health).

# Proliferation assay (WST-1 assay)

A cell proliferation assay kit (EZ-CYTOX, DAEILLAB SERVICE Co., Ltd., Seoul, Korea) was used to analyze the proliferation rates of CRC cells (SW480, SW620, HCT116, and HT29). Each well of 96-well plates were seeded with negative control cells and *CYP4X1*-siRNA treated cells (1x104 cells/well) and incubated at 37°C and 5% $CO_2$. Then EZ-CYTOX (DOGEN, Seoul, Korea) was applied 5 mg/mL into each well at 24, 48, and 72 hours. After adding EZ-CYTOX and reacting for 1 hour in 37°C incubation, the absorbance value was quantified at 450 nm using a microplate photometer. All analyzes were performed in triplicates.

# Transwell assay (migration and invasion assay)

The migration assay was performed using transwell and invasion assay was using transwell coated with 1 mg/mL Matrigel (Corning). For the post process of invasion assay, the same procedure as described for the migration assay was performed. Negative control cells and *CYP4X1*-siRNA treated cells were seeded into Transwell top chamber (8 um pore size) at $5 \times 10^5$ cells in serum-free RPMI1640. Furthermore, complete medium was added to the bottom chamber and incubated for 72 hours. The cells in the upper chamber were moved across the membrane to the lower chamber. After, the culture medium was removed from the chamber, and the chamber was washed three times with PBS. The chamber was then fixed with 3.7% formaldehyde (Biosesang, Seongnam, Korea) for 2 minutes. Then cells were treated to increase permeability with methanol for 20 minutes and stained with 0.005% crystal violet for 20 minutes. All the dyeing processes were performed at room temperature. The cells were counted by randomly selecting five locations.

# Soft agar colony formation assay

Bottom agar was used in RPMI1640 supplemented with 10% FBS and 0.5% agarose in a 6-well plate. The top agar was placed with the negative control cells and *CYP4X1*-siRNA treated cells ($2 \times 10^4$ cells) in RPMI1640 containing 10% FBS and 0.35% agarose. After 21 days, the agarose was stained with 0.005% crystal violet and five positions were randomly selected and photographed. Colonies larger than 30 μm in diameter were counted.

# Human colorectal carcinoma specimens

Formalin-fixed and paraffin-embedded TMA slides containing tissues from 243 colorectal cancer patients were purchased from Superbiochips (Seoul, Korea, https://www.tissue-array.com/). Patients with CRC included 153 men and 90 women (male: female ratio, 1:0.59), and the average age of the population is 63.2 years (range, 25–88 years). Clinicopathological data, including TNM classification and clinical stage, are presented in Table 1(Supplementary Table 1). The tumor stage was determined according to the TNM classification of the American Joint Committee.

Table 1
Information of tissues (TMA) used for immunohistochemistry staining

| Characteristic | N |
|---|---|
| Age | |
| ≤ 60 | 109 |
| > 60 | 134 |
| Sex | |
| Male | 133 |
| Female | 110 |
| pT | |
| T1 | 0 |
| T2 | 23 |
| T3 | 187 |
| T4 | 33 |
| pN | |
| N0 | 102 |
| N1 | 96 |
| N2 | 45 |
| Metastasis | |
| M0 | 222 |
| M1 | 21 |
| Pathologic stage | |
| Stage | 7 |
| Stage | 89 |
| Stage | 126 |
| Stage | 21 |
| Total | 243 |

# Immunohistochemistry staining

Colorectal patients TMA slides were deparaffinized with xylene for three times and rehydrated stepwise using ethanol from 100% to70%. Epitope retrieval was restored in the citrate buffer (pH 6.0) using a microwave for 20 minutes. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide for 30 minutes at room temperature. For blocking was used 3% BSA and washed with phosphate buffered saline containing 0.1% tween-20 (PBST). TMA slides were incubated with anti-*CYP4X1* rabbit polyclonal antibody (1:500, MyBioSource, Vancouver, Canada) overnight at 4°C and incubated with secondary anti-rabbit antibody (1:2000) for 4 hours at room temperature. After washing, tissues were stained with 3, 3'-diaminobenzidine tetrahydrochloride-chromogen (DAB) and counterstained with Mayer's hematoxylin and observed under a microscope. The negative control was stained using the same procedure, omitting the primary antibodies.

# Immunohistochemistry data analysis

*CYP4X1* expression in the TMA slides were evaluated by two observers (Chang Jin Kim and Dongjun Jeong) and scored based on staining intensity and percentage of positive cells. The percentage of positive cells was classified into four groups: score 0 (0–10%); 1 (11–30%); 2 (31–50%), and 3 (51–100%). The staining intensity was scored into four grades: score 0 (no expression), 1 (mild expression), 2 (moderate expression), and 3 (strong expression). The final score was obtained by multiplying the distribution of cells with the staining intensity score. The final score was classified into a group greater than 4 and a low group (high expression group $\geq 4$ and low expression group $< 4$).

# Statistical analysis

All data are presented as the average of triplicate values. Analysis was performed using SPSS v19 software (SPSS, Inc., Chicago, IL, USA) and statistical analysis was performed using an unpaired Student's t-test. For analysis of IHC scores, comparisons were made using the Mann Whitney U test followed by Kruskal–Wallis followed by Dunn or Steel–Dwass post hoc tests. Chi-squared analysis was used for clinicopathological factors of CRC patients according to *CYP4X1* expression. *CYP4X1* expression and 95% risk ratio were addressed by Cox regression analysis, and 5-year survival was assessed by Kaplan–Meier analysis between *CYP4X1* expression and patient outcome. A p-value $< 0.05$ was considered to indicate a statistically significant difference.

# Results

### Inhibition of CYP4X1 mRNA and protein expression in colorectal cancer cell lines and CYP4X1 siRNA effect

Small interfering RNA (siRNA) was used to explore the role in CRC cell function according to *CYP4X1* expression. The expression of *CYP4X1* was suppressed in colorectal cancer cell lines (SW480, SW620, HCT116, and HT29). *CYP4X1* expression was efficiently reduced by treatment with *CYP4X1*-siRNA at 30 nM and downregulation of *CYP4X1* gene expression was demonstrated using RT-PCR and western blot assays. *CYP4X1* expression was significantly downregulated at the mRNA levels in all colorectal cancer cell lines ($p < 0.001$, Fig. 1A-B). The average *CYP4X1* expression in the *CYP4X1* siRNA treated cell group

was reduced by 59.3 ± 3.7% compared to the negative control cell group (SW480; 57.5 ± 6.2%, SW620; 46.8 ± 3.3%, HCT116; 60.8 ± 2.4%, and HT29; 72.1 ± 3.2%). In addition, western blot assay indicated a decrease in the protein levels of *CYP4X1* expression in CRC cells ($p < 0.001$, Fig. 1C-D). Compared to the negative control group of each cell line, the *CYP4X1* protein expression levels of *CYP4X1* siRNA treated group was decreased as follows: SW480; 88.3 ± 7.3%, SW620; 69.9 ± 4.4%, HCT116; 69.4 ± 6.6%, and HT29; 82.6 ± 11.7%. Cells in which efficient *CYP4X1* inhibition was confirmed were then used for cancer function studies of proliferation, migration, invasion, colony formation.

### CYP4X1 expression was associated with human CRC cell proliferation

WST-1 assay was performed to investigate the proliferation patterns of human colorectal cancer cells according to *CYP4X1* expression. Proliferation of cells was compared for negative control cells and *CYP4X1* siRNA treated cells in human colorectal cancer. CRC cells in which *CYP4X1* expression was downregulated showed reduced cell proliferation compared to negative controls. The inhibitory effect on cell proliferation was significantly shown at 24, 48 and 72 hours after transfection ($p < 0.001$, Fig. 2A-D). At 24 hours, the proliferation rate of the *CYP4X1* siRNA treated group than the negative control group was SW480; 23.31 ± 7.5%, SW620; 47.81 ± 4.4%, HCT116; 43.17 ± 4.6%, and HT29; 67.32 ± 5.6%, decreased. And 48 hours; SW480; 19.03 ± 3.2%, SW620; 46.21 ± 4.6%, HCT116; 47.15 ± 3.8%, and HT29; 62.09 ± 6.3%, 72 hours; SW480; 32.64 ± 4.2%, SW620; 29.38 ± 7.0%, HCT116; 31.46 ± 2.2%, and HT29; 51.42 ± 6.6%, decreased. These results demonstrated that *CYP4X1* inhibition particularly inhibited the proliferation of CRC cell lines.

### CYP4X1 downregulation affected cell migration and invasion in CRC

To investigate the effect of *CYP4X1* expression on metastasis of CRC cells (SW480, SW620, HCT116, and HT29), the effect of cell migration and cell invasion were analyzed using transwell assay. Downregulated *CYP4X1* was associated with significantly reduced cell migration in CRC. *CYP4X1* siRNA treated cells had decreased migration ability to the bottom chamber compared with the negative control cells; SW480; 58.4 ± 18.4%, SW620; 67.1 ± 6.7%, HCT116; 53.2 ± 12.2%, and HT29; 85.5 ± 6.7% ($p < 0.001$, Fig. 3A, 3C). And downregulated *CYP4X1* expression was associated with notably decreased cell invasion. *CYP4X1*-downregulated cells had lower ability to invade the bottom chamber than the negative control cells; SW480; 72.7 ± 14.4%, SW620; 79.8 ± 7.5%, HCT116; 62.3 ± 13.2%, and HT29; 93.9 ± 4.5% ($p < 0.001$, Fig. 3B, 3D). These results indicate that *CYP4X1* is significantly in migration and invasion.

### CYP4X1 knockdown reduced the tumorigenicity of CRC

To determine whether *CYP4X1* affects tumorigenesis, we performed a colony forming assay using CRC cells. *CYP4X1* downregulated cells demonstrated lower colonizing ability than the negative control cells; SW480; 77.7 ± 12.2%, SW620; 84.5 ± 9.7%, HCT116; 79.8 ± 13.3%, and HT29; 75.0 ± 14.0% ($p < 0.001$, Fig. 4A-B). These findings indicate that *CYP4X1* knockdown markedly reduced colony formation in CRC cells.

### CYP4X1 is associated with poor prognosis in patients with colorectal cancer

The expression of *CYP4X1* was compared and analyzed by immunohistochemistry staining in the normal colon tissues (adjacent to cancer) and colorectal cancer tissues obtained from 243 colorectal cancer patients. *CYP4X1* positive expression was mostly detected on the cell membrane and was stained brown. *CYP4X1* is overexpressed in CRC tissues compared normal colon tissues (Fig. 5A). *CYP4X1* low expression was observed in 108 CRC tissues in 243 tissues of CRC patients, whereas high *CYP4X1* levels were expressed in 135 CRC tissues, and the results were shown in Table 2. *CYP4X1* overexpression significantly correlated with pN ($p < 0.001$) and clinical stage ($p < 0.001$). *CYP4X1* expression and colorectal cancer patients clinical relationships were also investigated. The 5-year overall survival rate according to *CYP4X1* expression was compared and analyzed using Kaplan-Meier analysis. The result was showed that patients with high *CYP4X1* expression had shorter survival times than patients with low *CYP4X1* expression (Log-rank test, $p < 0.001$, Fig. 5B). In addition, as a result of analyzing the overall survival rate of CRC patients in clinical stage ， (low) and clinical stage ， (high) according to the expression of *CYP4X1*, the 5-year overall survival rate of low stage and the difference between *CYP4X1* expression there was no significant difference ($p = 0.150$, Fig. 5C). However, high stage of CRC patients was relationship between the overall survival rate and the expression of *CYP4X1* ($p < 0.001$, Fig. 5D). Cox regression analysis related to clinicopathological factors for patient prognosis was performed (Table 3). Univariate analysis indicated that the following factors were specifically related to overall survival (Hazard ratio (95%/CI)): pT: 0.470(0.271–0.813), $p = 0.007$; pN: 11.833(5.959–23.497), $p < 0.001$; metastasis: 3.949(2.364–6.597), $p < 0.001$; clinical stage: 16.627(7.277–37.988), $p < 0.001$ and *CYP4X1* expression: 6.708(3.873–11.619), $p < 0.001$. Multivariate analysis also demonstrated that the following factors were significantly associated with overall survival (Hazard ratio (95%/CI)) pT: 0.339(0.188–0.609), $p < 0.001$, metastasis: 4.106(2.287–7.370), $p < 0.001$ and *CYP4X1* expression: 3.237(1.699–6.167), $p < 0.001$. These results revealed the association of *CYP4X1* with the poor prognosis of patients with CRC.

Table 2
Clinicopathological factors related to *CYP4X1* expression in patients with CRC

| Characteristics | *CYP4X1* expression | | Total | *p* value |
|---|---|---|---|---|
| | Low (n = 108) | High (n = 135) | (n = 243) | |
| Age, years, mean (SD) | | | | |
| Sex, N (%) | | | | 0.818 |
| Male | 60(45.1) | 73(54.9) | 133 | |
| Female | 48(43.6) | 62(56.4) | 110 | |
| pT, N (%) | | | | 0.327 |
| T1 & T2 | 8(34.8) | 15(65.2) | 23 | |
| T3 & T4 | 100(45.5) | 120(54.5) | 220 | |
| pN, N (%) | | | | **< 0.001** |
| N0 | 78(76.5) | 24(23.5) | 102 | |
| N1 & N2 | 30(21.3) | 111(78.7) | 141 | |
| Metastasis, N (%) | | | | 0.878 |
| M0 | 99(44.6) | 123(55.4) | 222 | |
| M1 | 9(42.9) | 12(57.1) | 21 | |
| Clinical stage, N (%) | | | | **< 0.001** |
| Stage & Stage | 72(75.0) | 24(25.0) | 96 | |
| Stage & Stage | 36(24.5) | 111(75.5) | 147 | |

Table 3
Univariate and multivariate analysis of the clinicopathological factors in patients with CRC

| Characteristics | Variable | Univariate analysis | | Multivariate Analysis | |
|---|---|---|---|---|---|
| | | Hazard ratio (95%/CI) | *p* value | Hazard ratio (95%/CI) | *p* value |
| Age | < 60 year vs. ≥60 yr | 1.270(0.858–1.879) | 0.232 | 1.108(0.742–1.653) | 0.617 |
| Gender | Male vs. Female | 0.984(0.669–1.448) | 0.935 | 0.840(0.565–1.250) | 0.391 |
| pT | T1-T2 vs. T3-T4 | 0.470(0.271–0.813) | **0.007** | 0.339(0.188–0.609) | **< 0.001** |
| pN | N0 vs. ≥N1 | 11.833(5.959–23.497) | **< 0.001** | 2.133(0.533–8.539) | 0.284 |
| Metastasis | M0 vs. ≥M1 | 3.949(2.364–6.597) | **< 0.001** | 4.106(2.287–7.370) | **< 0.001** |
| Clinical stage | Stage - vs. Stage - | 16.627(7.277–37.988) | **< 0.001** | 4.346(0.938–20.130) | 0.060 |
| CYP4X1 expression | Low vs. High | 6.708(3.873–11.619) | **< 0.001** | 3.237(1.699–6.167) | **< 0.001** |

# Discussion

Despite advances in the diagnosis and treatment of colorectal cancer over the past decades, the mortality rate for patients with stage IV CRC remains high. The mortality rate in patients with CRC is associated with metastasis, regional lymph node metastasis, and recurrence [4]. In the Republic of Korea, the survival rate of patients with regional lymph nodes is 79.2%; however, that of patients with distant metastasis is significantly lower (14.6%) [1]. Therefore, targeted molecular therapy is important because the survival rate can be increased by predicting CRC recurrence. Targeted molecular therapy has enhanced the effectiveness of conventional therapies for CRC. Furthermore, prognostic biomarkers can assist oncologists in optimizing the treatment for challenging CRC cases. However, there is a lack of specific biomarkers.

This is the first study of *CYP4X1* in CRC. *CYP4X1* expression in CRC and colon normal tissues was determined using IHC, and the results demonstrated that *CYP4X1* expression was significantly higher in CRC tissues than normal tissues. Normal tissues had low or no expression. *CYP4X1* expression level was found to be related to TNM stage, tumor differentiation degree, infiltration depth of primary tumor, and lymph node metastasis. *CYP4X1* expression in CRC tissues significantly correlated with lymph node metastasis and the stage ($p < 0.001$). In addition, as a result of Kaplan-Meier survival analysis, CRC patients with high *CYP4X1* expression had shorter survival times than CRC patients with low *CYP4X1* expression ($p < 0.001$). This study indicated that *CYP4X1* expression was consistent with CRC

progression. Therefore, overexpression of *CYP4X1* may be an independent prognostic marker for CRC. We investigated *CYP4X1* expression using small interfering RNA technology in four CRC cell lines (SW480, SW620, HCT116, and HT29) and then validated *CYP4X1* expression in four cell lines. Consequently, *CYP4X1* downregulation significantly inhibited cell proliferation, invasion, migration, and colony formation. Therefore, *CYP4X1* can be a treatment target for patients with CRC. CYP4 family enzymes are prognostic markers known for breast cancer and are associated with poor overall survival [19]. The CYP4 family plays a significant role in tumorigenesis by metabolizing many carcinogens [8]. In addition, they play important roles in carcinogenesis and inflammatory diseases via the arachidonic acid sub-pathway [21]. Arachidonic acid derived epoxyeicosatrienoic acids (EETs) are associated with carcinogenesis owing to CYP polymorphisms. CYP-derived EETs regulate cell processes of progression and carcinogenesis, including cell survival, proliferation, invasion, angiogenesis, and metastasis. Upregulated EET accelerates the cell cycle and proliferation [22]. At the molecular level, it promotes tumorigenesis by phosphorylating the epidermal growth factor receptor (EGFR) and activating PI3K/AKT and MAPK signaling pathways. Conversely, CYP inhibition reduced the activity of the EGFR and PI3K/AKT pathways and reduced tumor cell adhesion, and metastasis [22]. Thus, CYP-derived EETs metabolites promote tumor growth, progression, and metastasis [23]. However, it remains unknown whether *CYP4X1* regulates EETs in CRC. Therefore, the *CYP4X1* increase in EETs requires further studies on the correlation between EGFR phosphorylation and PI3K/AKT and MAPK signaling. In this study, the overexpression of *CYP4X1* contributes CRC progression and can be an independent prognostic marker. The evaluation of *CYP4X1* expression in CRC can be a good modality for the treatment of CRC patients and a therapeutic target as well.

# Conclusions

Our study revealed that *CYP4X1* downregulation reduces cell proliferation, migration, invasion, and tumorigenesis in CRC cell lines. Owing to its overexpression in CRC, *CYP4X1* may be an important prognostic biomarker and therapeutic target. Therefore, *CYP4X1* inhibition is a potential marker for CRC prognosis and treatment.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

Anonymized trial data will be available from the corresponding author upon reasonable request for non-commercial research purposes.


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### Authors' contributions

Conceptualization, C.-J.K., D.J., and H.C.K.; Formal analysis, H.K., S.K., and I.H.; Investigation, I.H., S.K.,S.J., and M.L.; Supervision, D.J.; Writing-original draft, S.K. and I.H.; Writing-review and editing, D.J. and C.-J.K. All authors have read and agreed to the published version of the manuscript.

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# Figures

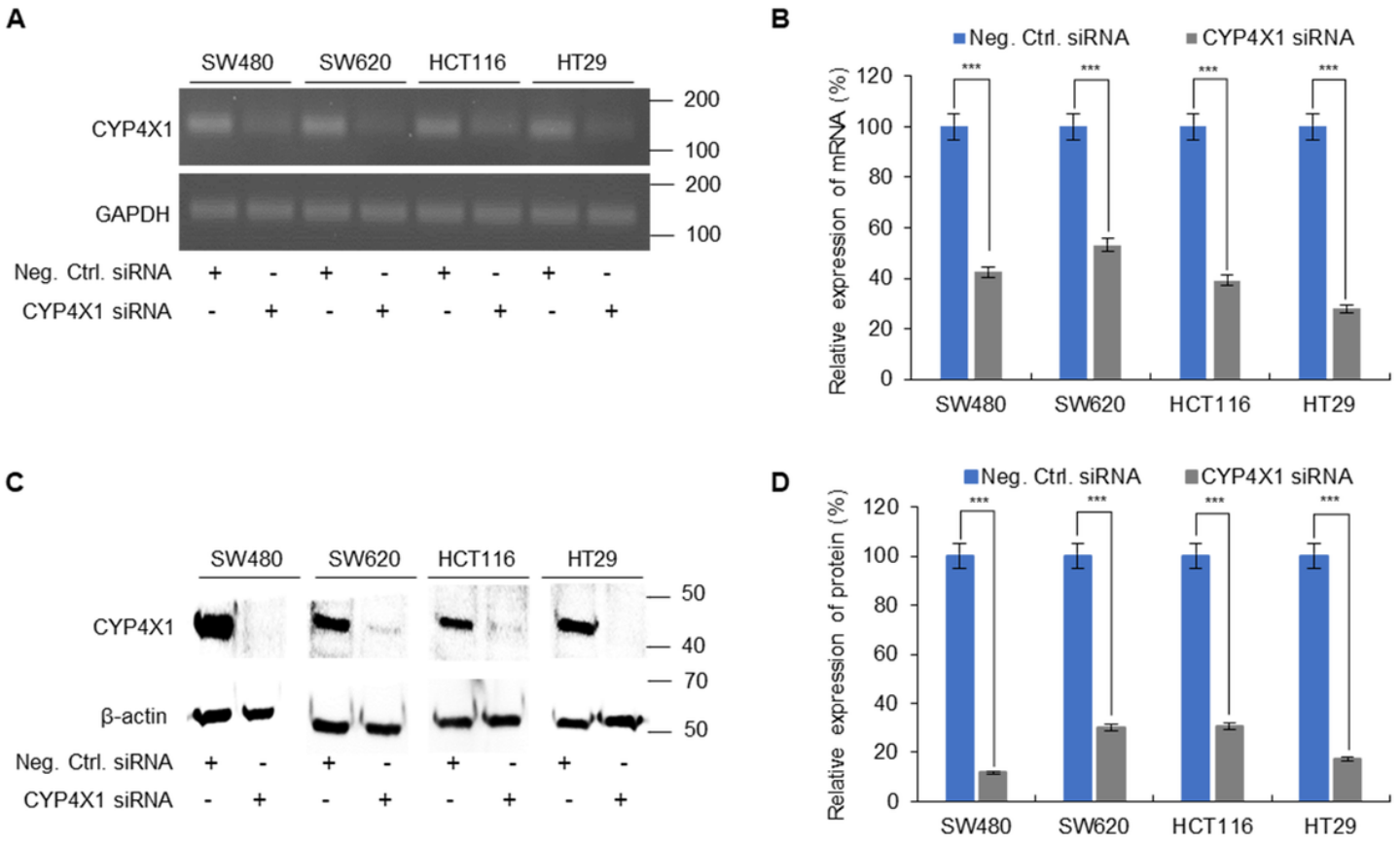
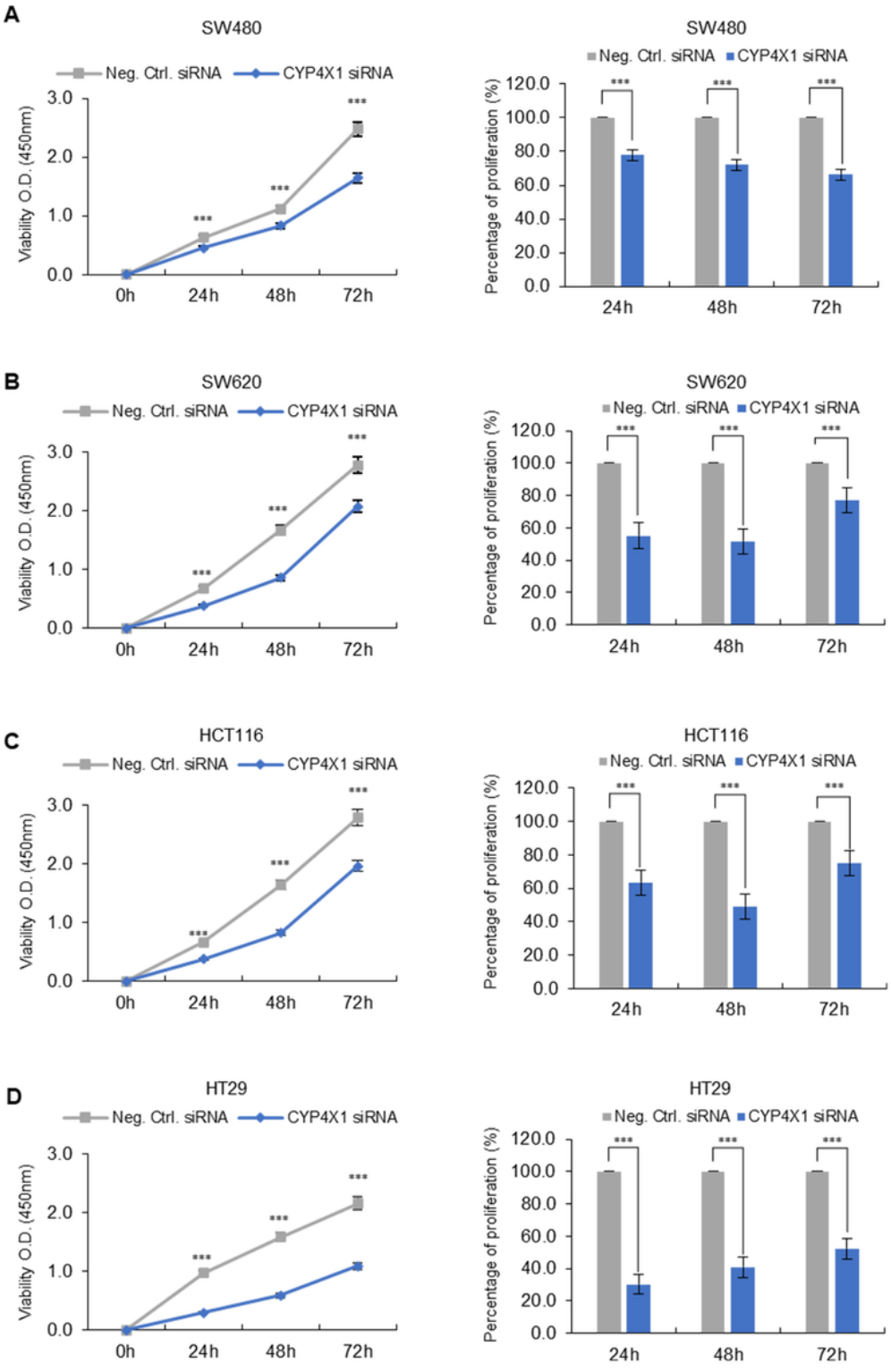



**Figure 1**

*CYP4X1* inhibition in human CRC cells using siRNA was confirmed using RT-PCR and immunoblotting. **A** Reverse transcription polymerase chain reaction (RT-PCR) products gel electrophoresis compared to mRNA levels in negative control and *CYP4X1* knockdown cell lines. **B** percentage of inhibition of *CYP4X1* expression by *CYP4X1*siRNA compared to negative control. **C** *CYP4X1* protein knockdown was confirmed

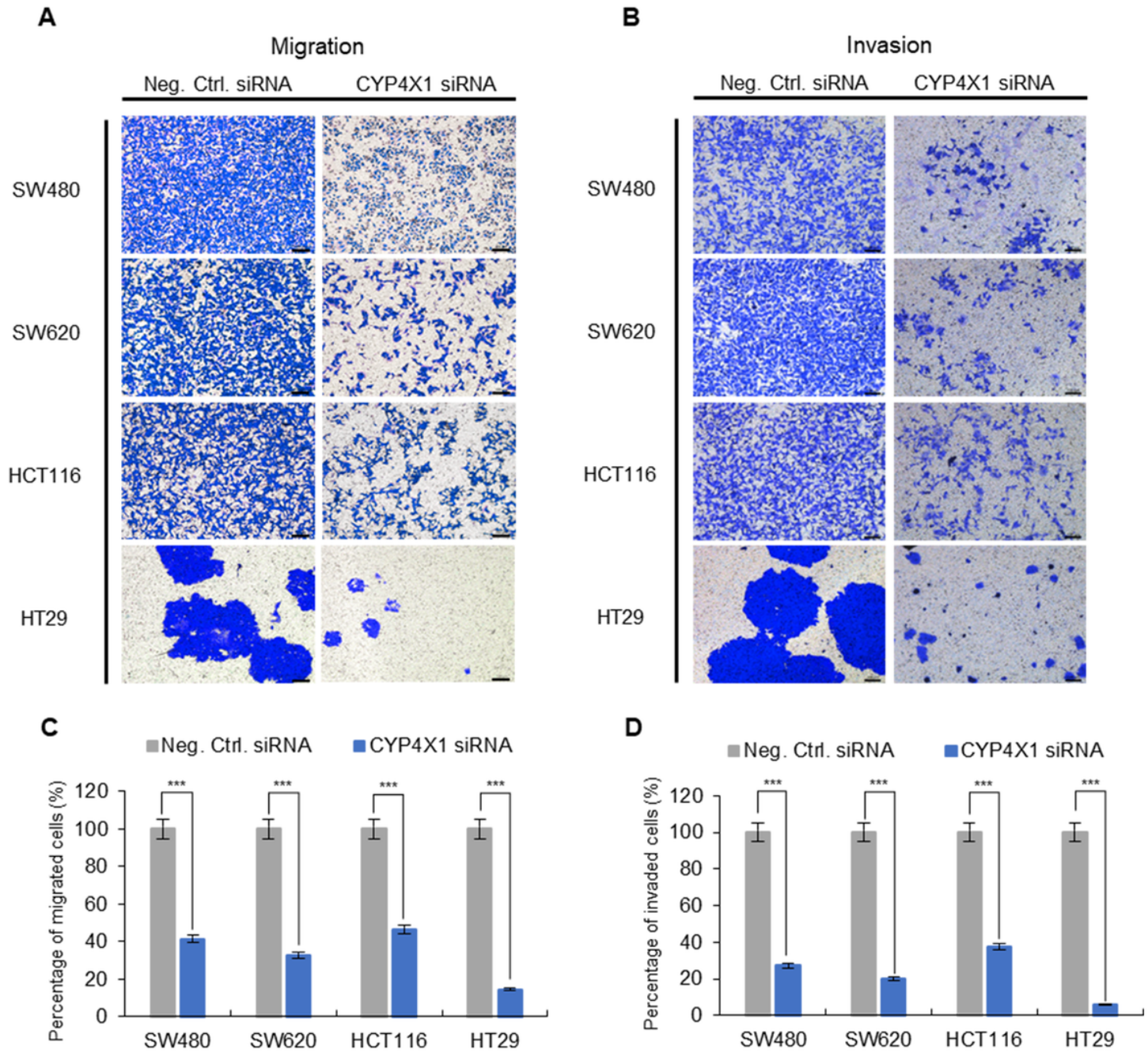
using immunoblotting analysis. **D** Relative protein expression of *CYP4X1* siRNA compared to negative control. ***$p < 0.001$




## Figure 2

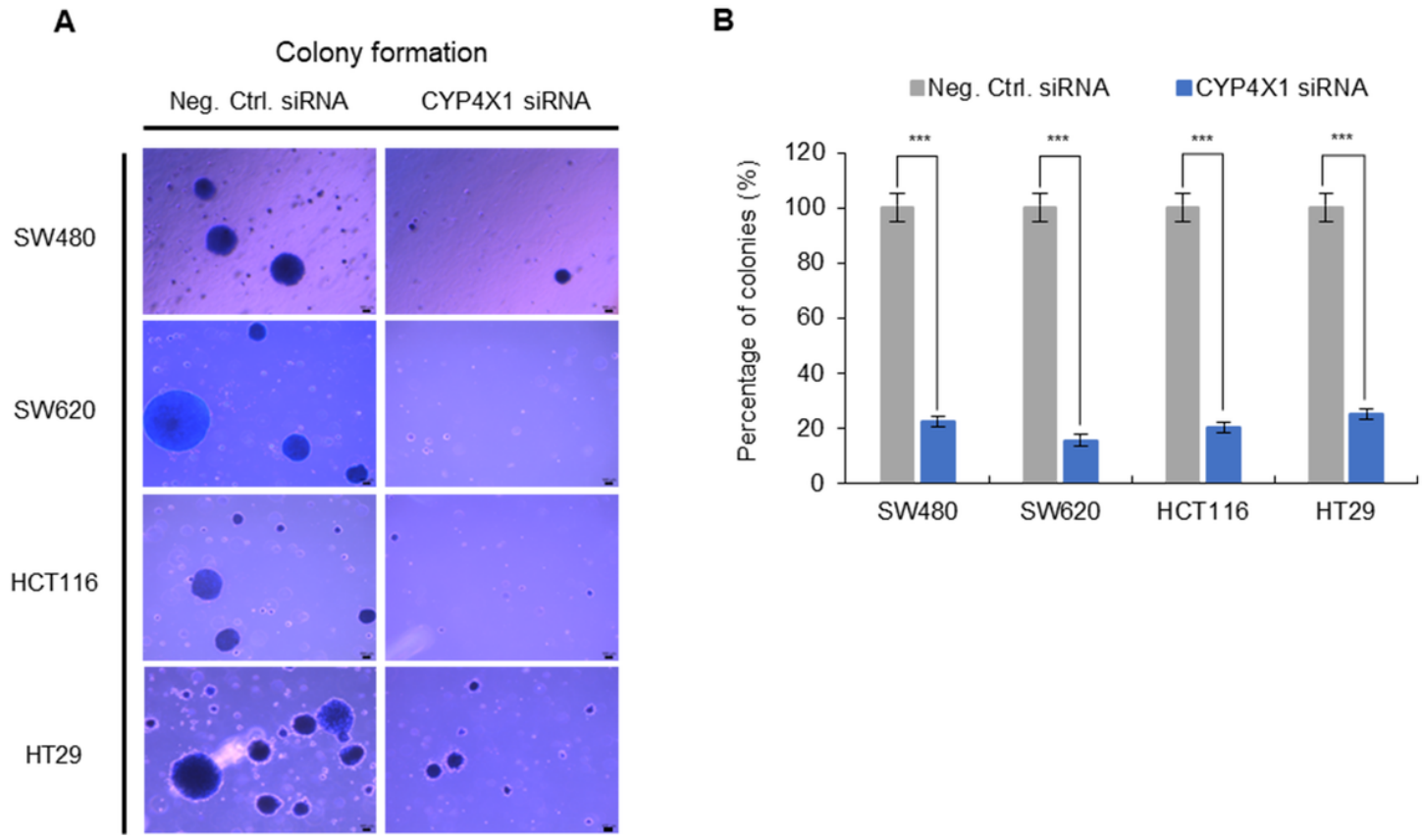
Inhibition of *CYP4X1* reduced the proliferative ability of CRC cells. **A-D** Cell proliferation of Neg. Ctrl. siRNA and *CYP4X1* siRNA cells were contrasted over time **A** SW480, **B** SW620, **C** HCT116, and **D** HT29.

***$p < 0.001$



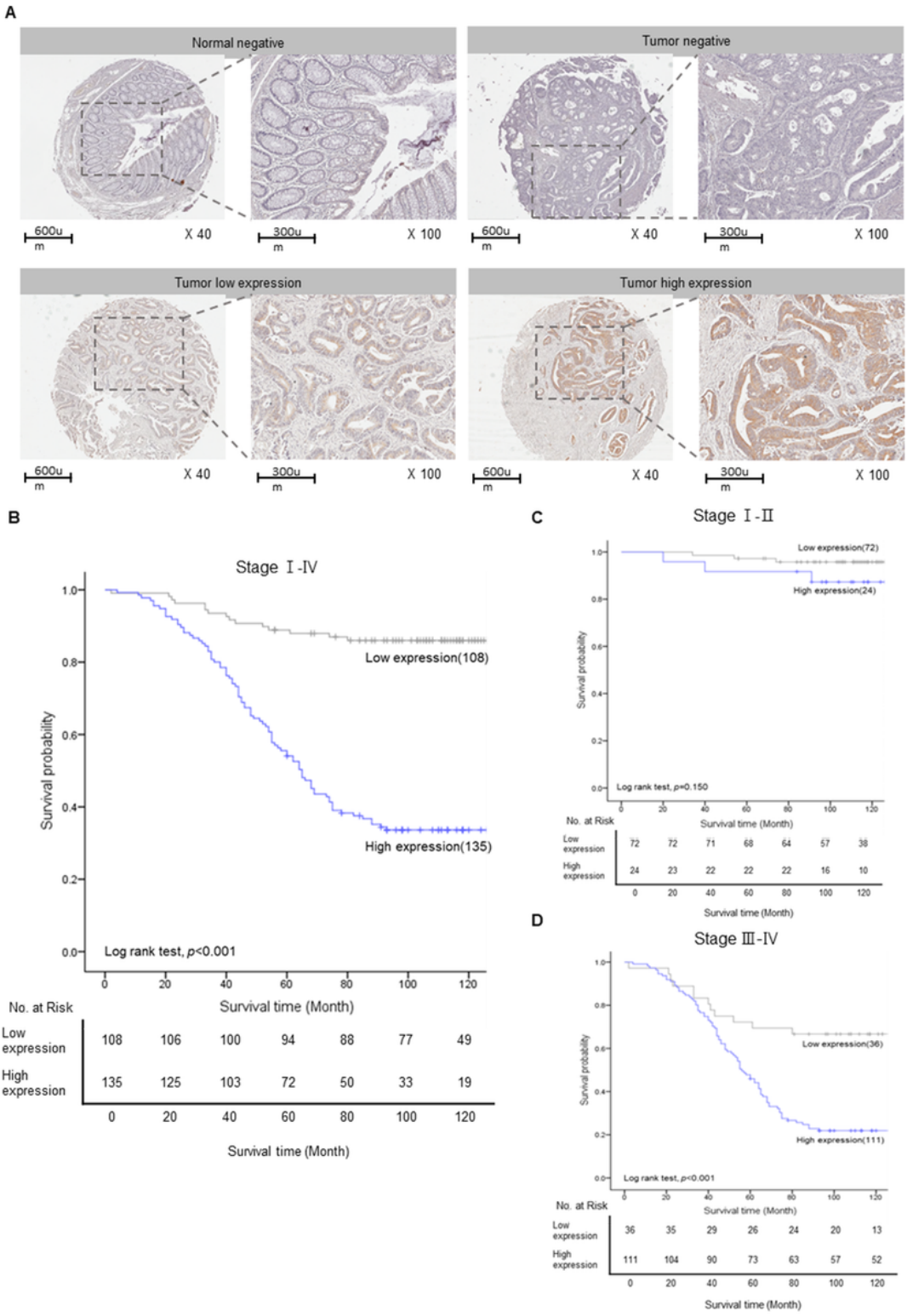

## Figure 3

The data of the Transwell assay concerning metastasis. *CYP4X1* downregulation suppressed migration and invasion. **A** Migration assay performed in Transwell chambers. Cells that migrated to the bottom chambers were stained and photographed (Magnification x40, Scale bar 100 um). **B** Cell that invaded to the bottom chambers were stained and photographed (Magnification x40, Scale bar 100 um). **C** Graph for migration data. **D** Invaded cells quantified in the graph. ***$p < 0.001$




## Figure 4

Soft agar assay for anchorage independent cell growth colony forming of tumorigenesis. *CYP4X1*inhibition reduced colonies. **A** Colony image (Magnification x40, Scale bar 100 um). **B** Colony reduction rate of negative control than *CYP4X1* siRNA in four CRC cell lines. ***$p$< 0.001




## Figure 5

Correlation between *CYP4X1* expression and survival rate of colorectal cancer patients. **A** *CYP4X1* expression was confirmed in CRC tissues using immunohistochemistry staining (IHC). Kaplan-Meier was analyzed in all CRC patient samples based on many variables and *CYP4X1* expression. **B** *CYP4X1*expression was associated with 5-year overall survival of CRC patients. 5-year survival rate of

CRC patients **C** in clinical stages I and II (low) and **D**in stage III and IV (high) according to the expression of *CYP4X1*. ***$p$< 0.001

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.xlsx
- CYP4X1480C480E620C620E116C116E29C29Eband.tif
- CYP4X14C4E6C6E1C1E2C2E.jpg
- GAPDH4C4E6C6E1C1E2C2E.jpg
- Bactin480C480E620C620E116C116E29C29Eband2.tif