THE RELATIONSHIP BETWEEN ARID1A AND EPITHELIAL-MESENCHYMAL TRANSITION-RELATED PROTEINS EXPRESSION IN DIFFERENTIAL HISTOLOGICAL GRADING OF COLORECTAL CANCER TISSUES

PHATTARAPON SONTHI

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
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has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Anatomy of Naresuan University

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ABSTRACT

AT-rich interactive domain-containing protein 1A (ARID1A) is an essential component of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complexes. ARID1A also belongs to the tumor suppressor family, which is involved in gene regulation during carcinogenesis. Previously, *ARID1A* mutations in colorectal cancer (CRC) resulted in loss of its expression level in CRC specimens and were associated with CRC-related clinicopathologic characteristics. Then, ARID1A has been proposed as a potential prognostic biomarker for CRC prognosis and diagnosis. Using the cBioPortal for cancer genomics database analysis, we found *ARID1A* mutations in 7.09% of CRCs, in which truncating and missense mutations were mostly found. The protein expression in the *ARID1A*-mutated group was lower than in the *ARID1A* non-mutated group. Furthermore, the epithelial-mesenchymal transition (EMT) process plays a crucial role in the progression and aggressiveness of CRC. The altered ARID1A expression is also involved in the EMT process in several cancers. However, the relationship between ARID1A and EMT-related protein expression in human CRC tissues still remains unclear. Thus, this study aimed to investigate the relationship between ARID1A and EMT-related protein expressions using immunohistochemistry (IHC). One hundred formalin-fixed, paraffin embedded (FFPE) blocks of CRC patients, including 65 well-differentiated, 23 moderately

differentiated, and 12 poorly differentiated adenocarcinomas, were acquired from Sawanpracharak Hospital, Nakhonsawan, Thailand. The CRC paraffin sections were immunostained with a specific antibody to observe the expression of ARID1A and EMT-related proteins, including epithelial proteins (epithelial-cadherin (E-cad) and zonula occludens-1 (ZO-1)) and mesenchymal proteins (vimentin and fibronectin). The staining intensity and percentage of ARID1A-positive cells were evaluated using a histological (H)-score. A quantitative analysis was performed to evaluate ARID1A and EMT-related protein expressions. The result demonstrated that the immunoreactivity signal of ARID1A was low in most of the cancerous areas of CRC samples (92.00%), while another 8.00% was unchanged. Quantitative analysis using ImageJ Fiji software revealed that the level of ARID1A protein was significantly decreased in the cancerous area when compared to the adjacent non-cancerous area in all three pathological differentiations of CRC ($p<0.001$). Moreover, the expressions of vimentin and fibronectin were increased, whereas E-cad and ZO-1 were decreased in CRC tissues with low ARID1A expression. The association of ARID1A protein expression with the pathological outcomes and prognosis of the patients was also investigated. The Fisher's exact test revealed that low expression of ARID1A protein was significantly associated with a greater number of positive lymph nodes, lymphovascular invasion, lymph node metastasis, lymph node ratio, and comorbidity. Moreover, the results of Kaplan-Meier analysis revealed that the 5-year progression-free survival (PFS) of CRC patients tended to be associated with ARID1A expression. Our results may be useful for the clinicopathological assessment and prognosis of patients with CRC, as well as confirm the involvement of ARID1A in cancer progression and EMT process induction.

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ABBREVIATIONS

AJCC	=	American Joint Committee on Cancer
Akt	=	Protein kinase B
AMACR/p504s	=	α-Methyacyl-CoA racemase
APC	=	Adenomatous polyposis coli
ARID	=	AT-rich Interaction Domain
ARID1A	=	AT-rich interactive domain-containing protein 1A
ARID1B	=	AT-rich interactive domain-containing protein 1B
BAF	=	BRM/BRG1-associated factor
BAF250a	=	BRG1-associated factor 250a
BAF45b	=	Double PHD fingers (DPF)1/2/3
bp	=	Base pair
BRAF	=	V-raf murine sarcoma viral oncogene homolog B1
BRD7	=	Bromodomain containing 7
BRG1	=	Brahma-related gene 1
CapeOx	=	Capecitabine and oxaliplatin
CD	=	Cluster of differentiation
CDX2	=	Caudal type homeobox 2
CIMP	=	The CpG island methylator phenotype
CIN	=	Chromosomal instability
CIs	=	Confidence intervals
CKs	=	Cytokeratins
cm	=	Centimeter
COAD	=	Colon adenocarcinoma
CRC	=	Colorectal cancer
cSRCC	=	Signet ring cell carcinoma of the colon
CTC	=	Computed tomographic colonography
CUP	=	Cancer of unknown primary

ABBREVIATIONS (CONT.)

DAB	=	Chromogen 3,3'-diaminobenzidine
DFS	=	Disease-free survival
DM	=	Diabetes mellitus
DNA	=	Deoxyribonucleic acid
DW	=	Distilled water
E-cad	=	Epithelial cadherin, E-cadherin
ECM	=	Extracellular matrix
EGFR	=	Epidermal growth factor receptor
EMT	=	Epithelial-mesenchymal transition
EMT-TFs	=	Epithelial-mesenchymal transition- inducing transcription factors
ERK	=	Extracellular signal-regulated kinase
FFPE	=	Formalin-fixed, paraffin-embedded
FIT	=	Fecal immunochemical tests for hemoglobin
FS	=	Flexible sigmoidoscopy
FSH	=	Follicle-stimulating hormone
FSP1	=	Fibroblast-specific protein 1
FRBI	=	Follicle-stimulating hormone receptor binding inhibitor
gFOBT	=	Guaiac-based fecal occult blood tests
GI	=	Gastrointestinal tract
GLTSCR	=	Glioma tumor suppressor candidate region gene
H&E	=	Hematoxylin and eosin
H-score	=	Histological score
H_2O_2	=	Hydrogen peroxide
HER2	=	Human epidermal growth factor receptor 2
HIER	=	Heat-induced epitope retrieval
HPF	=	High power fields
IBD	=	Inflammatory bowel disease

ABBREVIATIONS (CONT.)

IDA	=	Iron deficiency anemia
IgG	=	Immunoglobulin G
IHC	=	Immunohistochemistry
IR	=	Insulin resistance
JARID	=	Jumonji AT-rich interaction domain
KRAS	=	Kristen rat sarcoma viral oncogene homolog
LGL	=	Lethal giant larvae
LNM	=	Lymph node metastasis
MAC	=	Mucinous adenocarcinoma of colon and rectum
Mb	=	Megabyte
mCEA	=	Monoclonal carcinoembryonic antigen
mCRC	=	Mucinous carcinoma
MEK	=	Mitogen-activated protein kinase
MET	=	Mesenchymal–epithelial transition
MLH1	=	MutL homolog 1
mLNR	=	Metastatic lymph node ratio
MMPs	=	Matrix metalloproteinases
MMR	=	Mismatch repair system
mRNA	=	Messenger ribonucleic acid
MSI	=	Microsatellite instability
MSI-H	=	High level microsatellite instability
MSI-L	=	Low level microsatellite instability
MSS	=	Microsatellite stable
MUC	=	Mucous glycoproteins, Mucins
N-cadherin	=	Neural cadherin
NaN_3	=	Sodium azide
ncBAF	=	Non-canonical BAF

ABBREVIATIONS (CONT.)

NF-κB	=	Nuclear factor kappa B
OS	=	Overall survival
p	=	Pathological differentiation
p53	=	Tumor protein 53
PALS1	=	Protein associated with lin-seven 1
PATJ	=	PALS1-associated tight junction protein
PBAF	=	Polybromo-associated BAF
PBRM1	=	Protein polybromo-1
PBS	=	Phosphate buffered saline
PDAC	=	Pancreatic ductal adenocarcinoma
PFS	=	Progression-free survival
PHF 10	=	PHD finger 10
PI3K	=	Phosphatidylinositol 3-kinase
RAS	=	Rat sarcoma
RCC	=	Renal cell carcinoma
READ	=	Rectal adenocarcinoma
RFS	=	Recurrence-free survival
RFS	=	Relapse-free survival
ROD	=	Relative optical density
S100	=	S100 calcium-binding protein
SD	=	Standard deviation
SEM	=	Standard error of the mean
SMAD4	=	Mothers against decapentaplegic homolog 4 (Drosophila)
SMARCA	=	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member
SNAIL	=	Zinc finger protein SNAI1

ABBREVIATIONS (CONT.)

SWI/SNF	=	SWItch/sucrose non-fermentable
TCGA	=	The Cancer Genome Atlas
TGF-β	=	Transforming growth factor β
TNM	=	Tumor-node-metastasis
TP53	=	Tumor suppressor protein 53
TWIST	=	Twist-related protein
UICC	=	Union for International Cancer Control
VEGF	=	Vascular endothelial growth factor
WHO	=	World Health Organization
Wnt	=	Wingless-related integration site
ZEB	=	Zinc finger E-box binding homeobox
ZO-1	=	Zonula occludens-1
5-FU	=	5-fluorouracil
α	=	Alpha
α-SMA	=	Alpha-smooth muscle actin
β	=	Beta
°C		Degree Celsius
κ	=	Kappa
μg	=	Microgram
μl	=	Microliter
μm	=	Micrometer, Micron

CHAPTER1

INTRODUCTION

Rationale of the study

The World Health Organization (WHO) reported that cancer is potentially the greatest cause of mortality among people under the age of 70 (Sung et al., 2021). The global incidence of colorectal cancer (CRC) is dramatically growing, as similar as its mortality rate. CRC incidence is expected to rise by 60%, with more than 2.2 million new cases and 1.1 million fatalities by 2030 (Arnold et al., 2017). CRC is ranked as the second most prevalent cause of cancer-related death in both genders in the United States (Siegel et al., 2020). In Thailand, CRC is the third most frequent cancer in men and the fourth most frequent in women (Bray et al., 2018). The incidence and mortality of CRC are approximately 25% higher in men than in women (Sung et al., 2021). Patients with distant metastases of CRC did not respond well to the standard therapies and had an unsatisfactory 5-year survival rate and a worse prognosis (Brenner et al., 2014; Manfredi et al., 2006). Early screening, detection, and diagnosis have resulted in a significant and considerable reduction in both CRC morbidity and mortality (Gellad & Provenzale, 2010; Mundade et al., 2014). Therefore, an accurate and reliable prognostic indicator for early CRC diagnosis and better prognostication should be identified to improve the pathological outcomes of patients with CRC.

AT-rich interactive domain 1A (*ARID1A*), also known as BAF250a, or p270, is a subunit of the human switch/sucrose non-fermenting (SWI/SNF) chromatin remodeling complexes. *ARID1A* is also a key constituent of the BRG1-associated factor (BAF) subclass of the human SWI/SNF chromatin remodeling complexes (Hurlstone et al., 2002; Wang et al., 2004; Wilsker et al., 2005). *ARID1A* has been recognized as a tumor suppressor gene that is involved in cell cycle regulation, apoptosis promotion, and genomic instability inhibition(Wu et al., 2014). However, the *ARID1A* gene is the most frequently mutated subunit of the SWI/SNF chromatin remodeling complexes that has been reported in various types of human malignancies, such as gynecological cancers, gastric carcinoma, cholangiocarcinoma, and bladder urothelial carcinoma

(Mathur, 2018; Wu et al., 2014). The majority of *ARID1A* mutations were inactivating mutations, leading to loss of expression of ARID1A at the protein level that can be detected by immunohistochemistry (Wang et al., 2021). Decreasing or loss of ARID1A expression has been increasingly found in various types of human cancers, especially gastrointestinal cancers (Wang et al., 2021). such as gastric cancer (Abe et al., 2012; Inada et al., 2015), hepatocellular carcinoma (He et al., 2015), cholangiocarcinoma (Namjan et al., 2020), and also in CRC (Chou et al., 2014; Erfani et al., 2020; Kishida et al., 2019; Lee et al., 2016; Wei et al., 2014; Ye et al., 2014). Several studies have suggested that ARID1A may serve as a prognostic biomarker for cancer diagnosis and prognosis (Lichner et al., 2013; Samartzis et al., 2012; Wei et al., 2014; Wiegand et al., 2014). In addition, previous studies revealed that decreasing or loss of ARID1A protein expression in CRC was significantly associated with the severity of clinicopathological features, such as gender, poor pathological grading, late tumor-node-metastasis (TNM) staging, distant metastasis, and lymphovascular invasion (Lee et al., 2016; Wei et al., 2014). However, alterations of ARID1A expression did not correlate with overall, disease-specific, or recurrence-free survival in patients with CRC (Chou et al., 2014; Erfani et al., 2020; Lee et al., 2016). The study of the relationship between ARID1A protein expression and clinical significance in CRC is limited. This still requires further investigations to elucidate the significance of ARID1A as one of the promising prognostic indicators that may be useful for a precise prognosis of CRC.

Furthermore, a biological process known as an epithelial-mesenchymal transition (EMT) is implicated in cancer growth and metastasis (Thiery, 2003).). EMT occurs during embryonic development, tissue remodeling, wound healing, and cancer progression and metastasis (Kalluri & Weinberg, 2009). Previous studies have demonstrated that EMT contributes to the proliferation, invasion, and metastasis in various epithelial tumors (Arias, 2001; Fantozzi et al., 2014). Moreover, EMT plays a crucial role in the progression and aggressiveness of CRC (Barker & Clevers, 2001; Bates, 2005; Brabletz et al., 2005; Hur et al., 2013). Recently, several studies have revealed that *ARID1A* knockdown exhibited an increase of cell proliferation, migration, and invasion in various cancer cell lines, including renal cell carcinoma (RCC), pancreatic ductal adenocarcinoma (PDAC), breast cancer, and CRC (Erfani et al., 2021; Somsuan et al., 2019; Tomihara et al., 2021; Wang et al., 2020).). In addition, *ARID1A*

knockdown exhibited the upregulated expression of mesenchymal markers (vimentin and fibronectin) and the downregulated expression of epithelial markers (E-cad and ZO-1) in RCC and PDAC (Somsuan et al., 2019; Tomihara et al., 2021). Thus, loss of *ARID1A* expression may promote cancer metastasis through decreased EMT-related protein (Erfani et al., 2021). Nevertheless, the investigation of ARID1A and EMT-related proteins has not been reported in human CRC tissues.

In this study, we aimed to investigate alterations of ARID1A protein expression and EMT-related protein in human CRC tissues. Furthermore, the relationship between ARID1A and EMT-related protein expression and the severity of clinicopathological characteristics and pathological outcomes in CRC patients was evaluated in order to provide a better understanding, clarification, and elucidation of the clinical significance of ARID1A expression in human CRC.

Objectives of the study

1. To investigate the expression of ARID1A protein in cancerous area compared with adjacent non-cancerous area in each pathological differentiation of CRC

2. To determine the expressions of EMT-related protein in cancerous area compared with adjacent non-cancerous area in each pathological differentiation of CRC

3. To compare the alterations of ARID1A expression on EMT-related protein expression in CRC

4. To consider the ARID1A and EMT-related protein expressions and their association with the severity of clinicopathological characteristics and pathological outcomes in CRC patients.

The research hypothesis

1. Expression of the ARID1A protein may decrease in the cancerous areas of CRC tissues as compared with adjacent non-cancerous areas.

2. The expression of epithelial and mesenchymal proteins may be different in the cancerous areas as compared with adjacent non-cancerous areas of CRC tissues.

3. Loss or decrease of ARID1A protein expression may be associated with EMT-related protein expression in CRC.

4. Altered expression of ARID1A and EMT-related proteins may be correlated with the severity of clinicopathological characteristics and worse pathological outcomes of CRC patients.

Scope of the study

This study protocol involving human subjects was approved by the Human Ethic Review Board of Sawan Pracharak Hospital (approval no. 16/2560) and the Naresuan University Ethical Committee for Human Research (NU-IRB) (approval no. 0504/62; COE no. 436/2019). In a retrospective design, the formalin-fixed, paraffin embedded (FFPE) blocks of CRC patients, composed of cancerous and adjacent non-cancerous areas, and demographic and clinicopathological information of patients who were diagnosed with the different pathological differentiations of CRC during 2017–2021 were obtained from the Unit of Pathology, Sawan Pracharak hospital, Nakhon Sawan province, Thailand. Demographic information was included by maintaining privacy and confidentiality provisions to protect the patient's information.

The expression of ARID1A and EMT-related proteins, including epithelial proteins (E-cad and ZO-1), and mesenchymal proteins (vimentin and fibronectin), was investigated using immunohistochemistry (IHC). The immunoreactivity of ARID1A was examined by pathologists and research investigators using the histological (H)-score, which evaluates both the grading assessment of ARID1A stained intensity and the percentage of positive cells of ARID1A staining. The IHC intensity of EMT-related protein was also investigated using ImageJ (Fiji) image analysis software. Accordingly, the association between the expressions of ARID1A and EMT-related protein with the clinicopathological characteristics was analyzed using the Fisher's exact test. In addition, the pathological outcomes of CRC patients with ARID1A and EMT-related protein expressions were analyzed by the Kaplan-Meier analysis and compared statistically using the log-rank test (Figure 1).

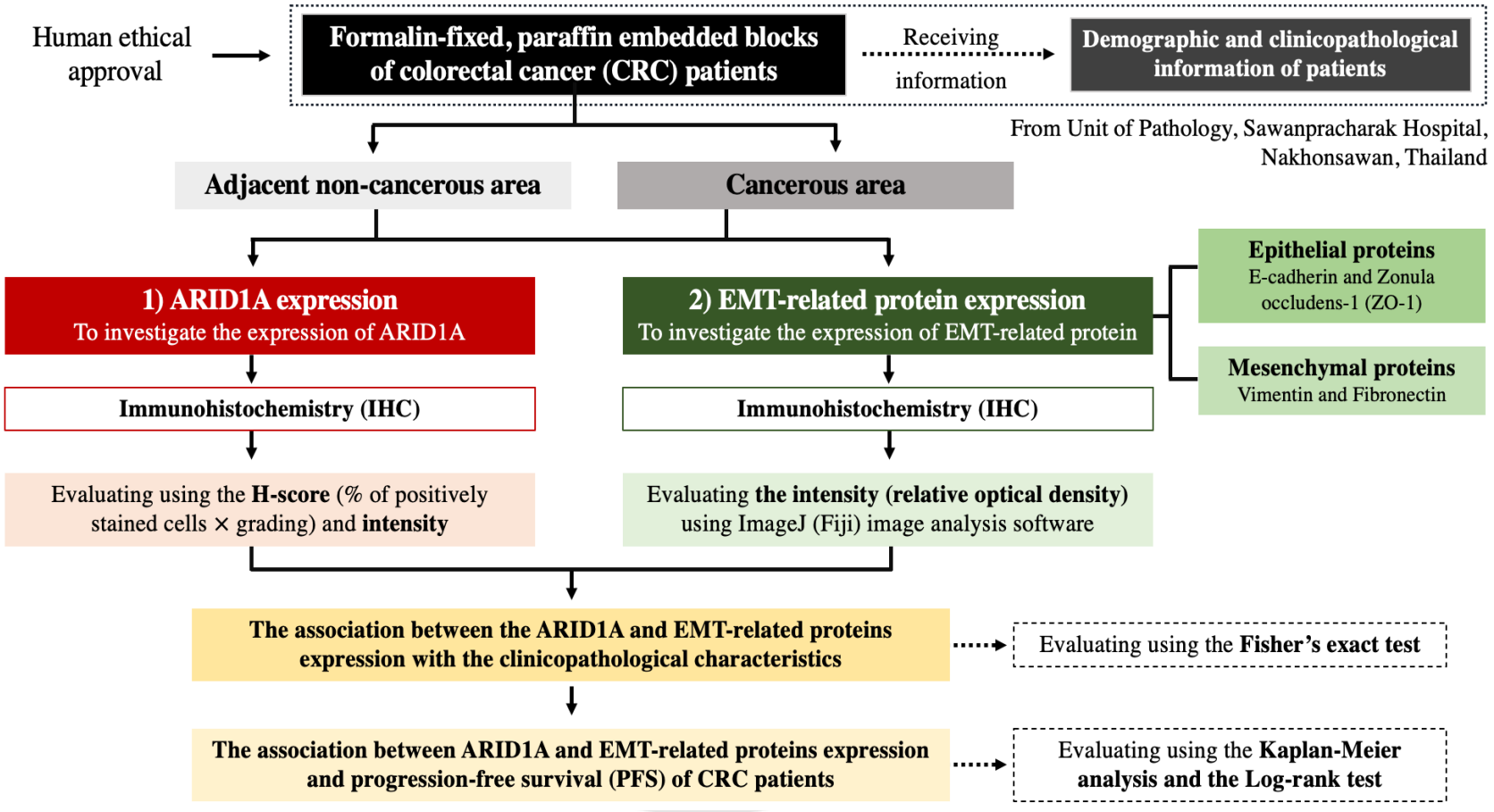

Figure 1 **Scope of the study**

CHAPTER II

LITERATURE REVIEW

Colorectal cancer (CRC)

1. Incidence of colorectal cancer

Colorectal cancer (CRC) accounts for approximately 10% of all cancer diagnoses and cancer-related deaths worldwide every year (Bray et al., 2018). According to the GLOBOCAN 2020 statistics, CRC is the third most prevalent and mortality-occurring cancer in men and the second most commonly occurring cancer in women (Sung et al., 2021). Incidence and mortality in men are approximately 25% higher than in women. These rates also vary geographically, with the highest rates found in developed countries rather than developing countries (Dekker et al., 2019). In 2020, there were more than 1.9 million new CRC diagnoses (Siegel et al., 2020).

CRC is the second most deadly cancer worldwide, with an estimated 881,000 deaths in 2018. Colon cancer is the fifth most deadly cancer, with 551,000 deaths projected for 2018, comprising 5.8% of all cancer deaths. Concurrently, rectal cancer is the tenth most deadly, with 310,000 deaths, which constitutes 3.2% of all cancer deaths (Rawla et al., 2019). Recently, the worldwide burden of cancer prevalence and mortality rate of CRC have been rapidly increasing. By 2030, the global incidence of CRC is expected to rise by 60%, with more than 2.2 million new cases and 1.1 million fatalities, and more than 2.5 million new cases in 2035 (Arnold et al., 2017; Bray et al., 2018). The highest rates of incidence of CRC are found in developed countries with a western lifestyle. Then, life expectancy, including health-related behaviors (food consumption, alcohol, smoking, obesity, and less exercise), and social factors (education, income, and government expenditure on health), are considered as the driving factors that may contribute to the development and increase the worldwide incidence of CRC (Chetty et al., 2016; Fidler et al., 2016).

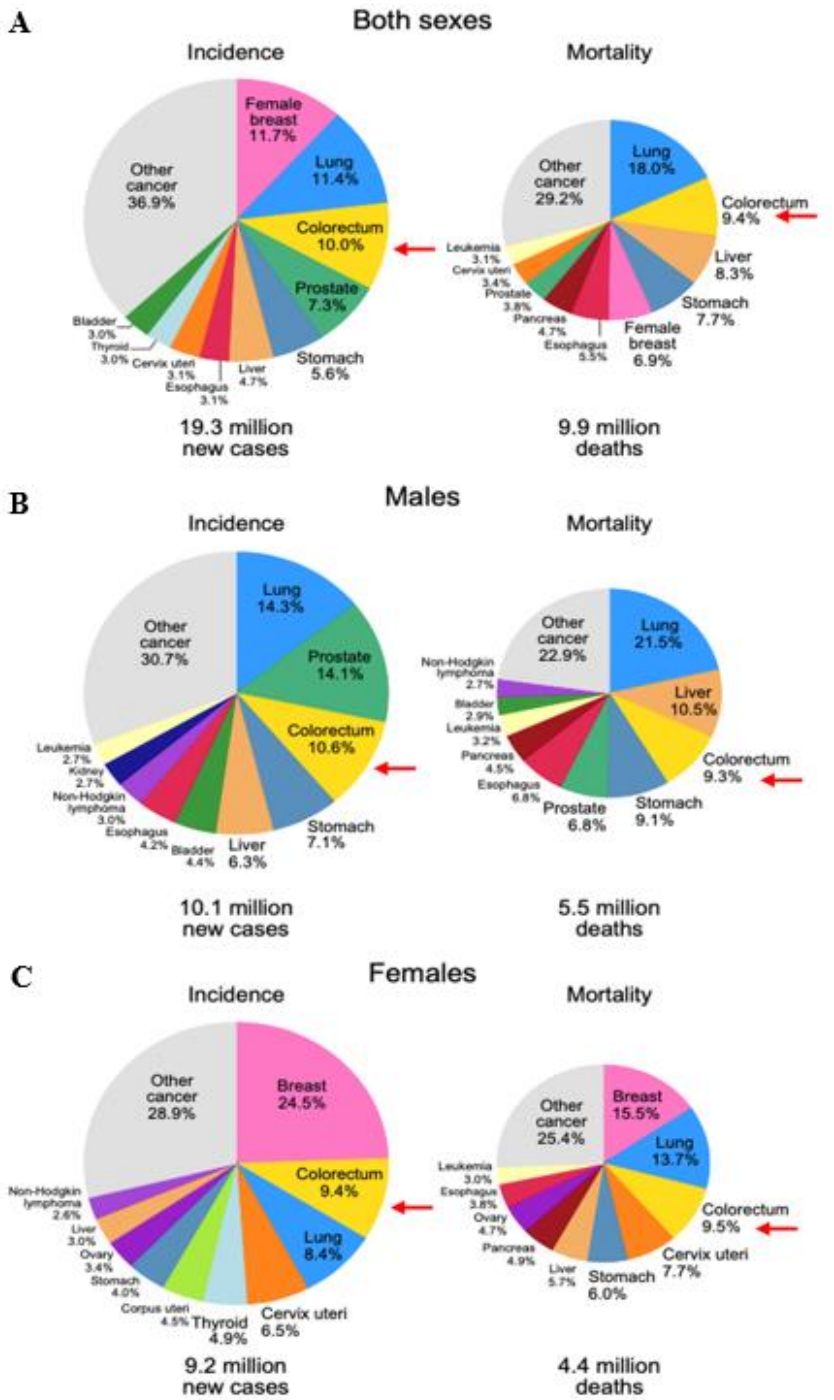

Figure 2 **Cancer incidence and mortality rates in 2020**
(A) in both genders, B) in men, and C) in women; the red arrow indicates CRC statistics)

Source Sung et al., 2021 from Global cancer statistics (GLOBOCAN) 2020

2. Risk factors of colorectal cancer

Several factors, including modifiable and non-modifiable risk factors, have been involved in the carcinogenesis and development of CRC (Figure 3). Individual factors, including race and ethnicity, male gender, older age, hereditary mutations, inflammatory bowel disease (IBD), and personal medical history**,** are the non-modifiable factors that may influence and develop CRC (Dekker et al., 2019).

Moreover, lifestyle and environmental factors play significant roles in the etiology of CRC (Sawicki et al., 2021). These are the modifiable risk factors, including obesity and overweight, less physical activity, types of food consumption (such as red and processed meats, and fruit and vegetable intake), smoking, alcohol consumption, and some medications. The diabetes mellitus (DM) type II and insulin resistance (IR) are also the independent risk factors for CRC (Dekker et al., 2019; Rawla et al., 2019; Sawicki et al., 2021).

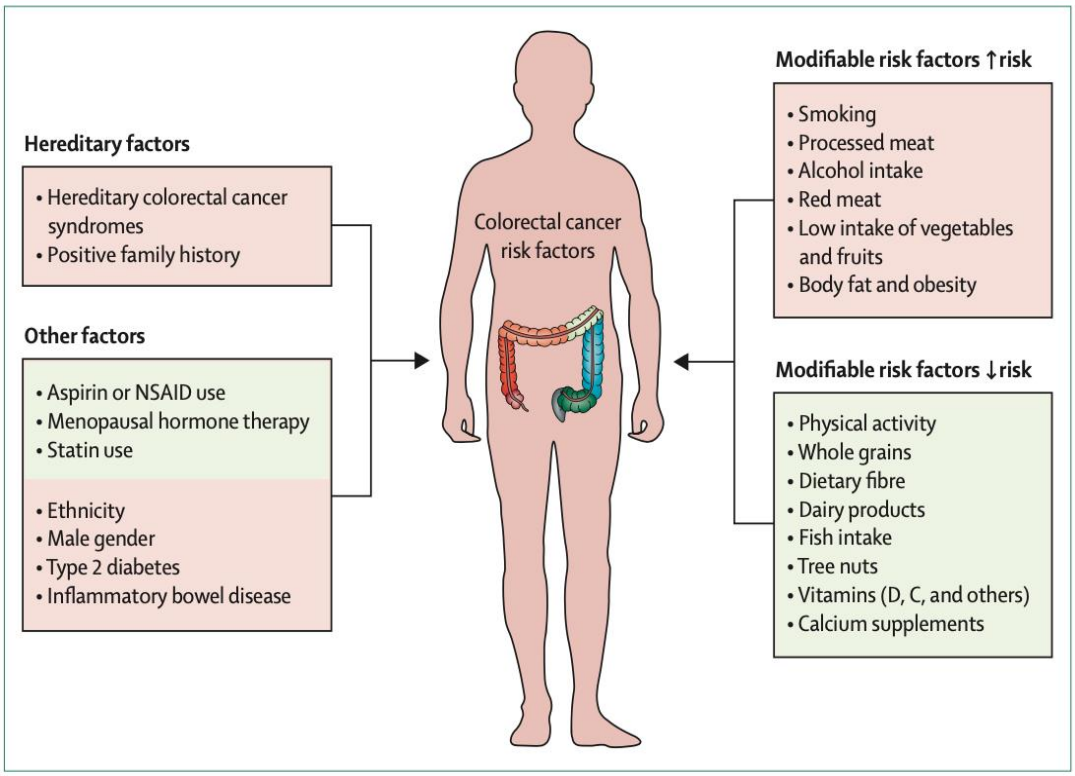

Figure 3 **The modifiable and non-modifiable risk factors for CRC**

Source Dekker et al., 2019

3. Pathogenesis of colorectal cancer

CRC is associated with a wide range of neoplasms, from benign growths to aggressive and invasive malignancies. The development of CRC begins in the inner layer of the colon and/or rectum as a tissue called a polyp slowly grows through some or all of its layers. A particular type of polyp called the adenomatous polyp or adenoma is a benign tumor that may undergo malignant transformation into cancer. This malignant transformation occurs when essential regulator genes are mutated or deleted, generating hyperplasia, adenoma, carcinoma, and then metastasis (Chung & Fleshman, 2004; Mundade et al., 2014) (Figure 4).

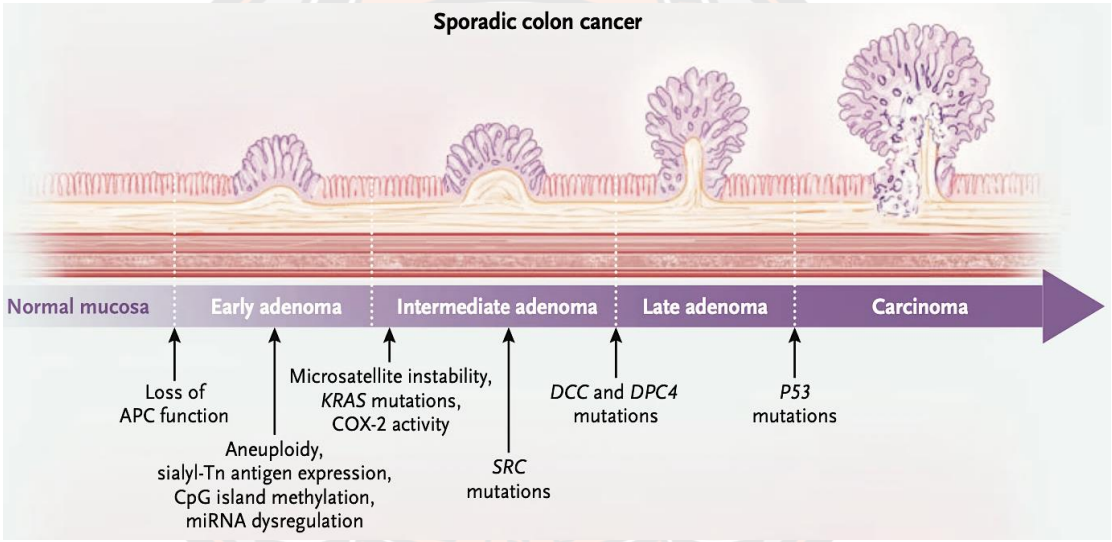

Figure 4 **Molecular stages of pathogenesis in sporadic CRC**

Source Modified from Beaugerie, & Itzkowitz, 2015

The pathogenesis of CRC includes the stages of initiation, promotion, and progression. The initiation stage implicates irreversible genetic damage that predisposes damaged epithelial cells in the intestinal mucosa to neoplastic transformation (Tanaka, 2009). In the promotion phase, the initiated cells multiply and generate abnormal growth to cause cancer. As opposed to this, benign cancer cells turn into malignant ones during the progression stage and acquire aggressive features and metastatic potential (Gandomani et al., 2017). The presence of a benign precursor lesions, including a

polyp, adenomatous polyps, or serrated polyps, are the significant antecedents of most malignancies. These precursor lesions are important features of most CRC carcinogenesis pathways (Rawla et al., 2019; Rosty et al., 2013).

There are three major distinct precursor lesion pathways through the alterations of genetic and epigenetic mechanisms involved in CRC, including adenoma-carcinoma sequence or chromosomal instability (CIN), microsatellite instability (MSI), and serrated or the CpG island methylator phenotype (CIMP) pathways as demonstrated in Figure 5 (Dekker et al., 2019; Mundade et al., 2014; Sawicki et al., 2021).

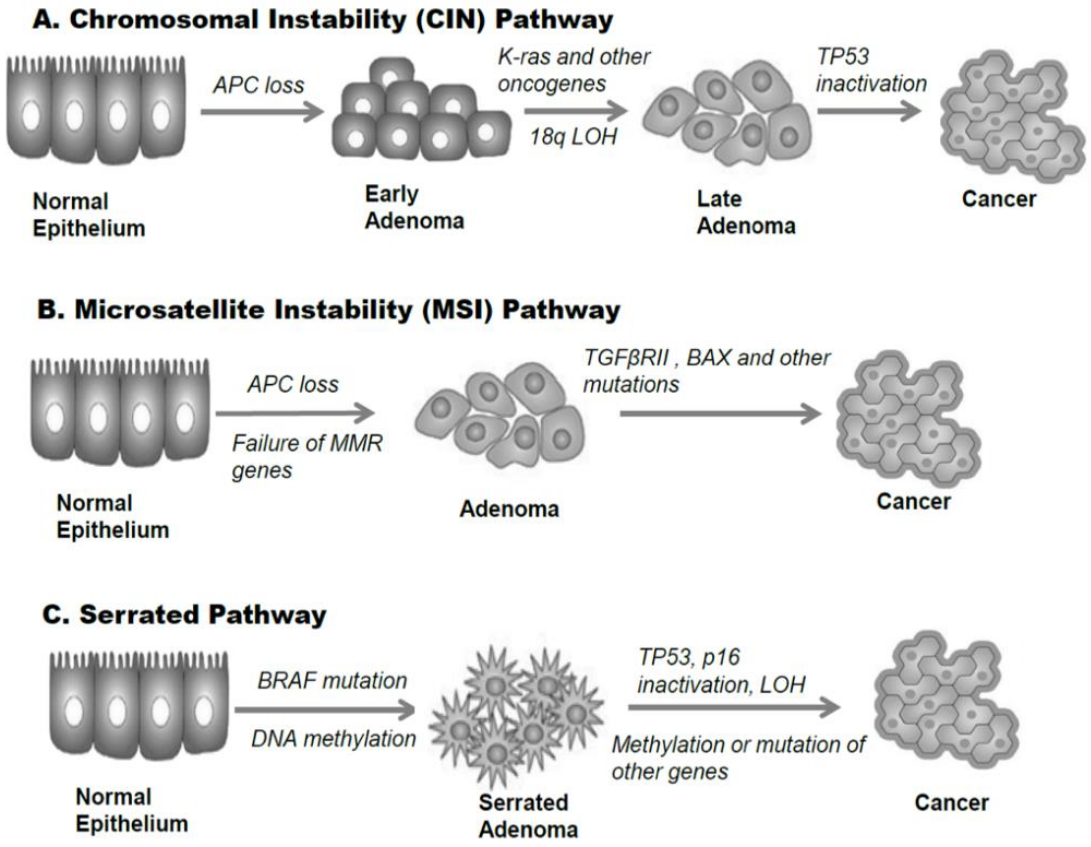

Figure 5 **Key molecular pathways in CRC development**

Source Mundade et al., 2014

3.1 Adenoma-carcinoma sequence or chromosomal instability (CIN) pathway

The CIN pathway is responsible for 70–90% of all CRC occurrences. The most commonly affected genes are *adenomatous polyposis coli* (*APC*), *tumor suppressor p53* (*TP53*), and *Kirsten rat sarcoma virus* (*KRAS).* Alterations of these genes can contribute to mutational activation of oncogenes or inactivation of tumor suppressors, which consequently causes malignant transformation (Armaghany et al., 2012; De Palma et al., 2019; Pino & Chung, 2010). The loss of the *APC* is the first occurrence in the progression of CRC. The hypermethylation of the *APC* promoter leads to Wnt/-catenin signaling activation, which is the essential event for adenoma initiation (Esteller et al., 2000; Powell et al., 1992). *TP53* is significantly involved in the control of the cell cycle and apoptosis. The *TP53* gene mutation is commonly found in CRC and consequently causes uncontrolled cell growth of cancer cells (Fearon & Vogelstein, 1990; Vogelstein et al., 1988). Moreover, *KRAS* is one of the *rat sarcoma* (*RAS*) gene families. *KRAS* mutations occur in 30%–50% of CRC gene mutations. RAS proteins play essential roles as regulators in cell division, differentiation, and apoptosis. One of the best characterized pathways regulated by the RAS family is the Raf–mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway. The MEK-ERK pathway is involved in cell cycle progression. *KRAS* mutation disrupts the RAS signaling pathway leading to tumorigenesis (Pruitt & Der, 2001; Schubbert et al., 2007; Tan & Du, 2012).

3.2 Microsatellite instability (MSI) pathway

The MSI pathway is responsible for approximately 10–15% of all CRC cases. MSI is the phenotypic evidence that DNA mismatch repair (MMR) is abnormally functioning, such as insertions and deletions, in microsatellites located in DNA coding regions, resulting in frameshift mutations and, ultimately, CRC carcinogenesis (Geiersbach & Samowitz, 2011). Inactivation of *MMR* genes occurs either through aberrant methylation of promoter CpG of the *MutL homolog 1* (*MLH1*) gene or point mutations. As a result, MSI cancers more readily acquire mutations in important cancer-associated genes

(Armaghany et al., 2012). MSI can be categorized as MSI-high, MSI-low, and microsatellite stable (MSS). Patients with MSI-H tumors had the best long-term prognosis among the MSI-L and MSS tumors (Boland & Goel, 2010; Fang et al., 1999; Geiersbach & Samowitz, 2011). The distinctive features of CRC with MSI include a tendency to arise in the proximal colon, lymphocytic infiltrate, poorly differentiated, and mucinous or signet ring appearance (Boland & Goel, 2010).

3.3 Serrated or the CpG island methylator phenotype (CIMP) pathway

The CIMP pathway is characterized by the presence of *protein kinase B-Raf* (*BRAF*) mutation and epigenetic silencing of genes involved in cell differentiation, DNA repairing, and cell-cycle regulation without APC gene involvement (Aran et al., 2016; De Palma et al., 2019; Jass et al., 2002; Leggett & Whitehall, 2010; Simon, 2016). *BRAF (V600)* point mutation increases MEK/ERK signaling, resulting in uncontrolled cell proliferation, immune response evasion, angiogenesis, tissue invasion, metastasis (via upregulation of several proteins involved in migration, integrin signaling, and cell contractility), and resistance to apoptosis (Ascierto et al., 2012; Rustgi, 2013).

4. TNM classification and AJCC staging of colorectal cancer

The most important prognostic factor is the stage of the disease at the time of diagnosis. Patients diagnosed with CRC have a 5-year relative survival rate of 90% for patients with localized disease, 69% for patients with regional spread, and less than 12% for patients with metastatic disease (Siegel et al., 2012).

CRC staging is classified by the TNM classification (Table 1) and assigned staging by the American Joint Committee on Cancer (AJCC) system (Table 2 and Figure 6). In this system, stages are assigned on the basis of the characteristics of the primary tumor (T), the extent of regional lymph node involvement (N), and distant metastasis (M). Moreover, metastasis may be defined clinically or pathologically, on the basis of preoperative clinical assessment (c) or pathologic evaluation of metastatic tissue (p) (Edge et al., 2010; Weiser, 2018).

Table 1 TNM classification of CRC

Classification	Definition
Primary tumor (T)	TX: Primary tumor cannot be assessed T0: No evidence of primary tumor Tl: Tumor invades submucosa T2: Tumor invades muscularis propria T3: Tumor invades through the muscularis propria into the subserosa, or into non-peritonealized pericolic or perirectal tissues T4: Tumor directly invades other organs or structures, and/or perforates visceral peritoneum In AJCC 8th edition, tumors that invade the serosal surface (visceral peritoneum) are referred to as T4a. Meanwhile, tumors that directly invade or adhere to adjacent organs or structures are considered T4b.
Regional lymph nodes (N)	NX: Regional lymph nodes cannot be assessed N0: No regional lymph node metastasis N1: Metastasis in 1 to 3 regional lymph nodes N2: Metastasis in 4 or more regional lymph nodes N2a: Metastasis in 4 to 6 regional lymph nodes N2b: Metastasis in 7 or more regional lymph nodes
Distant metastasis (M)	MX: Presence of distant metastasis cannot be assessed M0: No distant metastasis M1: Distant metastasis, divides into 3 subtypes; M1a: Metastases to one distant site or organ M1b: Metastases to more than one organ M1c: Peritoneal metastases

Source Modified from Edge et al., 2010; Weiser, 2018

Table 2 **AJCC staging of CRC**

Staging	TNM classification
Stage 0	Tis – N0 – M0
Stage I	T1 - N0 – M0 or T2 - N0 – M0
Stage IIA	T3- N0 – M0
Stage IIB	T4- N0 – M0
Stage IIIA	T1-T2 – N1 – M0
Stage IIIB	T3-T4 – N2 – M0
Stage IIIC	Any T – N2 – M0
Stage IV	Any T – Any N – M1

Source Modified from Edge et al., 2010

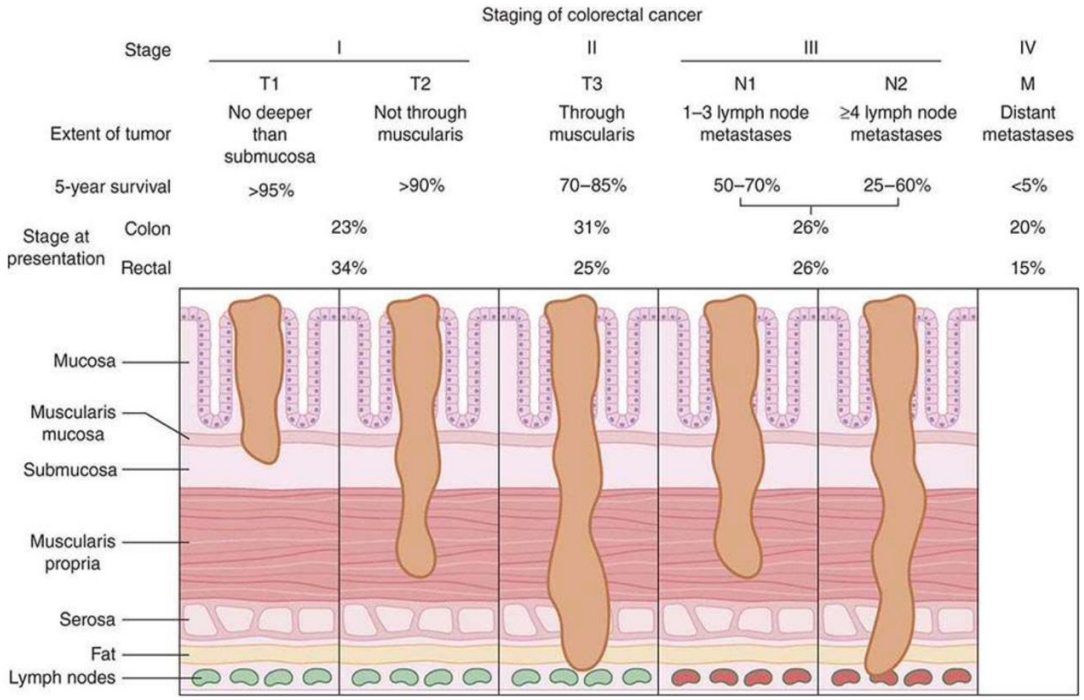

Figure 6 **Staging, prognostic factors, and spreading patterns of CRC**

Source Jameson et al., 2020

5. Histological grading of colorectal cancer

Histological grading of CRC is analyzed by the level of cell differentiation and growth rate when observed under a microscope. Cell differentiation is an important factor in determining how likely the tumor is to grow and spread to other organs of the body. Most cancers are graded by comparing them with their normal cells. A tumor grade typically ranges from 1 (well-differentiated) to 4 (undifferentiated or anaplastic). Grade 1 tumors are well differentiated, grow slowly and are considered the least aggressive. Grades 3 or 4 are described as undifferentiated and the most aggressive in behavior (Greene et al., 2002) (Figure 7).

Several criteria for CRC grading have been reported. The most widely accepted and uniformly used standard for grading is defined on the basis of the degree of gland formation (Ueno et al., 2012). In the TNM classification, grade (G) 1-4 tumors are defined as well-differentiated, moderately differentiated, poorly differentiated, and undifferentiated, respectively (Brierley et al., 2017) (Figure 8). Tumor grading is conventionally based on the assessment of the most unfavorable tumor differentiation (Compton, 2002; Hamilton, 2000). Although histological grading of tumor differentiation has repeatedly been shown by multivariate analysis to be a stage-independent prognostic factor (Fisher et al., 1989; Freedman et al., 1984; Greene et al., 2002), a significant degree of interobserver variability exists (Blenkinsopp et al., 1981; Deans et al., 1994; Thomas et al., 1983).

Conventional colorectal adenocarcinoma is characterized by glandular formation, which is the basis for histological tumor grading. In well- and moderately differentiated adenocarcinomas, are more than 95% and 50-95% of tumor gland formation. Poorly differentiated adenocarcinoma is mostly solid with less than 50% gland formation. In practice, approximately 70% of colorectal adenocarcinomas are diagnosed as moderately differentiated. Well- and poorly differentiated carcinomas account for 10% and 20%, respectively (Fleming et al., 2012). Tumor grade is generally considered as a stage-independent prognostic variable, and high grade or poorly differentiated histology is associated with a poor survival rate (Blenkinsopp et al., 1981; Compton et al., 2000; Jass et al., 1986).

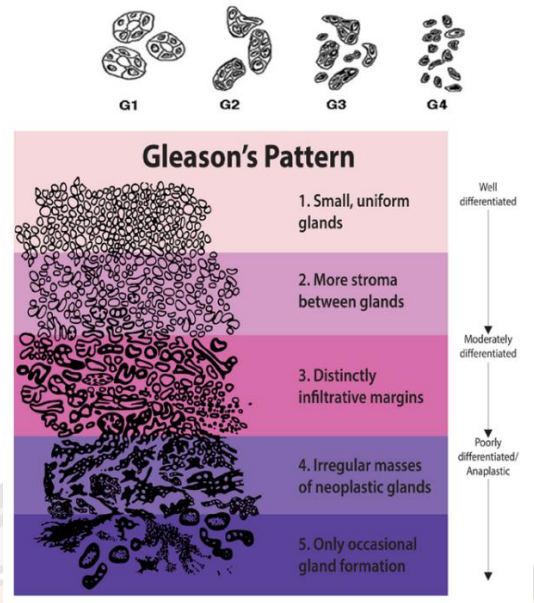

Figure 7 **Tumor grading by Gleason's pattern using in prostate cancer**

Source Modified from Humphrey, 2004

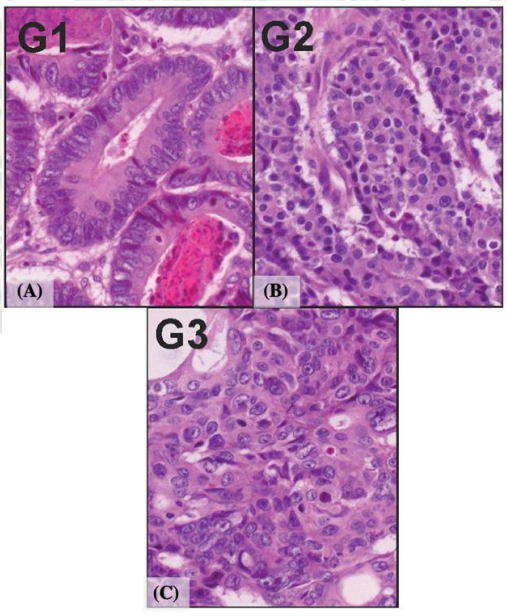

Figure 8 **Histological grading of CRC**
(A) Well-differentiated, B) Moderate differentiated, and C) Poor differentiated adenocarcinoma)

Source Kuepper et al., 2016

6. Histological variants of colorectal cancer

More than 90% of CRCs are adenocarcinomas that originate from epithelial cells of the mucosal layer of the colorectal mucosa and form glands. Currently, the WHO classifies tumors of the digestive system, including other histological types such as mucinous, signet-ring cell, medullary, micropapillary, adenosquamous, serrated, cribriform comedo-type, spindle cell, and undifferentiated adenocarcinomas (Hamilton, 2000). Some of the histological variants were discussed and represented in Figure 9, including;

6.1 Mucinous adenocarcinoma

Mucinous adenocarcinoma is defined by more than 50% of the tumor volume being composed of extracellular mucin (Hamilton, 2000). 10-50% of tumors with a significant mucinous component are usually termed adenocarcinoma with mucinous features or mucinous differentiation. Mucinous adenocarcinoma typically shows large glandular structures with pools of extracellular mucin. A variable number of individual tumor cells, including signet ring cells, may be found. The prognosis of mucinous adenocarcinoma in comparison with conventional adenocarcinoma has been controversial among different studies (Kang et al., 2005; Verhulst et al., 2012). Many mucinous adenocarcinomas occur in patients with hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) and thus represent high-level MSI (MSI-H) tumors (Leopoldo et al., 2008). These tumors are expected to behave in a low-grade fashion. In contrast, mucinous adenocarcinomas with MSS tumors are expected to behave more aggressively, particularly when detected at an advanced stage (Figure 9A).

6.2 Signet-ring cell carcinoma

This variant of adenocarcinoma is defined by the presence of more than 50% of tumor cells with prominent intracytoplasmic mucin (Sasaki et al., 1998). Nevertheless, signet-ring cell carcinoma is rare in the colorectum, representing less than 1% of all CRC cases (Fleming et al., 2012). The typical signet-ring cell has a large mucin vacuole that fills the cytoplasm and displaces the nucleus. Signet-ring cells can occur in the mucin pools of mucinous adenocarcinoma or

in a diffusely infiltrative process with minimal extracellular mucin. By definition, signet ring cell carcinoma is poorly differentiated (high grade) and carries a worse outcome than conventional adenocarcinoma (Chen et al., 2010; Kang et al., 2005; Makino et al., 2006). However, some signet ring cell carcinomas may be MSI-H tumors and thus may behave as low-grade tumors biologically (Hamilton, 2000) (Figure 9B).

6.3 Medullary adenocarcinoma

Medullary adenocarcinoma is an extremely rare variant, accounting for about 5-8 cases out of every 10,000 CRC diagnoses, with a mean annual incidence of 3.47 (0.75) per 10 million population (Thirunavukarasu et al., 2010). This histological variant is characterized by sheets of malignant cells with vesicular nuclei, prominent nucleoli, and abundant pink cytoplasm exhibiting prominent infiltration by intraepithelial lymphocytes (Jessurun et al., 1999). Medullary carcinoma is a distinctive histological subtype that is strongly associated with MSI-H (Alexander et al., 2001; Hinoi et al., 2001). It usually has a favorable prognosis despite its poorly differentiated or undifferentiated histology(Fleming et al., 2012) (Figure 9C).

6.4 Micropapillary adenocarcinoma

This histological variant is an uncommon subtype of colonic adenocarcinoma with distinctive behavior. 9-19% of CRC diagnoses may have micropapillary features (Haupt et al., 2007; Kim et al., 2006). Microscopic features of micropapillary adenocarcinoma are characterized by small clusters of malignant cells with abundant eosinophilic cytoplasm and pleomorphic nuclei. Micropapillae inhabit lacunar-like spaces and demonstrate a reverse polarity configuration, with apical surfaces facing the periphery rather than the center. Additionally, lymphovascular invasion is commonly present. The morphology of the tumor is similar to micropapillary carcinomas of other organs (Nassar, 2004) (Figure 9D).

6.5 Adenosquamous carcinoma

These unusual tumors show features of both squamous carcinoma and adeno-carcinoma, either as separate areas within the tumor or admixed. The lesion is classified as adenosquamous and is found to have numerous small foci of squamous differentiation. Pure squamous cell carcinoma is very rare in the large bowel (Hamilton, 2000) (Figure 9E).

6.6 Undifferentiated carcinoma

These rare tumors lack morphological evidence of differentiation beyond that of an epithelial tumor and have variable histological features (Tortola et al., 1999). Despite their undifferentiated appearance, these tumors are genetically distinct and typically associated with MSI-H (Hamilton, 2000) (Figure 9F).

6.7 Other variants

Carcinomas that include a spindle cell component are best termed spindle cell carcinoma or sarcomatoid carcinoma. The spindle cells are, at least focally, immunoreactive for cytokeratin. The term "carcinosarcoma" applies to malignant tumors containing both carcinomatous and heterologous mesenchymal elements. Other rare histopathological variants of CRC include pleomorphic (giant cell), choriocarcinoma, pigmented, clear cell, stem cell, and Paneth cell-rich (crypt cell carcinoma). Mixtures of histopathological types can be found.

6.7.1 Carcinosarcoma

Carcinomas that include a spindle cell component are suitable to be termed sarcomatoid carcinomas or spindle cell carcinomas. The spindle cells are, at least focally, immuno-reactive for cytokeratin. The term "carcinosarcoma" applies to malignant tumors containing both carcinomatous and heterologous mesenchymal elements (Hamilton, 2000).

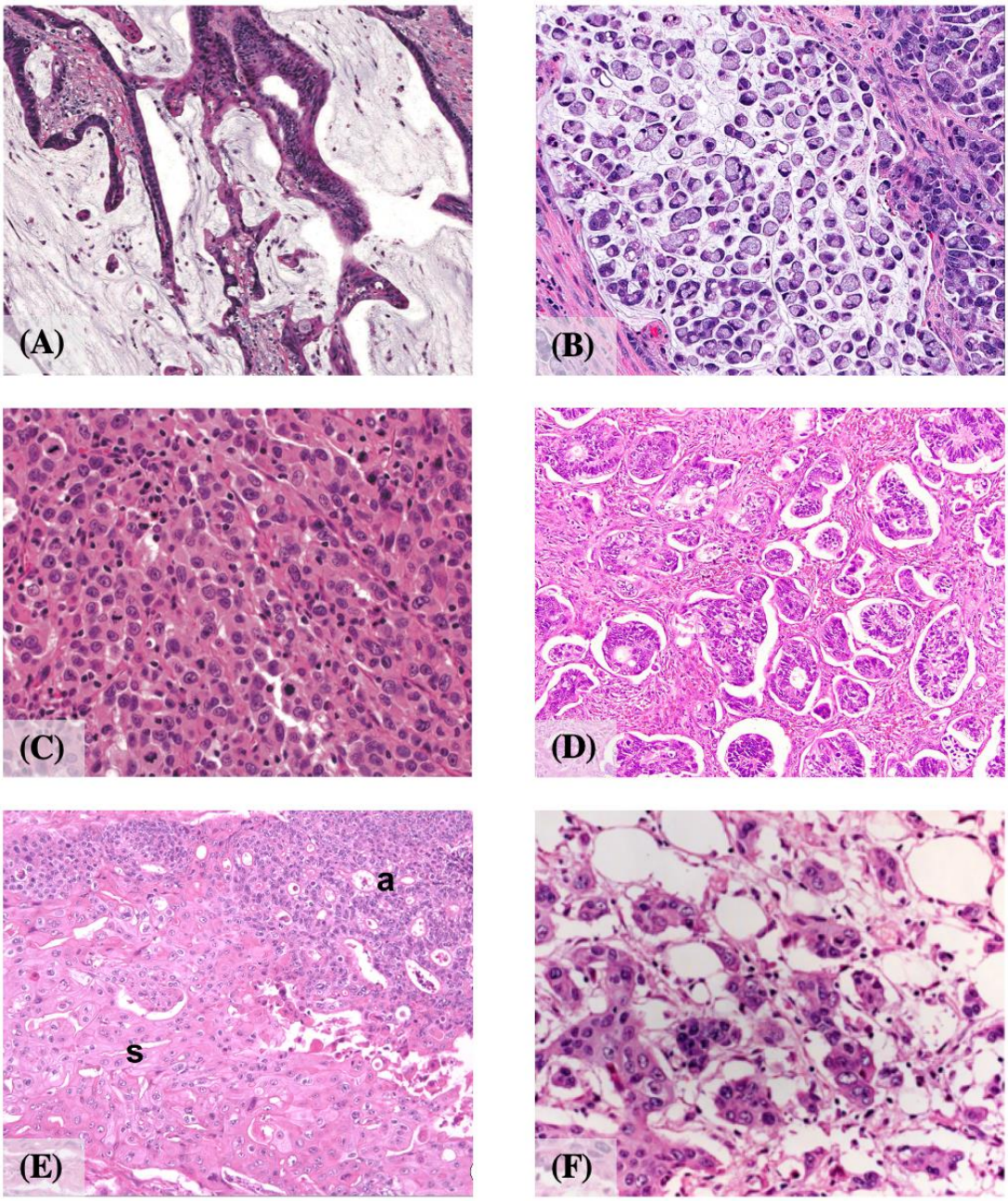

Figure 9 **Microscopic appearances of the histological variants of CRC (A) Mucinous adenocarcinoma, B) Signet-ring cell carcinoma, C) Medullary adenocarcinoma, D) Micropapillary adenocarcinoma, E) Adenosquamous carcinoma, and F) Undifferentiated carcinoma of the colon)**

Source Fleming et al., 2012; Kang et al., 2011; Bonetti et al., 2016

7. Signs and symptoms of colorectal cancer

CRC patients can present with a broad range of signs and symptoms and may be suspected of having some symptoms of the lower gastrointestinal (GI) tract (Dekker et al., 2019; Sawicki et al., 2021). Common signs of CRC include rectal bleeding, a change in bowel habits, and abdominal pain. Rectal bleeding is the most common sign of CRC. Rectal bleeding with bright red blood (70%), dark blood (22%), and darker burgundy or maroon (8%) can be found. Changes in bowel habits are associated with various symptoms, such as diarrhea, constipation, a change in frequency of defecation, a change in consistency and shape of stool, and unexplained weight. Also, abdominal pain may be considered as a part of IBD (Agréus et al., 1993; Fine et al., 1999; Longstreth et al., 2006; Summerton et al., 2003).

Moreover, common symptoms of CRC include palpable masses in the rectum and abdomen, iron deficiency anemia (IDA), and acute and metastasized disease at presentation. IDA is a classical indicator of CRC (Goodman & Irvin, 2005; John et al., 2011). Additionally, some non-site-specific symptoms, such as unexplained appetite loss and deep vein thrombosis, should be considered (Poston et al., 2011).

8. Diagnosis of colorectal cancer

Signs and symptoms of CRC are usually asymptomatic during the early stages. Screening at an early stage of CRC has contributed to a significant decrease in both the number of CRC incidences and the number of CRC deaths (Gellad & Provenzale, 2010; Mundade et al., 2014).

There are several recommended methods for screening and diagnosis of CRC. All of these methods have a comparable ability to improve survival if performed appropriately. Diagnostic and screening techniques are commonly used for CRC, including visual examinations such as colonoscopy, computed tomographic colonography (CTC), and flexible sigmoidoscopy (FS). Colonoscopy is the most widely used screening test in the United States. Furthermore, recommended techniques for colorectal cancer screening and diagnosis can be performed using stool examinations, such as fecal immunochemical tests for hemoglobin (FIT), high-sensitivity guaiac-based fecal occult blood tests (gFOBT), and multi-targeted stool DNA tests (Cologuard®) (American Cancer Society, 2020; Centers for Disease Control and Prevention, CDC, 2012).

9. Immunohistochemistry application as the diagnostic biomarkers of colorectal cancer

CRC pathogenesis has heterogeneous characteristics at the molecular and biological level. The identification of biomarkers that can assist in CRC early detection or monitoring may enable the development of personalized management, improve the survival rates of patients, and increase the burden on pathologists to accurately identify the site of tumor origin. Recently, IHC has become one of the available screening methods for CRC diagnosis (Oh & Joo, 2020; Taliano et al., 2013). There are some commonly used immunohistochemical markers in the diagnosis of colonic adenocarcinoma, including;

9.1 Cytokeratins (CKs)

CKs are members of the family of intermediate filaments along with glia filament, neurofilament, desmin, and vimentin. Expression of CKs proteins was detected by epithelial cells in the intracytoplasmic cytoskeleton (Moll et al., 1982). Chu and colleagues demonstrated that CK7/CK20 patterns have been well-documented observations, which CK7-/CK20+ pattern is exhibited in non-neoplastic colonic mucosa proximal to the rectum (Chu et al., 2000). Approximately 65-95% of CRCs have demonstrated a CK7-/CK20+ pattern, which is a typical method for metastatic CRC diagnosis (Figure 10A and 10B) (Bayrak et al., 2012; Bayrak et al., 2011).

9.2 Caudal type homeobox 2 (CDX2)

CDX2 is a transcription factor that is a member of the caudal subgroup of homeobox genes. CDX2 is involved in embryonic and lifelong maintenance of a cellular intestinal phenotype, the regulation of normal cell differentiation in the GI tract, and tumor suppression in the colon (Silberg et al., 2000). In a normal state, CDX2 is strongly expressed in various cells such as epithelial cells of the small intestine, appendix, colon, rectum, and pancreas (Moskaluk et al., 2003). However, the CDX2 protein was decreased expression in CRCs (Moskaluk et al., 2003; Werling et al., 2003).

9.3 Monoclonal carcinoembryonic antigen (mCEA)

mCEA is a subgroup of the carcinoembryonic antigen (CEA), which is a member-associated glycoprotein with variable roles in cell adhesion or signal transduction (Hammarström, 1999). mCEA is expressed in a broad variety of adenocarcinomas, including those originating from the colon, small intestine, stomach, pancreatic duct, biliary tract, cervix, and sweat gland secretory epithelium, as well as many urothelial and squamous cancers (Hammarström, 1999; Lau et al., 2002; Sheahan et al., 1990). Moreover, CEA levels in circulation were significantly associated with patient outcomes (Park et al., 1999).

9.4 β-Catenin

β-Catenin is an EMT-related marker that is involved in both cell adhesion and intracellular signaling. β-Catenin enables to simultaneously bind α-catenin and E-cad components of the cell membrane and cytoplasmic actin filaments, whereas the latter is accomplished through β-catenin's actions in the Wnt signaling pathway (Gao et al., 2014; Willert & Nusse, 1998). β-Catenin is one of the essential factors in the progression of CRC. The overexpression of nuclear β-Catenin was associated with late TNM stage, lymph node metastasis, poor histological differentiation, and poor prognosis outcomes in patients with CRC (Gao et al., 2014).

9.5 α-Methyacyl-CoA racemase (AMACR/p504s)

AMACR is a peroxisomal and mitochondrial enzyme that is involved in β-oxidation of branched-chain fatty acids through the racemization of α-methyl, branched carboxylic coenzyme A thioesters (Amery et al., 2000; Ferdinandusse et al., 2000). In a normal state, AMACR is expressed in various cells, such as hepatocytes, renal tubular epithelial cells, bronchial epithelial cells, and the gallbladder (Jiang et al., 2003). AMACR protein expression frequently reduces sensitivity in prostatic and colonic adenocarcinomas, particularly in poorly differentiated CRCs (Jiang et al., 2003; Kuefer et al., 2002; Zhou et al., 2002).

9.6 Mucous glycoproteins (Mucins)

Mucins are an essential structural component of mucus that can be secreted (gel-forming and non-gel-forming) or transmembrane (Forstner, 1978; Strous & Dekker, 1992). MUC is a core protein of mucins (Jonckheere & Van Seuningen, 2010; Joshi et al., 2014). The normal colon comprises a mixture of neutral mucin, sialomucin, and sulphomucin. MUC2 and MUC4 are expressed in both goblet and columnar cells, whereas MUC3 is expressed within enterocytes. In addition, MUC1, MUC5AC, and MUC6 are not expressed in the normal colonic mucosa (Byrd & Bresalier, 2004; Cao et al., 1997; Swallow et al., 1987). MUC2 expression was found to be increased in mucinous carcinomas of various cancers, including CRC, ovarian carcinoma, breast cancer, and pancreatic cancer (Figure 10C) (Hanski et al., 1997). Alteration of MUC2 expression was associated with the MSI and MMR, as well as the prediction of chemotherapy resistance, and poor prognosis of CRC patients (Kang et al., 2011; Lugli et al., 2007; Park et al., 2006; Perez et al., 2008).

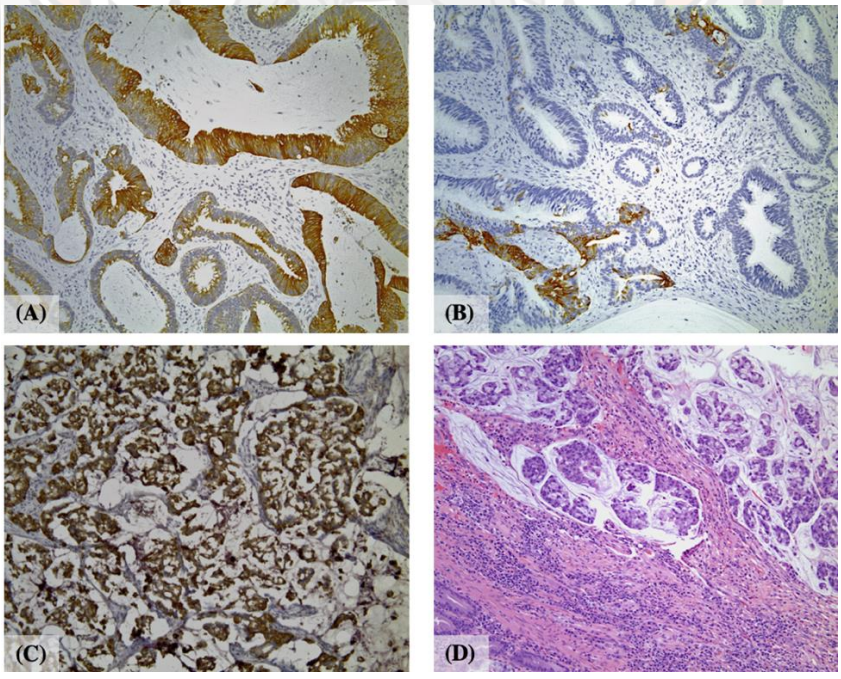

Figure 10 **Immunohistochemical markers in the diagnosis of CRC (A) CK20, B) CK7, C) MUC2, and D) H&E staining)**

Source Modified from Taliano, LeGolvan, & Resnick, 2013

10. Immunohistochemical markers in the diagnosis of colorectal cancer and its subtypes and variants

10.1 Usual type of CRC

This CRC type refers to lacking mucin production, intratumoral lymphocytes, or Crohn-like response (Greenson et al., 2009). The panel markers for evaluating this tumor should include CK7, CK20, mCEA, AMACR, CDX2, β-Catenin, MUC1, MUC2, and MUC5AC (Taliano et al., 2013).

10.2 Rectal adenocarcinoma

CK20 expression is positively present in most cases of rectal adenocarcinoma. CK7 and CDX2 are also frequently expressed in rectal cancer (Saad et al., 2009; Zhang et al., 2003).

10.3 Mucinous carcinoma (mCRC)

mCRC is associated with MSI. CK7, CK20, and CDX2 exhibit increasing variability in this variant of CRC. Also, MUC2 and MUC5AC expression are shown in approximately 90% and 50% of mCRC cases, respectively (Ajioka et al., 1996; Park et al., 2006).

10.4 Signet ring cell carcinoma of the colon (cSRCC)

In this variant, MUC1 and MUC5AC, which are mucins markers, are present, whereas MUC2 is absent (Chu et al., 2000; Chu & Weiss, 2004; Goldstein et al., 2000; Nguyen et al., 2006). Additionally, decreased expression of CDX2 was demonstrated in 47% of cSRCC tumors (Baba et al., 2009).

10.5 Micropapillary carcinoma

Previous studies reported that MUC1 and villin were demonstrated in micropapillary carcinoma. These immunohistochemical markers were expressed on the basal-lateral aspects of the neoplastic cells at the tumor-stroma interface. CK7, CK20, mCEA, and CDX2 were also expressed in micropapillary carcinoma (Kuroda et al., 2007; Sakamoto et al., 2005; Wen et al., 2008).

11. Management and treatment of colorectal cancer

Treatments for CRC have advanced rapidly over the past several decades, particularly for advanced disease (Kennedy et al., 2014; Murphy et al., 2015). Management and treatment for patients with CRC depend on the stages and progression of the disease (Magrini et al., 2002; Mundade et al., 2014). Common management and treatment for CRC are summarized in Table 3.

Table 3 Management and treatment for CRC

Treatments for CRC	AJCC staging of CRC
Colectomy	Stage 0, Stage I, and early Stage II
Postoperative adjuvant chemotherapy	Stage III and some Stage II
Chemotherapy with multi-drug therapy including 5-fluorouracil (5-FU) and leucovorin, capecitabine and oxaliplatin (CapeOx), and irinotecan (Camptosar)	Stage II
Radiation therapy	Recurrent or advanced disease

Source Margrini et al., 2002; Suzuki et al., 2013; Mundade et al., 2014

11.1 Treatment for colon cancer

Most patients with colon cancer will have to undergo surgery for tumor removal, such as colectomy and polypectomy. Postoperative adjuvant chemotherapy can also be applied. Radiation therapy is used less frequently to treat colon cancer. Furthermore, different management depends on the involvement of tumors. Carcinoma in situ is a state of disease where malignant cancer has not spread yet. Then polypectomy, or more invasive surgery, will be performed. A surgical resection with adjacent lymph nodes will be performed in a localized stage, which refers to an invasive cancer that has penetrated the colonic wall but is not completely involved. Additionally, for the regional stage, in which cancers have grown through the colonic wall and/or

spread to nearby lymph nodes, surgery to remove the tumor, adjacent normal colonic tissue, and nearby involved lymph nodes will be performed. Moreover, adjuvant chemotherapy based on the drug 5-FU is typically used in patients with stage III or high-risk stage II. Oxaliplatin is often part of adjuvant chemotherapy as well (Sargent et al., 2009; Shah et al., 2016). Lastly, for the metastasis stage, which is a stage where cancers have spread to other organs, removing all of the tumors with surgery will be performed. Also, chemotherapy and targeted therapies, such as an inhibiting drug of the vascular endothelial growth factor (VEGF) and the epidermal growth factor receptor (EGFR), have been approved to treat metastatic colon cancer (American Cancer Society, 2020).

11.2 Treatment for rectal cancer

The main treatment of rectal cancer is surgery, frequently accompanied by chemotherapy and radiation to decrease the risk of spread and recurrence of the disease. The chemotherapy (non-targeted drugs) and targeted drugs used in the treatment of rectal cancer are broadly the same as those used for colon cancer (Table 4 and Table 5).

Different management and treatment options for rectal cancer depend on the involvement of tumors. For carcinoma in situ, polypectomy, local excision, or full-thickness rectal resection will be carried out. For localized stage, which is a state that cancers have grown through the first layer of rectum into the deeper layers but have not spread throughout the rectal wall, surgery may be involved in the removal of tumors and adjacent normal tissues. Additionally, for regional stage cancers that have grown through the rectal wall and/or spread to nearby lymph nodes or other organs, before and after surgery, chemotherapy and radiation (chemoradiation) have been applied (Bosset et al., 2014; Kulaylat et al., 2017; Maas et al., 2015). Lastly, for the metastasis stage, surgery will be performed by removing all of the tumors. Palliative treatments, which are given treatments to relieve the symptoms and decrease the suffering caused by cancer and other life-threatening diseases, such as surgery, chemotherapy, and/or radiation therapy, are also treated for metastasized rectal cancer patients. In addition, targeted therapies, including VEGF and EGFR inhibitors, have also been approved to treat select metastatic rectal cancers (American Cancer Society, 2020).

Table 4 **Commonly U.S. Food and Drug Administration (FDA) (Approved chemotherapy treatment (non-targeted drugs) for CRC)**

Non-targeted therapy drugs	Details of non-targeted therapy drugs
1. Capecitabine (Xeloda®)	• Capecitabine is an adjuvant chemotherapy that has been used as a first-line treatment for patients with metastatic CRC. • Capecitabine exhibited a superior safety profile compared with 5-FU/leucovorin, with a significantly lower incidence (p<0.001) of side effects (Twelves, 2002).
2. 5-Fluorouracil (5-FU)/ Leucovorin (Adrucil®)	• Adrucil is a chemotherapy treatment for adenocarcinoma of the rectum or the colon. • 5-FU treatment can improve the survival of patients with various cancers, especially CRC (Pardini et al., 2011).
3. Oxaliplatin (Eloxatin®)	• Oxaliplatin is an adjuvant chemotherapy for advanced CRC patients who have had a resection of the primary tumor. • Oxaliplatin has demonstrated modest activity in metastatic CRC patients (Comella et al., 2009).
4. Irinotecan (Camptosar®)	• A first- and second-line therapy with 5-FU and leucovorin for patients with metastatic colon and rectum carcinoma. • Irinotecan has an acceptable tolerability profile and is not associated with cumulative toxicities in patients with metastatic CRC (Fuchs, Mitchell, & Hoff, 2006)
5. Trifluridine and Tipiracil (Lonsurf®)	• For patients with CRC who have previously been treated with fluoropyrimidine, irinotecan, oxaliplatin chemotherapy, anti-VEGF therapy, and, if *RAS* wild-type, anti-EGFR therapy. • It is possible to work against wild-type *KRAS*.

Source Modified from National Comprehensive Cancer Network, NCCN, 2017

Table 5 **Targeted therapies for management of advanced CRC**

Targeted therapy drugs	Details of targeted therapy drugs
1. Bevacizumab (Avastin®)	• Bevacizumab was the first VEGF inhibitor approved for use in CRC. • Bevacizumab with leucovorin treatment improved the median duration of progression-free survival (PFS) in CRC patients (p<0.001) (Hurwitz et al., 2004).
2. Ramucirumab (Cyramza®)	• Ramucirumab is an anti-VEGF therapy for the treatment of patients with stage IV metastatic CRC. • Ramucirumab with FOLFIRI treatment increased the overall survival (OS) rate of patients (Tabernero et al., 2015).
3. Ziv-aflibercept (Zaltrap®)	• Patients with CRC who received ziv-aflibercept (ant-VEGF) with FOLFIRI treatment had a better OS rate and a longer PFS rate (Van Cutsem et al., 2016).
4. Cetuximab (Erbitux®)	• Cetuximab is an anti-EGFR targeted treatment approved for use in CRC. • Cetuximab with irinotecan combination treatment demonstrated a longer median time to disease progression than cetuximab monotherapy (Cunningham et al., 2004).
5. Panitumumab (Vectibix®)	• Panitumumab (anti-EGFR) significantly reduced the relative risk of CRC progression by 46% (Van Cutsem et al., 2007).
6. Regorafenib (Stivarga®)	• Regorafenib is a multi-kinase inhibitor that affects several signaling pathways. Regorafenib inhibits VEGF signaling. • Regorafenib increased the OS rate of patients with metastasized CRC (p<0.0001) (Grothey et al., 2013).

Source Modified from Bai, 2017

12. Prognosis factors for colorectal cancer patients

CRC represents one of the most common malignancies and a leading cause of cancer-associated morbidity and mortality worldwide. In spite of evidence of a 5-year survival rate of 90% when CRC is diagnosed at an early stage, less than 40% of cases are diagnosed when the cancer is still localized (Brenner et al., 2014; Oh & Joo, 2020). The survival rate is the best indicator of determining the effectiveness of healthcare, diagnostic, and remedial interventions in CRC patients. To improve survival rates, accurate and dependable prognostic factors should be identified to provide the highest-quality information to patients (Lang & Jacqmin, 2003; Rasouli et al., 2017).

Prognostication of new diagnosticians of CRC predominantly depends on the stage or anatomic extent of disease based on the International Union Against Cancer (UICC-TNM) and AJCC staging classifications (Brierley et al., 2017; Frederick et al., 2002). The most important morphological prognostic factors for CRC included the local extent of tumor assessed pathologically (the pT category of the TNM classification), lymph node status, tumor histological grade, and the assessment of lymphatic and venous invasion. Additionally, tumor budding and tumor border configuration should be considered as additional histological parameters (Compton et al., 2000; Zlobec & Lugli, 2008). However, several molecular features, such as chromosomal loss at 18q (LOH18q) and *TP53* mutation, have shown promising results in terms of their prognostic value. Furthermore, approaches to the reliable prognostic protein markers identified such as EGFR or VEGF by IHC should be developed (Zlobec & Lugli, 2008). Novel tissue prognostic biomarkers for the diagnosis and prognosis of CRC have been reported, including MSI, CIMP, *BRAF*, *APC*, *TP53*, and *SMAD4* mutations (Oh & Joo, 2020). The mutations or altered expression of these tissue prognostic biomarkers are associated with poor prognosis by decreasing the disease-free survival (DFS), relapse-free survival (RFS), and OS rates of CRC patients (Chen et al., 2013; Guastadisegni et al., 2010; Jia et al., 2016; Oh & Joo, 2020; Sepulveda et al., 2017). Then, precision and accuracy of prognostic biomarkers may be effective for early diagnosis, well-management, and, in particular, improving survival and lowering mortality rates of CRC patients.

AT-rich interactive domain-containing protein 1A (ARID1A)

1. SWI/SNF chromatin remodeling complexes

The human Switch/Sucrose Non-Fermentable (SWI/SNF) complexes are an evolutionarily conserved multi-subunit chromatin-remodeling complex that uses the energy of ATP hydrolysis to mobilize nucleosomes and remodel chromatin, and thereby regulate transcription of target genes. The SWI/SNF complexes are composed of approximately 12–15 protein subunits encoded by 26 genes (de la Serna et al., 2006; Roberts & Orkin, 2004). These complexes contain three main groups, including BRM/BRG1-associated factor (BAF), polybromo-associated BAF (PBAF), and non-canonical BAF (ncBAF). They have several common subunits. The BAF complex includes the specific subunits including ARID1A/BAF250a, ARID1B/BAF250B, and double PHD fingers (DPF)1/2/3, or BAF45b. The PBAF complex contains ARID2/BAF200, PHD finger 10 (PHF 10), bromodomain containing 7 (BRD7), and polybromo-1 (PBRM1)/BAF180 as the specific subunits. Moreover, glioma tumor suppressor candidate region genes (GLTSCR) 1/1L and BRD9 are the specific subunits of the ncBAF complex (Figure 11) (Mashtalir et al., 2018; Tsuda et al., 2021).

SWI/SNF complexes have been discovered to serve key roles in transcriptional regulation that play a role in chromatin remodeling at both promoters and enhancers, which is regulated lineage-specific differentiation (Figure 12), and as tumour suppression (Alver et al., 2017; Hu et al., 2011; Kowenz-Leutz & Leutz, 1999; Mathur & Roberts, 2018; Tolstorukov et al., 2013). Many previous studies support the role of these complexes in cancer development. Most mutations in some subunits of the human SWI/SNF complexes are loss-of-function mutations that indicate the role of these subunits as tumor suppressors. Although in synovial carcinoma studies, a gain-of-function mutation has been demonstrated, which indicates an oncogenic function (Clark et al., 1994; Kadoch et al., 2013). Additionally, several recent studies have shown that the most frequently mutated subunit in the human SWI/SNF chromatin remodeling complexes is *ARID1A*. *ARID1A* is mutated in more than 8% of human cancers, whereas other subunits such as *ARID2*, *PBRM1*, *SMARCA4*, *ARID1B*, and *SMARCA2* are mutated in approximately 2% of all cases. Therefore, the human SWI/SNF complexes are the most commonly mutated chromatin modulators in human cancers (Cerami et al., 2012; Gao et al., 2013; Hoadley et al., 2018; Kadoch & Crabtree, 2013; Tsuda et al., 2021).

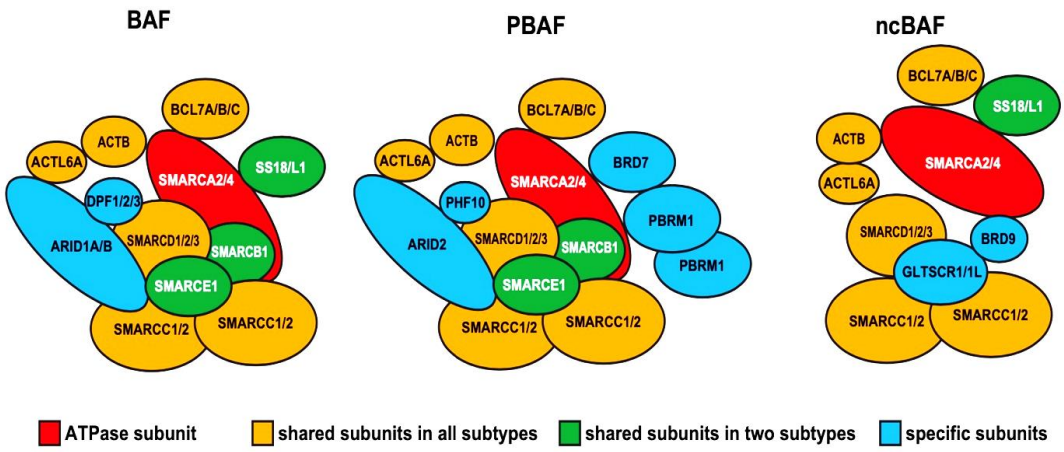

Figure 11 **Major subunits of the SWI/SNF chromatin remodeling complexes (Including BRM/BRG1-associated factor (BAF), polybromo-associated BAF (PBAF), and non-canonical BAF (ncBAF) complexes)**

Source Tsuda et al., 2021

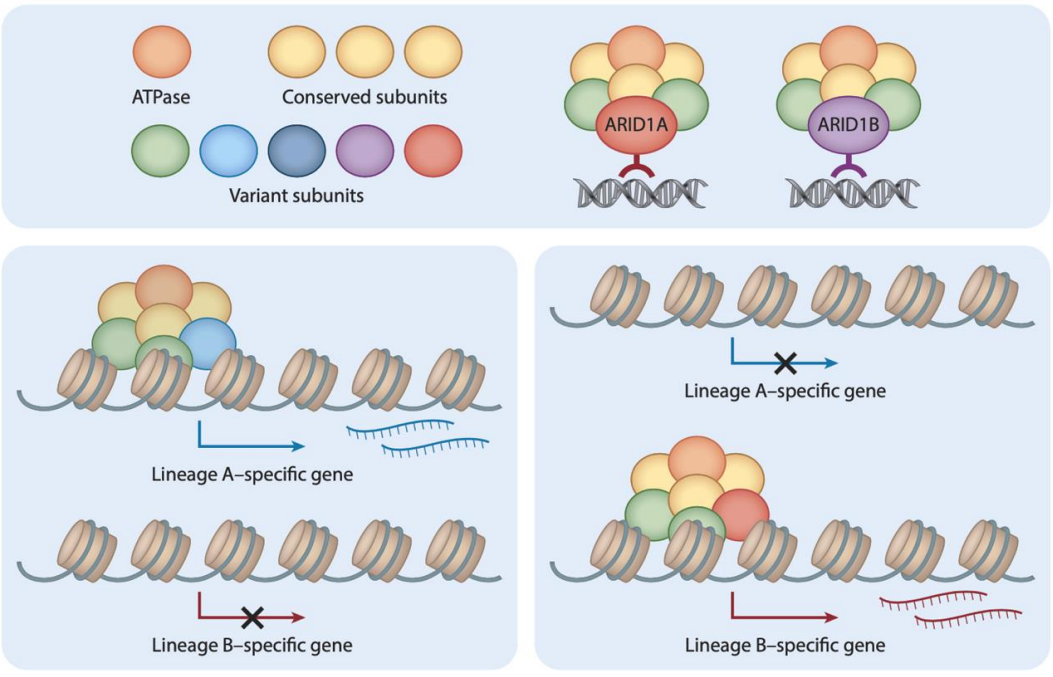

Figure 12 **The human SWI/SNF chromatin remodeling complexes function in the regulation of lineage-specific differentiation.**

Source Mathur, & Roberts, 2018

2. The human AT-rich interaction domain (ARID) family and ARID1 subfamily

The AT-rich Interaction Domain (ARID) is a helix–turn–helix motif-based DNA-binding domain and is sustained in all sequenced higher eukaryotic genomes. The ARID was first discovered as a DNA-binding domain in the mouse B cell-specific transcription factors in Bright and the Dead Ringer Protein of *Drosophila melanogaster* (Gregory et al., 1996; Herrscher et al., 1995; Kortschak et al., 2000; Wilsker et al., 2002). The human ARID family consists of seven subfamilies that are divided based on the degree of identification of sequences between individual members. The seven subfamilies included ARID1, ARID2, ARID3, ARID4, ARID5, Jumonji AT-rich interaction domain 1 (JARID1), and JARID2. All fifteen members of the ARID family contain a DNA-binding domain that was initially found to interact with AT-rich DNA elements (Figure 13) (Lin et al., 2014; Patsialou et al., 2005; Wilsker et al., 2005). AT-rich binding was not an intrinsic property of ARID and that members of the ARID family might be involved in a broader range of DNA interactions, which play a role as transcriptional regulators that are involved in cell differentiation and proliferation (Wilsker et al., 2002). Recent advanced roles of the ARID family members that may be involved in various human cancers have been discussed and reported by Lin et al. in 2014. The ARID family members are involved in cancer-related signaling pathways, highly mutated or differentially expressed in tumor tissues, and act as predictive factors for cancer prognosis or therapeutic outcomes (Lin et al., 2014).

This study focused on the ARID1 subfamily, especially the ARID1A member. The ARID1 subfamily has two members, including ARID1A and ARID1B. The *ARID1A* and *ARID1B* genes are located on chromosome 1 at 1p36.11 and on chromosome 6 at 6q25.3, respectively. These members are exclusive subunits of the BAF subclass, which is one of the human SWI/SNF chromatin remodeling complexes that is involved in ligand-dependent transcriptional activation by nuclear receptors (Hurlstone et al., 2002; Wang et al., 2004; Wilsker et al., 2005). *ARID1A* and *ARID1B* share 66% overall similarity in structure but have some particular functions that are different from each other (Nagl et al., 2005). *ARID1A* and *ARID1B* play a role as tumor suppressors and also inhibit colony formation in cancer cells. Nevertheless, these members have opposite roles in the cell cycle, where *ARID1A* is essential for cell cycle

arrest, whereas *ARID1B* has been shown to activate the cell cycle in pancreatic cancer cells (Khursheed et al., 2013; Mamo et al., 2012; Nagl et al., 2007; Nagl et al., 2005; Van Rechem et al., 2009). Furthermore, *ARID1A* is highly mutated and decreases expression at the protein level in various types of cancer that may be associated with poor prognostic outcomes of patients (Lin et al., 2014).

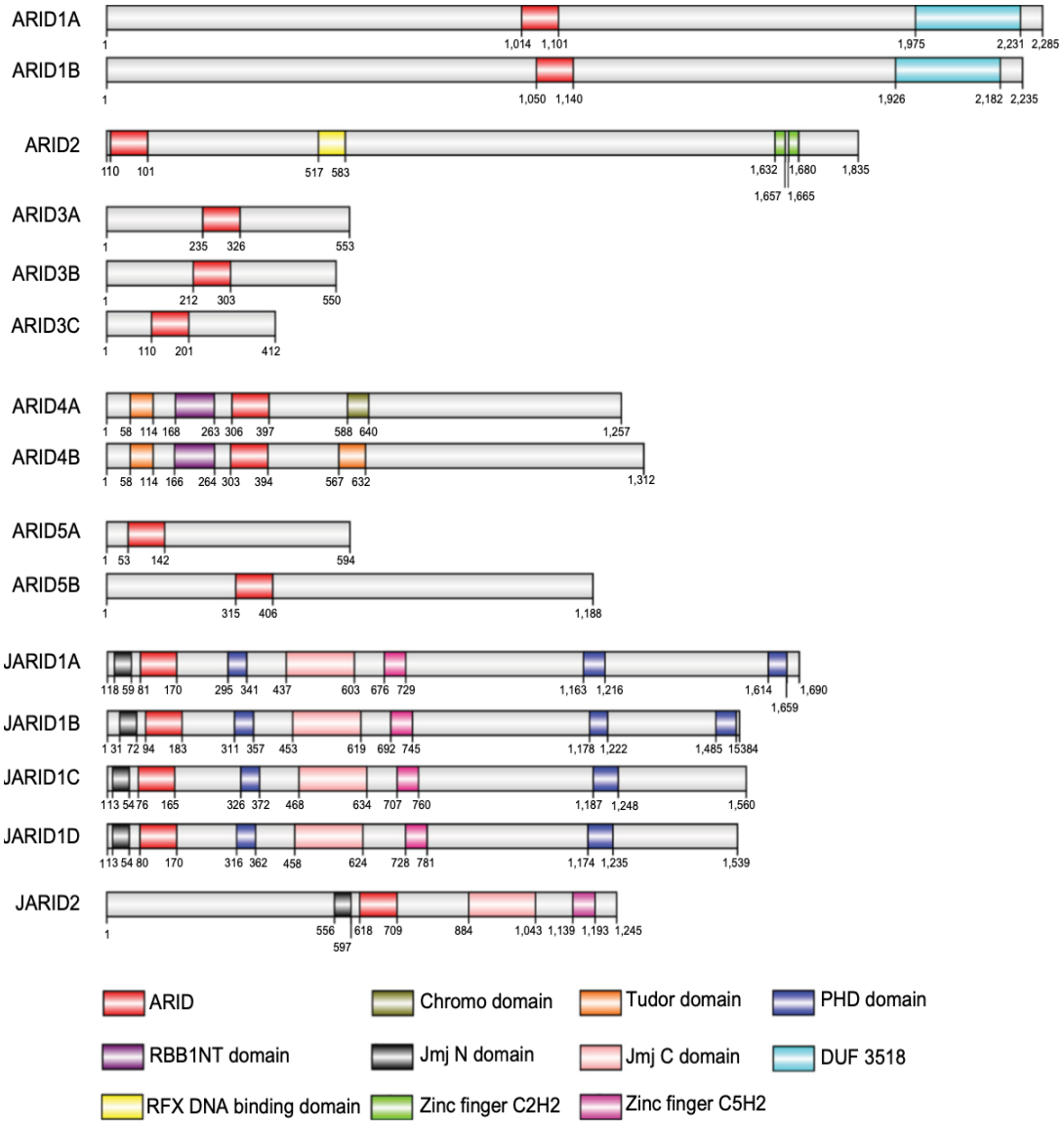

Figure 13 **Schematic overview of the human ARID family and the domains in each member**

Source Lin et al., 2014

3. Structure and expression of ARID1A

ARID1A, also known as BAF250a, p270, and SMARCF1, is one of the members of the ARID1 subfamily. *ARID1A* is a key component of the BAF subclass of the human SWI/SNF chromatin remodeling complexes (Hurlstone et al., 2002; Wang et al., 2004; Wilsker et al., 2005). The *ARID1A* gene is encoded by twenty exons spanning 86,08 Mb on chromosome 1p36.11 (Figure 14A) (Suryo & Wang, 2014). The human chromosome 1p36 region is frequently deleted in various human cancers (Erfani et al., 2020; Lotem et al., 2015). Human *ARID1A* has two transcript variants, including the long and short variants (Figure 14B). The long variant, or isoform 1, is transcribed into 8,585 bp of mRNA. The coding sequence of isoform 1 is from 374-7,231 bp. In contrast, the short variant, or isoform 2 mRNA, is transcribed into 7,934 bp, and the coding sequence is 374-6,580 bp. The ARID1A protein has two encoded protein isoforms. The longer isoform consists of 2,285 amino acids with a predicted molecular weight (MW) of 242,04 kDa. The shorter isoform has 2,068 amino acids with a MW of 218,33 kDa. Both isoforms comprise a single ARID DNA-binding, glutamine-rich region and several LXXLL on C terminal regions that generally interact with nuclear hormone receptors, particularly the glucocorticoid receptor (Figure 14C). However, the relative expression and function of these two isoforms are under-investigated and need further studies to be clarified (Nie et al., 2000; Samartzis et al., 2013; Suryo & Wang, 2014).

The ARID1A protein is mainly located in both the nucleus and the cytoplasm but not in the nucleolus. Cytoplasmic ARID1A is more stable than nuclear ARID1A. Nuclear ARID1A is rapidly degraded by the ubiquitin-proteasome system (Guan et al., 2012; Lin et al., 2014). In the cell cycle, ARID1A protein was accumulated more during the G0/G1 phases, whereas it was significantly downregulated during the G2/M phases (Flores-Alcantar et al., 2011). As a subunit of SWI/SNF complexes, ARID1A is thought to contribute to specific recruitment of its chromatin remodeling activity by binding transcription factors and transcriptional coactivator or corepressor complexes (Nie et al., 2000). Wu and colleagues reported that the emerging role of ARID1A is involved in a tumor suppressor. ARID1A has gatekeeper properties, such as regulating cell cycle progression or promoting apoptosis, as well as caretaker properties, such as preventing genomic instability in cancers (Wu et al., 2014)

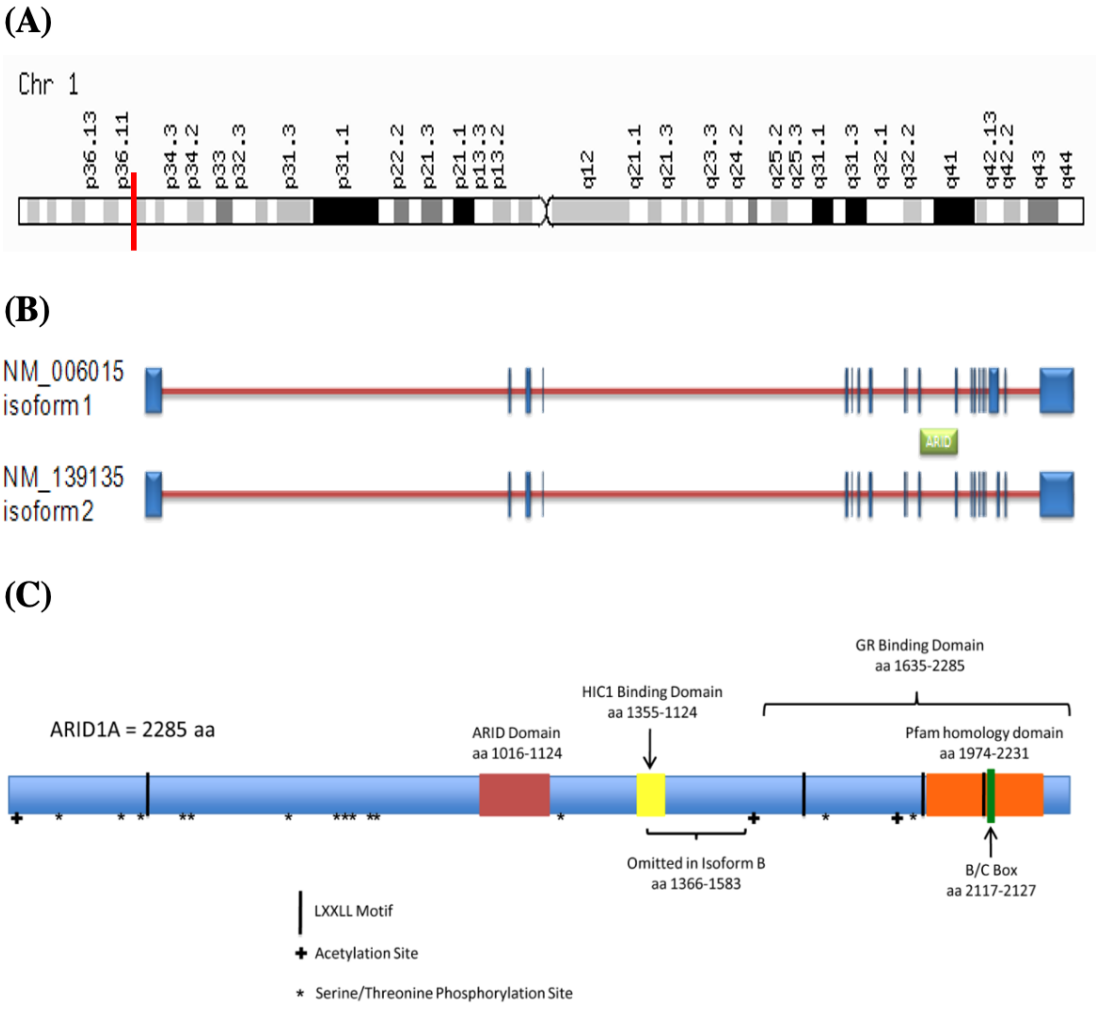

Figure 14 **Schematic overview of ARID1A structures**
(A) Chromosomal location for ARID1A (indicating in a red line),
B) DNA organization of ARID1A consists of the longer (isoform 1) and shorter variants (isoform 2), and C) Mapping of ARID1A)

Source Gene cards on human gene database. Accessed from https://www.genecards.org/cgi-bin/carddisp.pl?gene=ARID1A on date August 5, 2021; Suryo, & Wang, 2014; Wu, & Roberts, 2013

Furthermore, the ARID1A protein is ubiquitously expressed in various normal tissues. From the human protein atlas, it has been reported that ARID1A protein is highly expressed in organs or components of the central nervous system, respiratory system, urinary and female genital system, and lymphatic system. ARID1A protein expression is moderate in the GI tract (Figure 15) (Suryo, & Wang, 2014; https://v15.proteinatlas.org). Several studies have elucidated that decreased or loss of ARID1A protein expression is related to a variety of types of cancer (Lin et al., 2014).

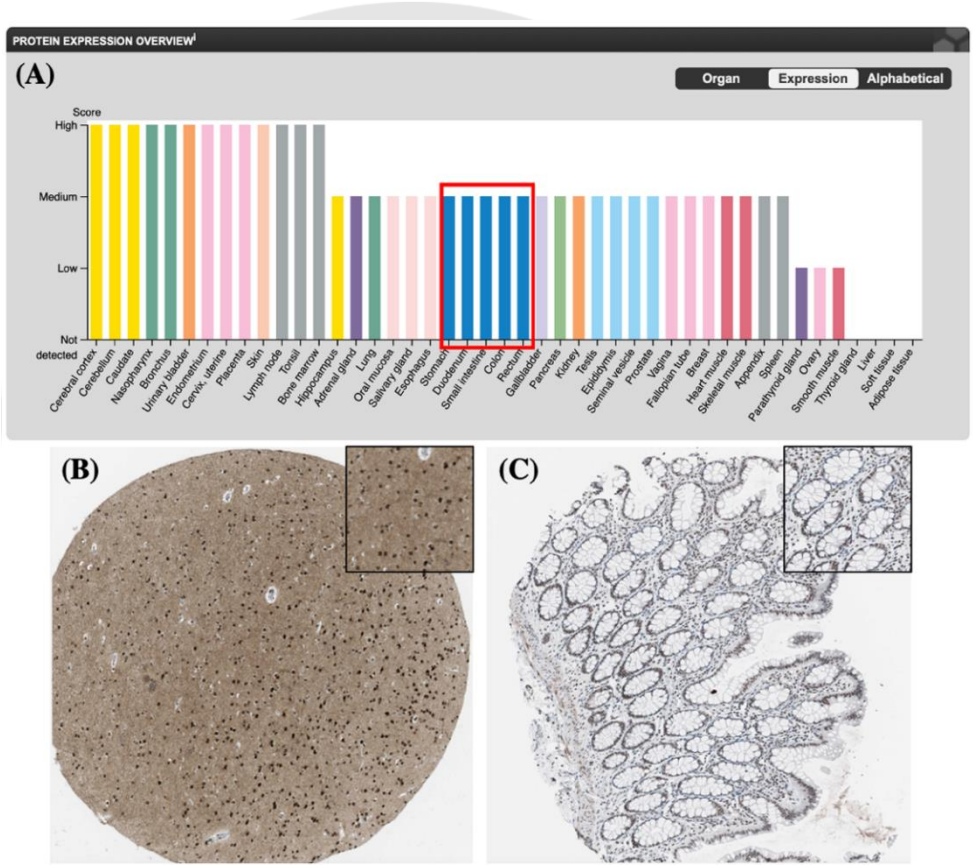

Figure 15 **ARID1A protein expression in human tissues**
(A) Summary of protein expression of ARID1A in human organs, which the gastrointestinal tract indicates in a red square, the represented images of ARID1A expression in (B) cerebral cortex, and (C) colon)

Source The human protein atlas. Accessed from https://www.proteinatlas.org/ENSG00000117713-ARID1A/tissue on date August 5, 2021

4. The ARID1A alteration in cancers

All human cancers, approximately 20% of which harbor mutations, are involved in some subunits of the human SWI/SNF chromatin remodeling complexes, including *ARID1A*, *ARID1B*, *ARID2*, *PBRM1*, *SMARCA4*, and others. The SWI/SNF complexes are one of the most frequently mutant epigenetic regulators in cancer, as well as one of the most frequently altered tumor suppressor genes in human malignancy (Kadoch et al., 2013; Shain & Pollack, 2013). *ARID1A* is one of the most frequently mutated tumor suppressor genes. It was identified as the first loss-of-function somatic mutations in endometriosis-associated ovarian cancers, including ovarian clear cell carcinoma and ovarian endometrioid carcinoma, which harbored *ARID1A* somatic mutations in 46–57% and 30%, respectively (Jones et al., 2010; Wei et al., 2014; Wiegand et al., 2010). Additionally, somatic mutations of *ARID1A* have been reported in other types of cancers, including uterine endometrioid carcinoma (39–44%), gastric carcinoma (8–29%), esophageal adenocarcinoma (9–19%), Waldenstrom macro-globulinemia (17%), pediatric Burkitt lymphoma (17%), hepatocellular carcinoma (10–16%), cholangiocarcinoma (14–15%), urothelial carcinoma of the bladder (12-15%), melanoma (11.5%), CRC (9.4%), and lung adenocarcinoma (8.2%) (Figure 16) (Jones et al., 2012; Kadoch et al., 2013; Wu et al., 2014). Recently, *ARID1A* mutations have been increasingly reported in malignant tumors of the GI tract (Wang et al., 2021). Mutations of *ARID1A* occur across the length of the gene, including truncating or frameshift (insertions and deletions) and nonsense mutations (Jones et al., 2010; Mathur, 2018). Namjan et al. discovered 89% of truncating mutations in cholangiocarcinoma (Namjan et al., 2020). *ARID1A* mutations have been found as a prognostic role in loss of *ARID1A* shortens time to cancer-specific mortality and cancer recurrence (Luchini et al., 2015; Mathur, 2018). The majority of *ARID1A* mutations were inactivating mutations, leading to loss of its expression at protein level (Wang et al., 2021).

Loss or reduction of ARID1A expression was associated with a variety of types of cancer, which is more frequently found in certain types of cancer, including ovarian endocervical-type mucinous borderline tumor (33%), cervical adenocarcinoma (24-31%), endometrial clear cell carcinoma (21-26%), endometrial carcinosarcoma (14%), anaplastic thyroid carcinoma (14%), Epstein-Barr virus-positive gastric carcinoma (34%), and aggressive phenotypes of breast cancer (Abe et al., 2012; Mamo et al., 2012; Wu et

al., 2014). ARID1A loss is associated with PI3K-Akt pathway activation in ovarian clear cell carcinomas, resistance to trastuzumab in HER2-positive breast carcinomas, and impairment in enhancer-mediated gene regulation in murine colorectal tumor models. In contrast, in hepatocellular carcinoma, ARID1A acts as an oncogene in tumor initiation but as a tumor suppressor in subsequent maintenance and metastasis (Berns et al., 2016; Bosse et al., 2013; Mathur et al., 2017; Sun et al., 2017). Importantly, loss of ARID1A expression is correlated with severe clinicopathological features such as large tumor size, high pathological grading, late TNM stage, distant metastasis, lymph node involvement, and worse prognosis of patients (Wu et al., 2014). Therefore, several studies have suggested that ARID1A may serve as a prognostic biomarker for cancer diagnosis (Lichner et al., 2013; Samartzis et al., 2012; Wei et al., 2014; Wiegand et al., 2014).

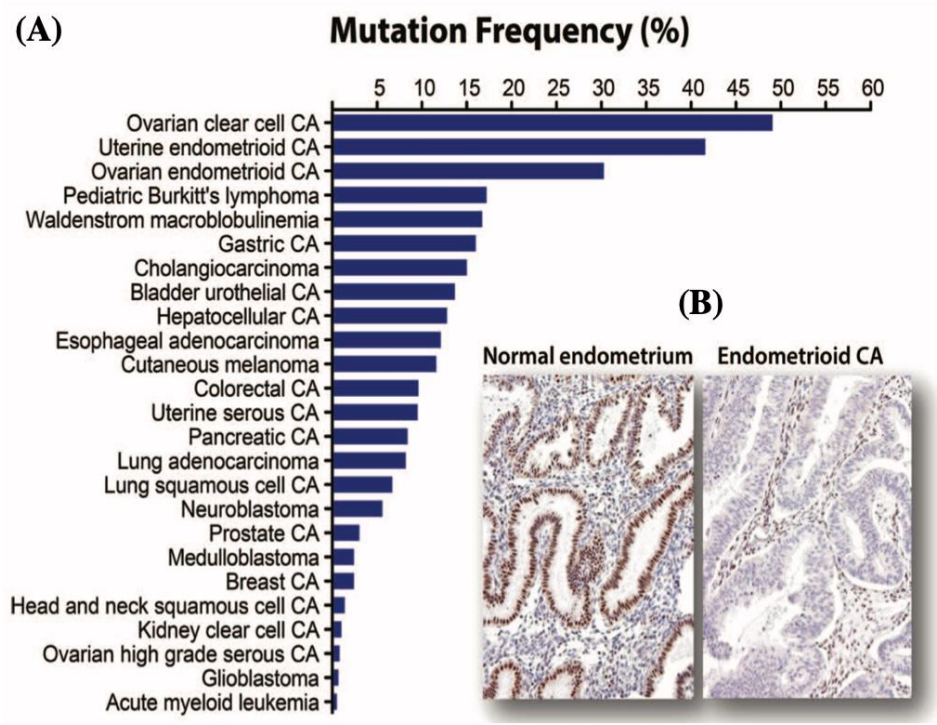

Figure 16 **Alteration of ARID1A expression in human cancers**
(A) ARID1A mutation frequency in human cancers, B) ARID1A expression in normal endometrium and uterine endometrioid carcinoma)

Source Modified from Wu, Wang, & Shih, 2014

5. The ARID1A alteration in colorectal cancer

Several studies have shown that *ARID1A* mutation, MMR deficiency, MSI, or promoter hypermethylation result in the loss or decrease of ARID1A expression in human CRC tissues and cell lines, which may be associated with poor pathological outcomes in CRC patients (Chou et al., 2014; Erfani et al., 2020; Mathur et al., 2017).

ARID1A is mutated in approximately 10% of all CRC cases, with mutations enhanced in cancers of the MSI type. Somatic mutations were also found in 12/119 CRC samples (Jones et al., 2012; Mathur, 2018). It has been reported that an inactivation of *ARID1A* drives the formation of invasive colon tumors that show features associated specifically with human colon cancers of the MSI type in a mouse model. These findings represent an advance in colon cancer modeling and implicate enhancer-mediated gene regulation as a principal tumor-suppressor function of *ARID1A* (Mathur et al., 2017). The dysfunction of MMR could contribute to MSI, which is related to the expression of ARID1A. Chou and colleagues reported that ARID1A deficiency was most commonly found in CRC with *BRAF V600E* mutations and MMR deficiency (Chou et al., 2014). Furthermore, *ARID1A* promoter hypermethylation at the CpG island reduced *ARID1A* mRNA levels in CRC cell lines (Erfani et al., 2020). Recently, decreasing or loss of ARID1A expression has been increasingly found in human CRC (Chou et al., 2014; Erfani et al., 2020; Kishida et al., 2019; Lee et al., 2016; Wei et al., 2014; Ye et al., 2014). Importantly, the alterations of ARID1A protein expression were significantly associated with the severity of clinicopathological characteristics, such as gender, poor pathological grading, late TNM staging, distant metastasis, and lymphovascular invasion (Lee et al., 2016; Wei et al., 2014). However, alterations of ARID1A expression did not correlate with OS, DSF, and recurrence-free survival (RFS) in patients with CRC (Chou et al., 2014; Erfani et al., 2020; Lee et al., 2016). The relationship between ARID1A protein expression and clinical significance in CRC is limited and understudied. Then it required further investigations to elucidate the significance of ARID1A as one of the promising prognostic indicators that may be useful for a precise prognosis of CRC.

Epithelial-mesenchymal transition (EMT)

1. Epithelial-mesenchymal transition (EMT)

EMT is an essential biological process that involves the differentiation of polarized epithelial cells that generally display apical–basal polarity. They are attached together by tight junctions, adherent junctions, and desmosomes. As well, they are tethered to the underlying basement membrane by hemi-desmosomes. Then they transform into mesenchymal cell phenotypes, which include enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased production of extracellular matrix (ECM) components (De Craene & Berx, 2013; Kalluri & Neilson, 2003). Inauguration of EMT induces the expression of the EMT-inducing transcription factors (EMT-TFs), including the ZEB, SNAIL and/or SLUG, and TWIST1 families. These EMT-TFs repress the expression of genes associated with the epithelial state, such as E-cad, occludins, claudins, α6β4 integrins, and cytokeratins. Concurrently, EMT-TFs induce the expression of genes associated with the mesenchymal state, for example, neural cadherin (N-cadherin), vimentin, fibronectin, β1 and β3 integrins, and matrix metalloproteinases (MMPs). These alterations of gene expression are resulting in cellular changes that include the disassembly of epithelial cell–cell junctions and the dissolution of apical–basal cell polarity via inhibition of proteins that specifically regulate tight junction formation and apical–basal polarity, including crumbs, PALS1-associated tight junction protein (PATJ), and lethal giant larvae (LGL). The loss of epithelial features is accompanied by the acquisition of a partial set of mesenchymal features with the retention of certain epithelial features. Mesenchymal cells display front-to-back polarity and an extensively reorganized cytoskeleton and express a distinct set of molecules and EMT-TFs that promote and maintain the mesenchymal state. During the EMT process, cells become motile and acquire invasive capacities. EMT is a reversible process, and mesenchymal cells have the reversible ability to reach the epithelial state by undergoing the mesenchymal–epithelial transition (MET) process (Dongre & Weinberg, 2019) (Figure 17). EMT and MET processes occur during the development of an embryo, tissue remodeling, wound healing process, as well as cancer progression and metastasis (Thiery, 2003).

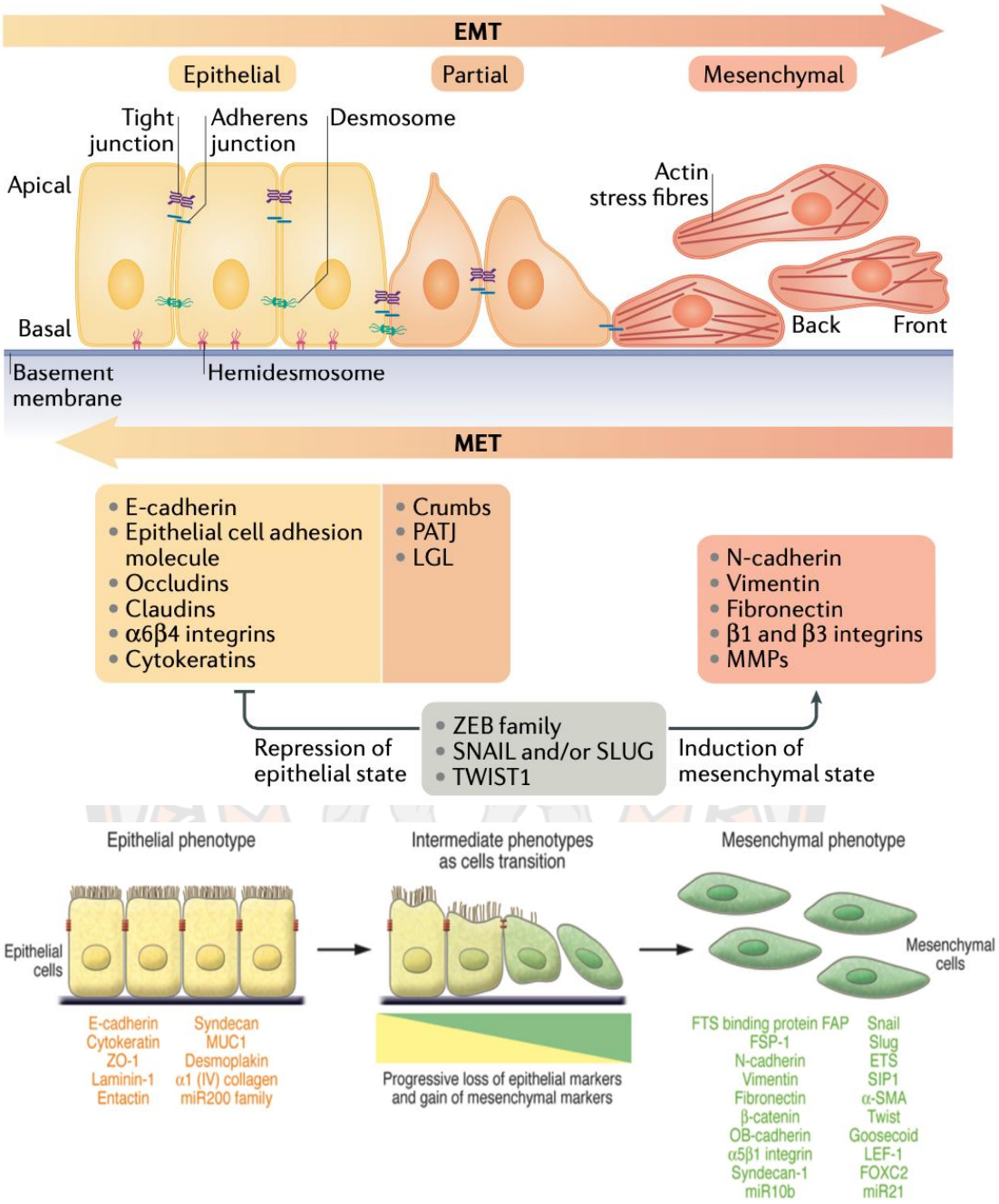

Figure 17 **An overview of the processes of EMT and MET**
(Including genes associated with the epithelial state (yellow box), genes associated with the mesenchymal state (orange box), and EMT-inducing transcription factors (EMT-TFs) (gray box))

Source Dongre, & Weinberg, 2019; Kalluri, & Weinberg, 2009

2. Different subtypes of the epithelial-mesenchymal transition

There are three different subtypes of EMT that occur in distinct biological processes that carry different consequences, including EMT during implantation, embryo formation, and organ development; EMT associated with tissue regeneration and pathological processes; and EMT associated with cancer progression, invasion, and metastasis (Figure 18) (Dongre & Weinberg, 2019; Kalluri & Weinberg, 2009).

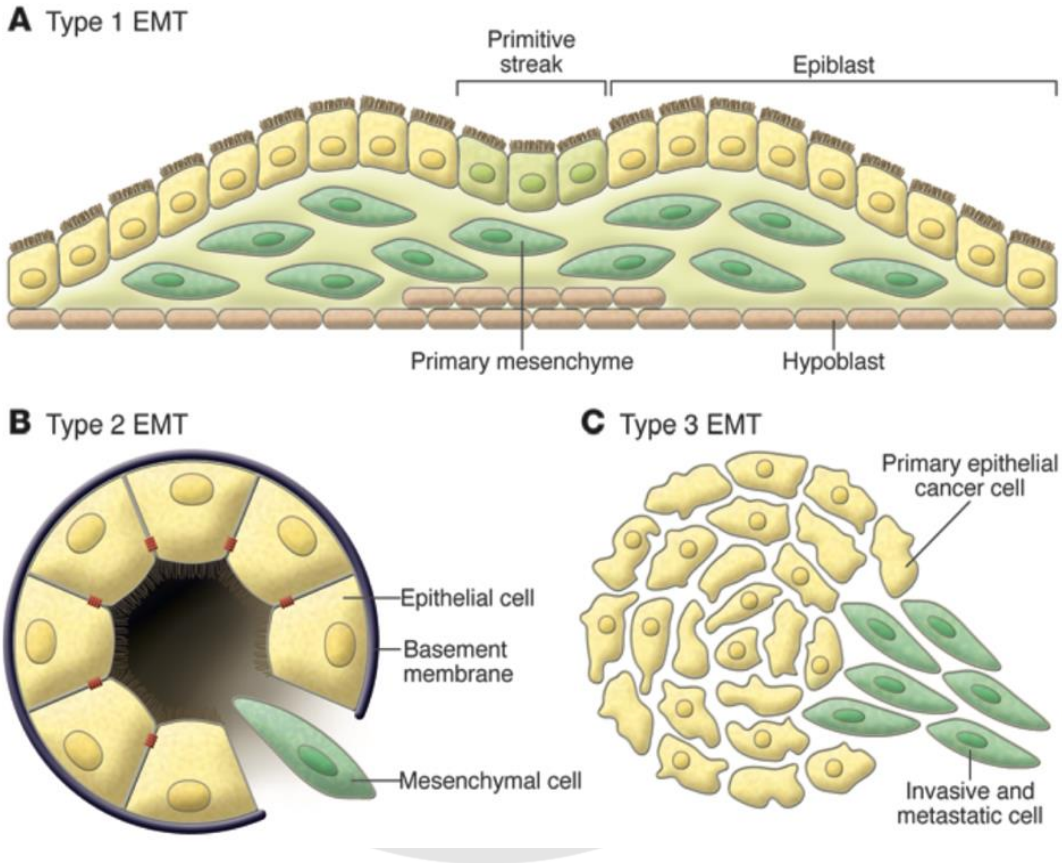

Figure 18 **Different EMT subtypes are involved in different biological processes**
(Including A) Type 1, EMT during implantation, embryogenesis, and organ development; B) Type 2, EMT associated with tissue regeneration and organ fibrosis; and C) Type 3, EMT associated with cancer progression, invasion, and metastasis)

Source Kalluri, & Weinberg, 2009

2.1 Type 1 EMT: EMT during implantation, embryogenesis, and organ development

EMT is fundamental for regulating the mesoderm formation during gastrulation and the cell migration that forms the neural crest from the neural tube (Nieto et al., 1994). EMT is involved in various specific morphogenetic events during development. During gastrulation, EMT promotes the generation of mesenchymal cells of the incipient mesoderm from the epiblast (Lim & Thiery, 2012; Oda et al., 1998; Schäfer et al., 2014). The Wnt signaling pathway was associated with EMT in the development of gastrulation, in which the embryo could not undergo gastrulation when Wnt3 deficiency occurred (Liu et al., 1999; Skromne & Stern, 2001). In addition, activation of EMT was found in neural crest cells and increased their migratory capacity, enabling their dispersion to multiple sites throughout the body of the developing chordate embryo (Clay & Halloran, 2014; Shoval et al., 2007; Simões-Costa & Bronner, 2015). EMT-TFs, especially the two members of the SNAIL family, have an important role in embryonic development (Barrallo-Gimeno & Nieto, 2005). Previous studies demonstrated that SNAIL and SLUG decreased expression of E-cad in mouse embryonic development (Arias, 2001; Aybar et al., 2003; Martínez-Álvarez et al., 2004).

2.2 Type 2 EMT: EMT associated with tissue regeneration and organ fibrosis

Organ fibrosis is mediated by inflammatory cells and fibroblasts that release a different set of inflammatory signals and components of the extracellular matrix, including collagen, laminins, elastin, and tenacins (Figure 19) (Kalluri & Weinberg, 2009). EMT is more specifically associated with organ fibrosis, which occurs in the liver, kidney, and small intestine (Kim et al., 2006; Potenta et al., 2008; Zeisberg et al., 2007). Previous studies have suggested that EMT is an essential precursor of fibroblasts that arise during the progression of organ fibrosis. Fibroblast-specific protein 1 (FSP1), S100 class of cytoskeletal protein, α-SMA, and collagen I have provided reliable markers to characterize the mesenchymal products generated by the EMTs that occur during the development of fibrosis in various organs (Okada et al., 1997; Rastaldi et al., 2002; Strutz et al., 1995; Zeisberg et al., 2003). EMT is an important process for tissue regeneration and repair during the wound healing process in adults (Dongre &

Weinberg, 2019). Moreover, SLUG has been involved in the regulation of the wound healing process. It has been reported that the overexpression of SLUG in human keratinocytes contributes to the increase in cell spreading and desmosome disruption that are generally observed at sites of wounding (Savagner et al., 2005).

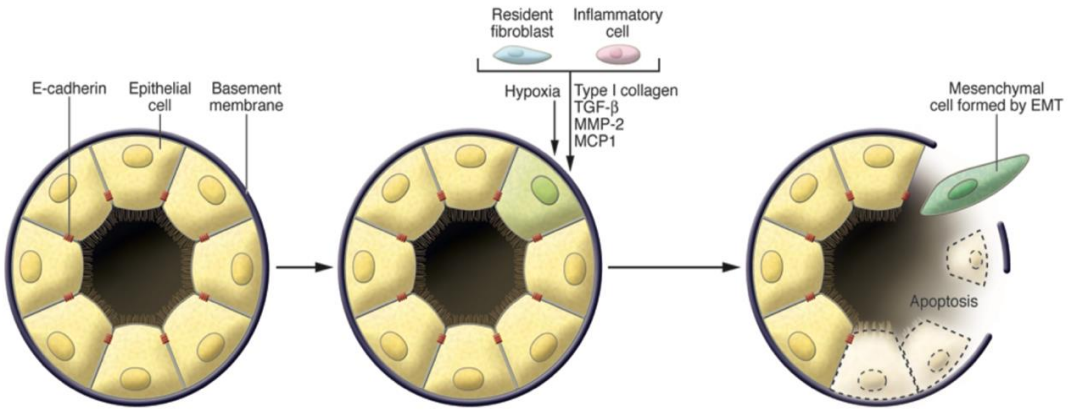

Figure 19 **Type 2 of EMT associated with organ fibrosis**
(Type 2 EMT is involved in organ fibrosis, which is associated with inflammation and the production of a variety of molecules by inflammatory cells and resident activated fibroblasts (myofibroblasts). These molecules disrupt the epithelial layers through degradation of the basement membrane. The epithelial cells lose polarity and either undergo apoptosis (the majority of cells) or EMT (the minority of cells))

Source Kalluri, & Weinberg, 2009

2.3 Type 3 EMT: EMT associated with cancer progression, invasion, and metastasis

Tumor metastasis is composed of sequential, interconnected, selective processes and various steps that are favored by conversions between two cellular states, including epithelial and mesenchymal phenotypes. EMT plays a critical and complicated role in promoting tumor invasion and metastasis in epithelium-derived carcinomas (Cao et al., 2015; Fidler, 2003). The subsequent steps of the invasion-metastasis cascade, initially

from tumor epithelial cells, lose their cell polarity and cell-cell adhesion, and transform into the mesenchymal phenotype. Tumors with mesenchymal phenotypes invade the local extracellular matrix (local invasion), penetrate into blood circulation (intrainvasion), and circulate through the bloodstream (systemic transportation) to distant organs (extravasation). Consequently, tumor cells establish micrometastases; this initial seeding of tumor cells at distant sites can occur rapidly, which is called the proliferation process. Subsequently, the colonization of tumor cells in distant organs requires the reversion of the EMT and/or activation of the MET process (Figure 20). For certain tumor types, the layout of the circulation may be the strongest determinant of metastatic tropism, such as the behavior of CRC, which has a strong preference for generating liver metastases (Cao et al., 2015).

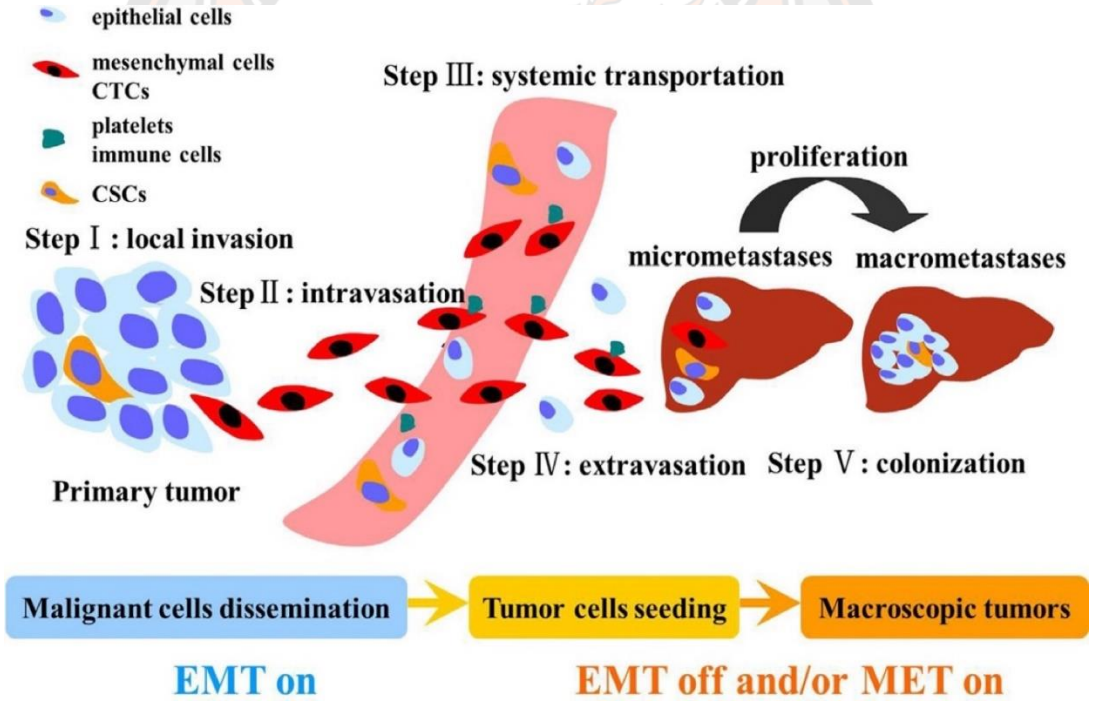

Figure 20 **Type 3 of EMT associated with cancer progression, invasion, metastasis (EMT contributes to cancer progression from normal epithelium to invasive carcinoma, which goes through several steps)**

Source Kalluri, & Weinberg, 2009; Cao et al., 2015

3. The alterations of EMT-related protein expression in colorectal cancer

EMT contributes to the proliferation, invasion, and metastasis in various epithelial tumors (Arias, 2001; Fantozzi et al., 2014). Previous studies have demonstrated that the EMT process plays a crucial role in the progression and aggressiveness of CRC (Barker & Clevers, 2001; Bates, 2005; Brabletz et al., 2005; Hur et al., 2013). Most CRC patients with distant metastasis did not show effectiveness to conventional treatment and exhibited a poor 5-year survival rate of less than 10% (Brenner et al., 2014; Manfredi et al., 2006). Therefore, a better understanding of molecular mechanisms underlying local invasion and distant metastasis is necessary to expedite the development of effective therapeutic strategies for metastatic CRC patients (Cao et al., 2015).

Approximately 85% of resected CRC samples have shown moderate to strong TWIST1 expression, which is notably more than either SNAIL1 or SLUG. Besides, SLUG and ZEB1 expression were significantly correlated with downregulated expression of E-cad and up-regulation of ZEB1 and ZEB2 at the invasion front, both correlated with the shorter survival times (Gomez et al., 2011; Kahlert et al., 2011; Kroepil et al., 2013; Larriba et al., 2009; Shioiri et al., 2006; Singh et al., 2011). Up-regulation of SLUG has emerged as an independent prognostic factor and a predictive marker of lymph node metastasis (LNM) and sprouting angiogenesis (Toiyama et al., 2013; Welch-Reardon et al., 2014). Moreover, TWIST1 overexpression was associated with nodal invasion, male sex, and unfavorable outcomes in CRC patients (Gomez et al., 2011; Okada et al., 2010; Valdés-Mora et al., 2009). Emerging evidence has indicated that many transcription factors and related signaling pathways are involved in EMT and CRC progression and metastasis (Figure 21) (Cao et al., 2015).

Decreasing of E-cad expression, a gene associated with the epithelial state, was correlated with the presence of LNM, distant metastasis, poor CRC pathological differentiation, and worse pathological outcomes of CRC patients (Aljafil et al., 2014; He et al., 2013; Peña et al., 2005). According to the role of E-cad as an essential gatekeeper of the epithelial state in carcinomas (Hay, 1995). Furthermore, the expression of occludin and ZO-1, which are epithelial state markers, was significantly downregulated in colorectal liver metastasis tissues (Orbán et al., 2008). On the contrary, increased vimentin expression, which is a gene associated with the mesenchymal state, was significantly associated with the presence of LNM and poor prognosis of CRC patients

(Toiyama et al., 2013). It was suggested that vimentin was able to increase the invasive ability of the tumor to affect tumorigenesis (Monteiro-Reis et al., 2019). Additionally, high expression of fibronectin, one of the mesenchymal state markers, was also correlated with poor prognosis of CRC patients as well as in CRC cell lines. Upregulation of fibronectin expression was associated with cell proliferation via the NF-κB/p53-apoptosis signaling pathway (Yi et al., 2016).

Furthermore, ARID1A is also involved in the EMT process. Previous *in vitro* studies have demonstrated that *ARID1A* knockdown exhibited increased cell proliferation, migration, and invasion in various cancer cell lines, including RCC, PDAC, breast cancer, and CRC (Erfani et al., 2021; Somsuan et al., 2019; Tomihara et al., 2021; Wang et al., 2020). Moreover, *ARID1A* knockdown also demonstrated the upregulated expression of mesenchymal markers (such as vimentin and fibronectin) and the downregulated expression of epithelial proteins (such as E-cad and ZO-1) in RCC and PDAC (Erfani et al., 2021; Somsuan et al., 2019). It has been suggested that *ARID1A* downregulation may promote CRC metastasis through decreasing EMT-related protein, in particular, E-cad, and promoting epithelial cell movement. Thus, ARID1A may be considered as a promising candidate therapeutic target for CRC (Erfani et al., 2021).

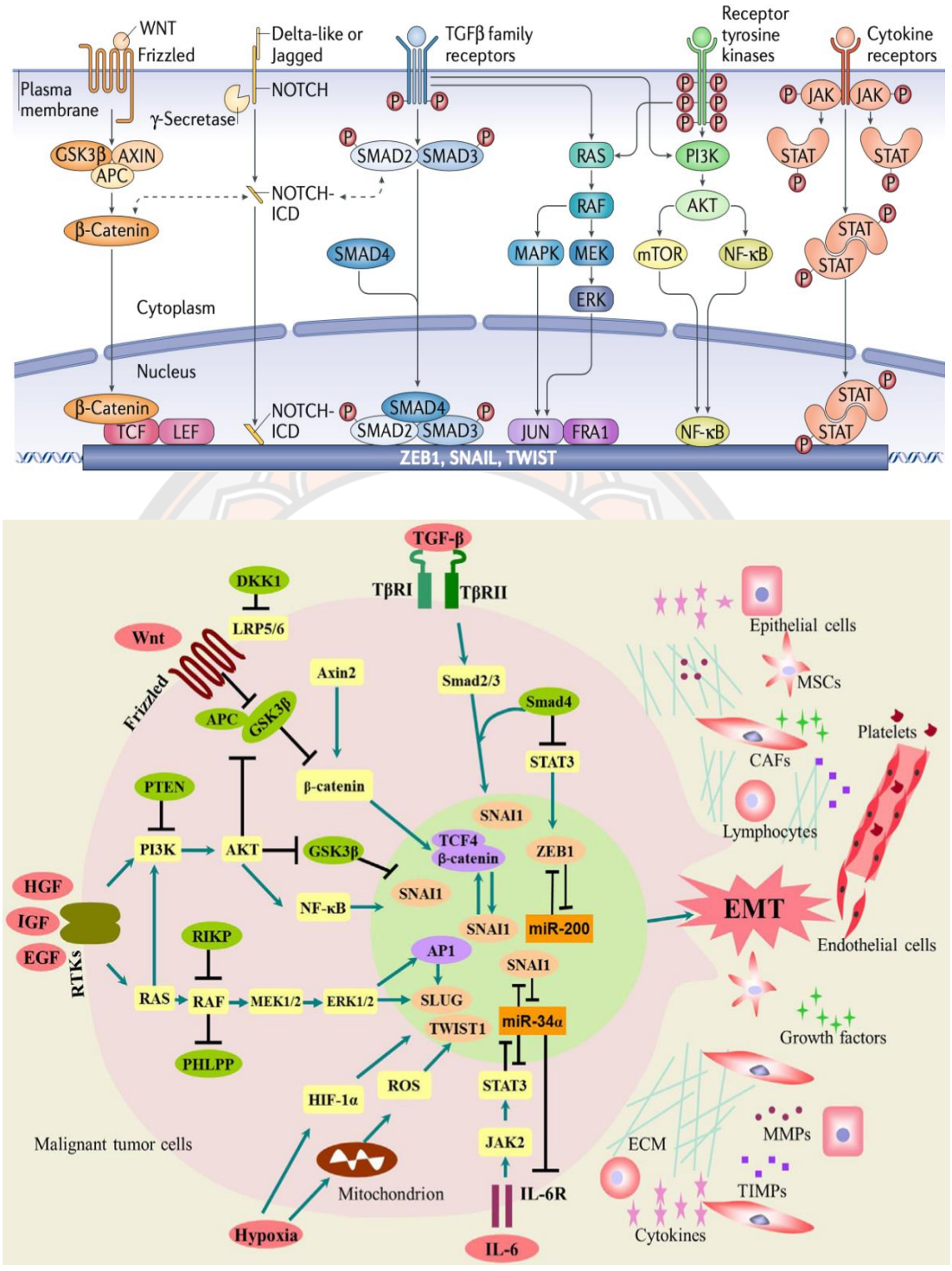

Figure 21 **Related signaling pathways and EMT-TFs of EMT in CRC**

Source Dongre, & Weinberg, 2019; Cao et al., 2015

CHAPTER III

RESEARCH METHODOLOGY

Bioinformatics analysis of *ARID1A* gene mutation in CRC

The cBioPortal for cancer genomics database (https://www.cbioportal.org/) was used to analyze *ARID1A* mutations and the frequent genetic mutations of *ARID1A* in CRC (Cerami et al., 2012). The Cancer Genome Atlas (TCGA) projects related to CRC, including Firehose Legacy, Nature, and the PanCancer Atlas projects, which recruited 1,506 patients (1,510 samples) from 3 studies (all information was accessed on December 17, 2021), were investigated. From this accessible information, the comparison of mRNA level and protein expression between mutated *ARID1A* and non-mutated *ARID1A* groups was also examined.

Ethics statement and the patient tissue's recruitment

All study ethics approvals were approved by the Human Ethic Review Board of Sawan Pracharak Hospital, Nakhon Sawan, Thailand (approval no. 16/2560) and the Naresuan University Ethics Committee for Human Research (NU-IRB) (approval no. P10181/64; COA no. 421/2021), and were undertaken following the ethical standards of the World Medical Association Declaration of Helsinki. All the patients in this study provided their written informed consent for their personal information.

FFPE blocks of CRC patients were used in this study. All patients were diagnosed with different pathological differentiation of CRC and had their tissue biopsy submitted during 2017- 2021 to the Unit of Pathology, Sawan Pracharak hospital, Nakhon Sawan province, Thailand.

Sample size

For the determination of sample size, the G*Power 3.1 analysis software (Faul et al., 2007) was performed to indicate the adequate number of CRC tissue FFPE blocks in this study. Therefore, after calculating the sample size, there were 100 FFPE samples of CRC patients included in this study by the clinical pathologist.

Inclusion and exclusion criteria

Inclusion criteria

1. FFPE blocks must be obtained from CRC patients aged 50 to 95 years. The young-age onset of CRC provides a clue to a possible relationship with the hereditary etiology of CRC. A previous study showed that the majority of CRC occurs in people older than 50 years old. The mean age at diagnosis of CRC is 72 years old in men and 75 years old in women (Kolligs, 2016).

2. FFPE blocks must be collected from CRC patients who underwent their biopsy during 2017–2021 because levels of protein expression may be affected by proteolysis after the long-term storage of FFPE blocks (Nuovo et al., 2013).

3. The relevant demographic and pathological information of patients must be available for access.

4. To avoid any potential problem due to an insufficient sample for further diagnosis and investigation, several FFPE blocks must be available.

5. Each FFPE block must be sufficient for performing of tissue sectioning at least 10-15 sections of 3-5-μm-thick section.

Exclusion criteria

1. Patients who were diagnosed with hereditary CRC syndromes, some inherited conditions, and aged less than 50 years were excluded.

2. Patients diagnosed with cancer of unknown primary (CUP) were excluded.

3. A patient who was diagnosed with CRC during pregnancy was excluded from this study. A previous study found that the follicle-stimulating hormone (FSH) receptor binding inhibitor (FRBI) influences ARID1A expression levels in ovarian cancers (Gong et al., 2019).

4. Patients undergoing pre-chemotherapy and pre-radiation treatment prior to surgery have been excluded.

5. Histological and/or immunohistochemical investigation cannot be clarified by a pathologist or researcher.

Conceptual framework

The FFPE blocks of CRC patients, composed of the cancerous and non-cancerous areas, and the demographic and clinicopathological information of patients who were diagnosed with the different pathological differentiations of CRC during 2017–2021, were obtained from the Unit of Pathology, Sawan Pracharak hospital, Nakhon Sawan province, Thailand. After that, FFPE blocks were transferred to the Department of Anatomy, Faculty of Medical Science, Naresuan University, to perform the experiments in this study.

Subsequently, FFPE blocks of CRC tissues were sectioned into 3-μm-thick tissue sections, and then ARID1A and EMT-related protein expressions, including epithelial proteins (E-cad and ZO-1), and mesenchymal proteins (vimentin and fibronectin), were performed using the indirect IHC method. After that, the immunoreactivity of ARID1A was examined by at least three investigators. The H-score was applied to evaluate the ARID1A immunoreactivity (Hirsch et al., 2003; John et al., 2009). Based on the H-score, the immunostained sections were categorized into two groups, including low and high ARID1A expressions. Furthermore, the IHC intensities of ARID1A and EMT-related protein were also investigated using ImageJ (Fiji) image analysis software (http://fiji.sc/Fiji) (Ruifrok & Johnston, 2001).

Accordingly, the association between the expression of ARID1A and EMT-related protein with the severity of clinicopathological characteristics was analyzed using the Fisher's exact test. In addition, the pathological outcomes of CRC patients with ARID1A and EMT-related protein expressions were analyzed by the Kaplan-Meier analysis and compared statistical data using the log-rank test. The p-value < 0.05 was considered as a statistically significant value.

Collection of tissue samples and clinicopathological information of CRC patients

A cohort of 100 patients who had their CRC surgically removed between January 2017 to January 2021 and submitted their removed tissue samples to the Unit of Pathology, Sawan Pracharak hospital, Nakhon Sawan, Thailand, was conducted. CRC tissues, including cancerous and adjacent non-cancerous areas, were obtained as FFPE blocks. Each case was diagnosed and examined by a clinical pathologist using hematoxylin and eosin (H&E) staining slides. The WHO classification criteria were used to classify the pathological differentiation of each specimen. Tumors were pathologically graded as well-differentiated adenocarcinoma (n=65), moderately differentiated adenocarcinoma (n=23), and poorly differentiated adenocarcinoma (n=12). Additionally, CRC staging was assessed according to the guidelines of the AJCC, TNM classification, 8th edition (Amin et al., 2017). Stage I (n=8), stage II (n=22), stage III (n=36), and stage IV (n=34) were included in the total number of CRC specimens.

Furthermore, the clinicopathological information of patients who were diagnosed with CRC was accessed by a clinical pathologist. Demographic and clinicopathological information of CRC patients, for instance, age, gender, location of tumor, tumor mass dimension, pathological differentiation, AJCC staging, tumor invasion, metastasis, recurrence, angiolymphatic invasion, number of examined and positive lymph nodes, patient's comorbidity, and follow-up period after operation were acquired and analyzed.

All FFPE blocks were labeled using a new research code. The confidential data of patients, such as name, identification number, and hospital number, were blinded to protect the patient's information. The FFPE blocks were transferred under control temperature at 4 ℃ and then collected at -20 ℃ until the experiments were performed. All of the information was scanned and recorded securely on the password-protected computer of the researcher. After the end of the experiments, all FFPE blocks were returned to the unit of pathology, Sawan Pracharak hospital, or kept at the Department of Anatomy, Faculty of Medical Science, Naresuan University. The flowchart of the patient tissue's recruitment and collection was represented in Figure 22.

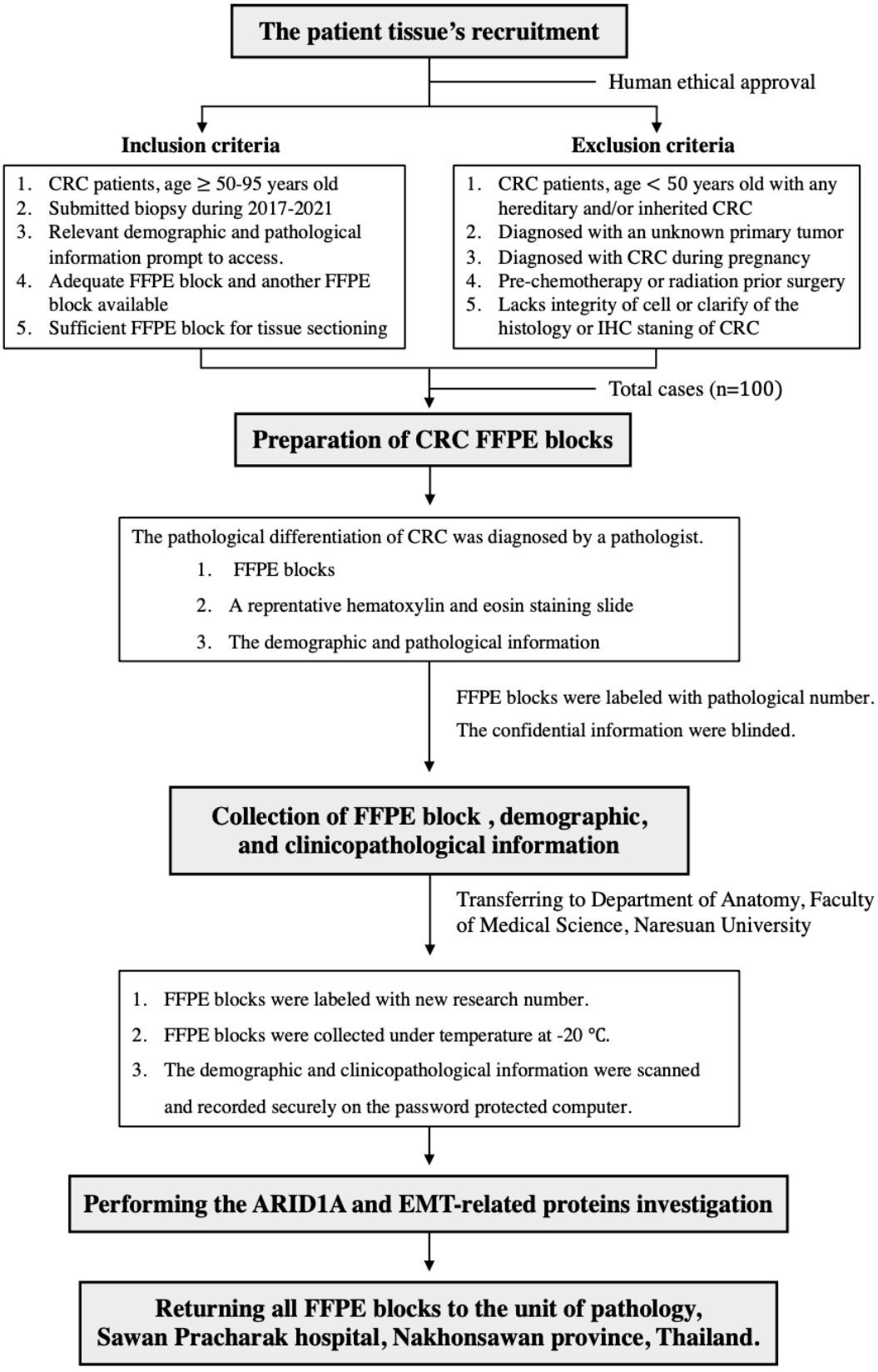

Figure 22 Flowchart of the patient tissue's recruitment and collection

Immunohistochemistry staining of ARID1A and EMT-related protein

IHC is a methodology that employs antibodies to detect antigens in cells within a tissue section. This application is used to locate specific antigens in tissue sections with labeled antibodies based on antigen-antibody interactions (Ramos-Vara, 2011). The principle of the indirect IHC method was demonstrated in Figure 23.

To investigate the expressions of ARID1A and EMT-related protein, the standard IHC procedure was applied using anti-ARID1A rabbit polyclonal antibody (1:400 dilution; HPA005456, Sigma-Aldrich, St. Louis, MO, USA), anti-E-cad rabbit monoclonal antibody (1:750 dilution; AB40772, Abcam), anti-ZO-1 rabbit polyclonal antibody (1:400 dilution; AB216880, Abcam), anti-vimentin rabbit monoclonal antibody (1:750 dilution; AB92547, Abcam), and anti-fibronectin rabbit polyclonal antibody (1:400 dilution; AB2413, Abcam, Cambridge, MA, USA). The details of primary antibodies used in this study were summarized in Table 6.

FFPE blocks, which were composed of cancerous and non-cancerous areas from patients with CRC, were sectioned at 3-μm-thick using a rotary microtome and placed on a silane/acetone-coated slide. The immunoreactivities of ARID1A and EMT-related proteins were assessed by IHC using an indirect method. In brief, all tissue sections were dried on a hot plate at 60°C for a half hour, deparaffinized in xylene, rehydrated through a graded series of ethanol/distilled water (DW) (from high to low concentration), and washed in DW. Subsequently, heat-induced epitope retrieval (HIER) was performed for antigen retrieval by incubating in a citrate buffer, pH 6.0, at 97°C. Tissue sections were cooled down at room temperature for 30 minutes, then immersed in 3% hydrogen peroxide (H_2O_2)/sodium azide (NaN_3) for 25 minutes to inhibit the endogenous peroxidase, washed in phosphate buffer saline (PBS), and incubated with 0.1% NaN_3 for 20 minutes to inhibit the non-specific protein. Then, the tissue sections were incubated with working primary antibodies, as previously described, in a humidified chamber for an hour at room temperature and then overnight at 4°C. As a negative control, the sections were treated with PBS. After washing with PBS three times, the tissue sections were treated with the biotinylated goat anti-rabbit IgG secondary antibody for 15 minutes, followed by incubation with streptavidin peroxidase (Ab64261, Abcam, Cambridge, MA, USA) at room temperature for 15 minutes. The chromogen 3,3'-diaminobenzidine (DAB) substrate was applied at a 1:50 dilution for

visualization, followed by rinsing in PBS to stop the DAB reaction. Sections were counterstained with Mayer's hematoxylin (C.V. Laboratories CO., LTD.) for nuclear staining, dehydrated with a stepwise increasing concentration of ethanol/DW, cleared in xylene, mounted using Permount® Mounting Medium (Permount, Fisher Scientific, Belgium), and then tissue sections were covered with a coverslip. Finally, the stained sections were visualized under a light microscope. The stained sections were observed and photographed using the ZEN program (Rushmore Precision Co., Ltd.) under the Axiocam 105 color ZEISS microscope (Carl Zeiss, Oberkochen, Germany). The summary of standard IHC procedures in this study was represented in Figure 24.

Table 6 The details of primary antibodies used in the human CRC study

Targeted proteins	Primary antibody	Corporation	Dilution	Secondary antibody
ARID1A	Rabbit polyclonal anti-ARID1A (HPA005456)	Sigma-Aldrich	1:400	Goat anti-rabbit (Ab64261, Abcam)
E-cadherin	Rabbit monoclonal anti-E-cadherin, intercellular junction protein (Ab40772)	Abcam	1:750	Goat anti-rabbit (Ab64261, Abcam)
Zonula occludens-1 (ZO-1)	Rabbit polyclonal, anti-ZO-1, tight junction protein (Ab216880)	Abcam	1:400	Goat anti-rabbit (Ab64261, Abcam)
Vimentin	Rabbit monoclonal anti-vimentin, cytoskeleton protein (Ab92547)	Abcam	1:750	Goat anti-rabbit (Ab64261, Abcam)
Fibronectin	Rabbit polyclonal anti-fibronectin (Ab2413)	Abcam	1:400	Goat anti-rabbit (Ab64261, Abcam)

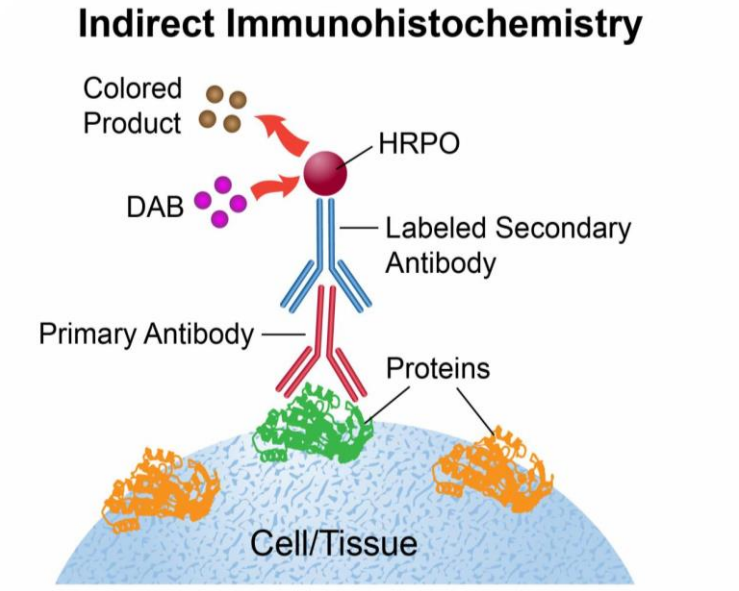

Figure 23 **An illustration of the indirect IHC method**

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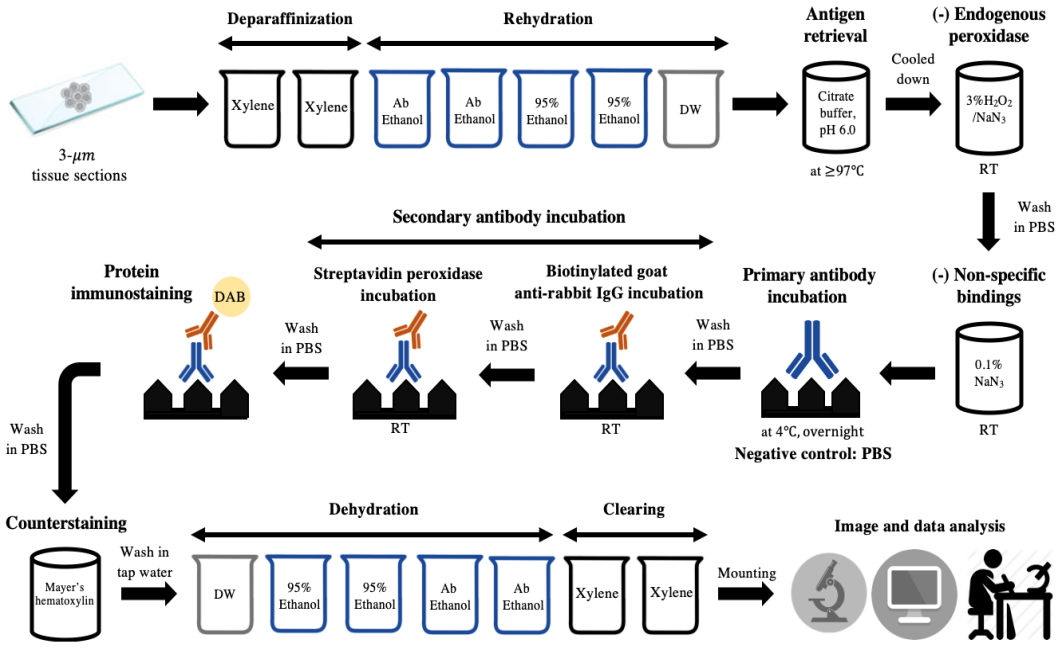


Figure 24 **The schematic summary of standard IHC procedures**

Assessment of ARID1A protein expression and quantitative analysis

Three independent investigators who were blinded to the demographic and clinicopathological information of CRC patients reviewed and evaluated the ARID1A immunostained sections. For assessment of ARID1A immunoreactivity, five independent areas of each section were imaged at high power fields (HPF) provided by 40× magnification of the objective lens using a ZEN program (Rushmore Precision Co., Ltd.) under an Axiocam 105 color ZEISS microscope (Carl Zeiss, Oberkochen, Germany) in both cancerous and adjacent non-cancerous areas of CRC tissues. The H-score, which is a semi-quantitative assessment to evaluate immunoreactivity in tumor samples (Hirsch et al., 2003), was applied to assess the expression of ARID1A protein. The H-score was evaluated based on the staining intensity and the percentage of positive cells of ARID1A staining. Three investigators evaluated the staining intensity of ARID1A and scored it as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong staining) in both cancerous and adjacent non-cancerous areas of CRC tissues (Figure 25). In addition, the percentage of the ARIDA-positive cells was detected and analyzed using ImageJ (Fiji) image analysis software (Ruifrok & Johnston, 2001; Schindelin et al., 2012). The summation of the H-score was calculated according to the formula:

H-score = [(0×% negative cells) + (1×%weakly positive cells) +
(2×%moderately positive cells) + (3×%strongly positive cells)] (Numata et al., 2013)

Consequently, the conceivable H-score ranges from 0 to 300. The 50% cut-off value of the H-score (150/300) has been used to classify ARID1A expression into two groups: low (less than 150) and high (equal to or more than 150) groups.

Furthermore, levels of ARID1A protein expression in the colonic epithelial cells were examined in the cancerous areas compared to the adjacent non-cancerous areas of CRC samples. ImageJ (Fiji) image analysis software (http://fiji.sc/Fiji) was conducted to measure the intensities of ARID1A protein expressions. The relative optical density (ROD) of protein contents from at least 100 nuclei was evaluated and calculated according to the following formula:

$$ROD = \log_{10} (\text{max intensity} / \text{mean intensity})$$

(https://imagej.nih.gov/ij/docs/menus/analyze.html)

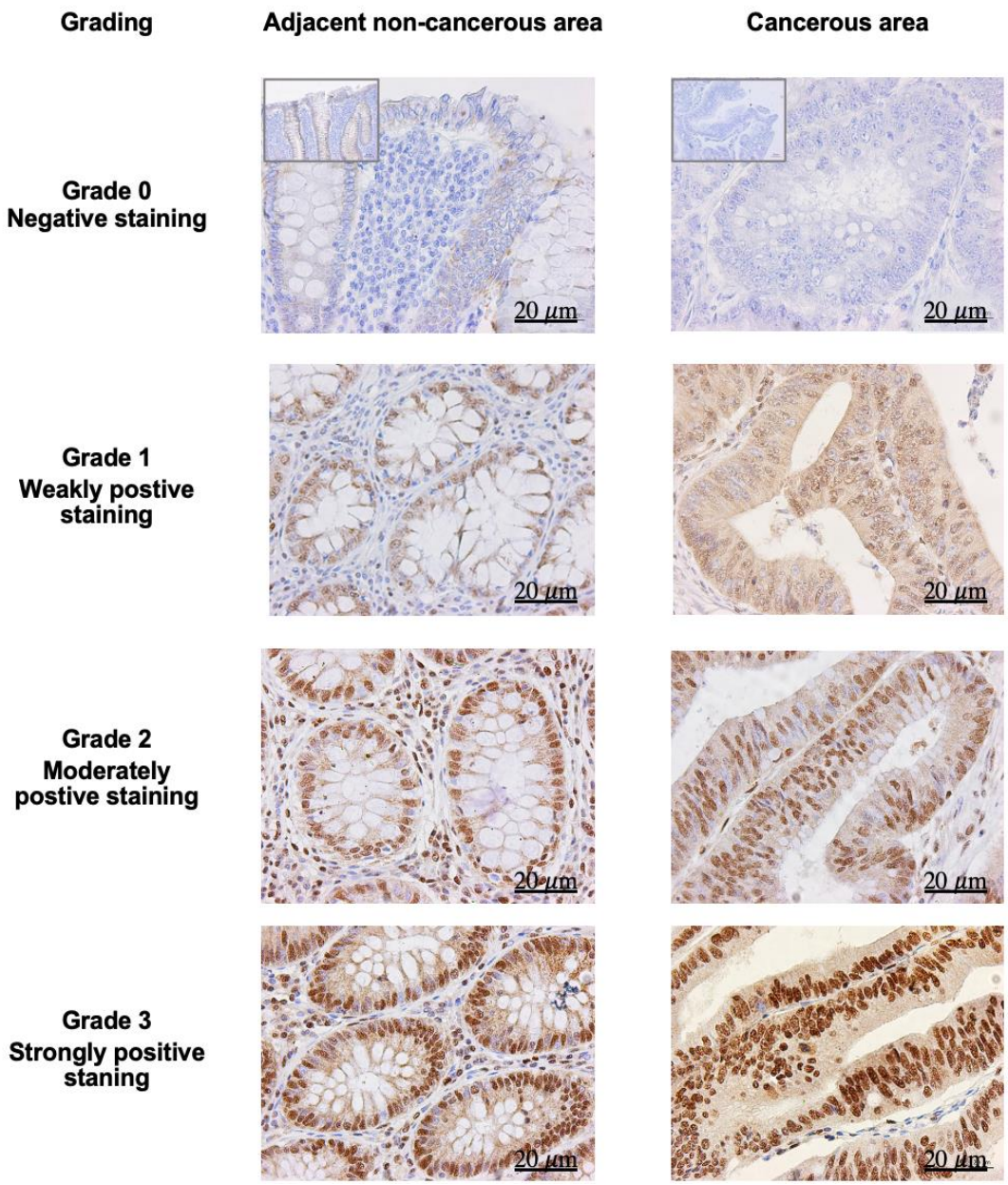


Figure 25 **Grading assessment for evaluating the intensity of ARID1A protein (Intensity of ARID1A staining was evaluated by three investigators as 0 (negative staining), 1 (weakly positive staining), 2 (moderately positive staining), and 3 (strongly positive staining) in both adjacent non-cancerous (left panel) and cancerous areas (right panel). The insets show the negative control for ARID1A-IHC staining. Original magnification power of 400× for all panels)**

Quantitative analysis of EMT-related protein expression

After investigation of ARID1A expression, CRC samples that had the ARID1A H-score of cancerous area less than 150, CRC patients who had distant metastasis, and positive for LNM were selected to investigate the alterations of EMT-related proteins using the indirect IHC method, as previously described. 10 samples from each CRC pathological differentiation; well-, moderately, and poorly differentiated adenocarcinoma, were taken for quantitative analysis.

To investigate the immunoreactivity of EMT-related protein, ten randomized areas of each stained section were imaged at 20× magnification of the objective lens using a ZEN program (Rushmore Precision Co., Ltd.) under an Axiocam 105 color ZEISS microscope (Carl Zeiss, Oberkochen, Germany) in both cancerous and adjacent non-cancerous areas of CRC tissues.

Subsequently, levels of EMT-related protein expression, including epithelial proteins (E-cad and ZO-1) expression in the intestinal epithelial cells and expressions of mesenchymal proteins (vimentin and fibronectin) in the stromal or interstitial area, were evaluated in the cancerous area compared to the adjacent non-cancerous area. The intensity of EMT-related protein expression was quantitated using ImageJ (Fiji) image analysis software (http://fiji.sc/Fiji). For quantitative analysis, the TIFF file format was adjusted using the color deconvolution algorithm "H DAB" to separate images of hematoxylin and DAB staining. Only DAB staining image was selected to evaluate the intensity of EMT-related protein expression. Thereafter, the DAB staining image was adjusted to the threshold for selecting the interesting area for analysis. The mean gray value in all ten randomized areas was measured and then calculated to the ROD value according to the following formula:

$$ROD = \log_{10} (\text{max intensity} / \text{mean intensity})$$

(https://imagej.nih.gov/ij/docs/menus/analyze.html)

Based on the mean IHC intensity in the cancerous area, individual EMT-related protein was divided into low intensity, where the mean intensity was less than the median value, and high intensity, where the mean intensity was equal to or greater than the median value.

Statistical analysis

All statistical analyses were conducted using the IBM SPSS statistical software version 25.0 for Mac (SPSS, Inc. Chicago, IL, USA) and GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, CA, USA). Mean±SEMs were used to represent quantitative data in this study. The student's t-test was carried out for statistical significance to compare the data in paired samples. Otherwise, statistical analysis of the unpaired samples was performed using the unpaired Student's t-test (when quantitative data was shown to be normally distributed) or Mann-Whitney U test (when data was not shown to be normally distributed). The association between the expressions of ARID1A and EMT-related protein with the clinicopathological characteristics of CRC patients was statistically analyzed using Fisher's exact probability and Pearson's chi-square tests. The cumulative 5-year PFS was interpreted by using the Kaplan–Meier analysis, and statistical significance was analyzed using the log-rank test. Cumulative PFS was defined as the time from the date of surgery to the diagnosed date of disease progression (metastasis). Additionally, the univariate and multivariate analyses of PFS were conducted using Cox proportional hazards regression analysis at 95% confidence intervals (CIs). The p-value < 0.05 was used as a statistically significant value in all data analysis.

CHAPTER IV

RESULTS

Mutation of *ARID1A* and its expression at mRNA and protein levels in CRC

The TCGA projects pertinent to CRC, including Firehose Legacy (colon adenocarcinoma; COAD, rectal adenocarcinoma; READ, mucinous adenocarcinoma of colon and rectum; MAC, and CRC), Nature (COAD, READ, and CRC), and the PanCancer Atlas projects. (COAD, READ, and MAC), were used to conduct bioinformatics analysis of *ARID1A* mutations and the frequent genetic mutations of *ARID1A* in CRC. The bioinformatics analysis revealed that mutations of *ARID1A* were found in 105 of the 1482 CRC patients, accounting for 7.09% of all the altered genes identified in all the affected cases (Figure 26A). A somatic mutation in the *ARID1A* gene was found in 6.6% of all CRC samples. As well, a total of *ARID1A* mutations related to CRC were detected in 109 of the 1510 queried CRC samples, including 69 truncating (63.30%), 37 missense (33.94%), 2 inframe (1.83%), and 1 splice mutation (0.92%), along ARID1A/BRIGHT DNA binding domain and in the SWI/SNF-like complex subunit BAF250/Osa (Figure 26B).

Furthermore, the expression of mRNA and protein in the *ARID1A*-mutated and *ARID1A* non-mutated groups was investigated. The investigation through the cBioPortal for cancer genomics database demonstrated that the mRNA expression in the *ARID1A*-mutated group was not different to the *ARID1A* non-mutated group (Figure 26C). In contrast, the protein expression in the *ARID1A*-mutated group showed a tendency to be lower than in the *ARID1A* non-mutated group (Figure 26C).

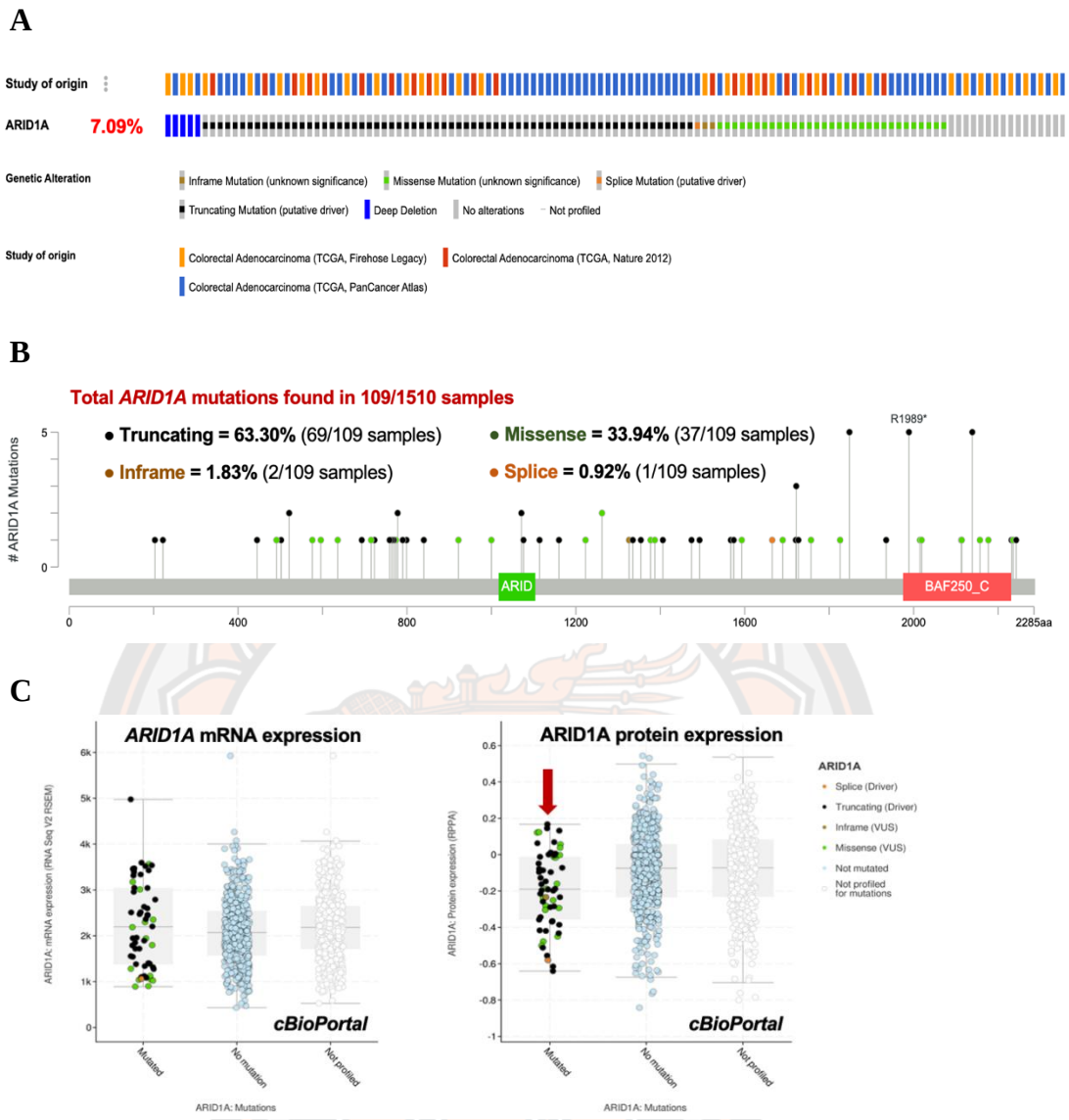


Figure 26 **Analysis of bioinformation of ARID1A mutations via cBioPortal (A) *ARID1A* mutations in CRC patients B) *ARID1A* mutations frequency and mapping C) mRNA and protein expression between *ARID1A*-mutated and non-mutated samples were shown. The red arrow indicates that the protein expression in the *ARID1A*-mutated group was lower than in the non-mutated group. The cBioPortal for cancer genomics database (https://www.cbioportal.org/) was used to analyze *ARID1A* mutations and the frequent genetic mutations of *ARID1A* in CRC)**

Clinicopathological characteristics of CRC patients

The clinicopathological characteristics of the patients with CRC (n=100) were shown in Table 7. The patients in this study belonged to the age group from 50 to 97 years old (median age, 66.0 years; mean age, 67.78±9.97 years). The demographics and medical condition of the patients included 46 males and 54 females. Fifty-three patients had a tumor that arose at the rectum or sigmoid colon. In addition, sixty-two patients had the largest tumors in the sample, with tumors larger than 4.50 cm (median dimension, 4.90 cm; average dimension, 5.22±2.07 cm). The pathological differentiation was graded as well-differentiated adenocarcinoma in 65 patients, moderately differentiated adenocarcinoma in 23 patients, and poorly differentiated adenocarcinoma in 12 patients. CRC staging was assessed using the TNM classification of the AJCC. Based on this classification scheme, 8 patients were at stage I, 22 patients at stage II, 36 at stage III, and 34 at stage IV. In particular, 88.00% of the patients had been diagnosed with CRC in the late stages of tumor invasion (pT3-pT4), whereas only 12.00% had been detected when they were in the early stages of tumor invasion (pT0-pT2). Moreover, in thirty-three patients, the CRC had metastasized to other organs such as the liver, peritoneum, and prostate gland, whereas for the remaining sixty-seven patients, CRC had not yet occurred. Additionally, seventy-five CRC patients presented with comorbidities, such as DM type II, hypertension, and dyslipidemia.

Furthermore, a greater number of positive lymph nodes (pN stage) and LNM were identified in 57 of the 100 patients with CRC, whereas the other 43 patients had not been identified. Also, 57.00% of the patients had been diagnosed with lymphovascular invasion. To evaluate the metastatic lymph node ratio (mLNR), the number of examined lymph nodes and positive lymph nodes were documented. The number of examined lymph nodes ranged from 2 to 70 nodes (median number of nodes, 16.00; the average number of nodes, 16.78±9.46) and the number of positive lymph nodes ranged from 0 to 24 nodes (median number of nodes, 1.00; the average number of nodes, 3.10±5.06). The mean value of mLNR was 0.21±0.31 and the median value was 0.05. According to the median value of mLNR, fifty patients with CRC had a high mLNR (≥0.05), whereas the other fifty patients had a low mLNR (<0.05).

Table 7 Clinicopathological characteristics in 100 patient samples of CRC

Clinicopathological characteristics	Value
Age (years)	
Age range (mean±S.D.)	50-97 (67.78±9.97)
Median of age	66.0
Gender (n (%))	
Male	46 (46.00)
Female	54 (54.00)
Location of tumor (n (%))	
Rectum/ sigmoid colon	53 (53.00)
Right-sided colon	36 (36.00)
Left-sided colon	11 (11.00)
The greatest dimension of tumor (cm)	
Size range (mean±S.D.)	1.8-12.5 (5.22±2.07)
Median of the greatest dimension of tumor	4.90
Pathological differentiation (n (%))	
Poor differentiation	12 (12.00)
Moderate differentiation	23 (23.00)
Well differentiation	65 (65.00)
AJCC CRC staging (n (%))	
Stage IV	34 (34.00)
Stage III	36 (36.00)
Stage II	22 (22.00)
Stage I	8 (8.00)
Depth of tumor invasion (pT stage) (n (%))	
Late stage (pT3-pT4)	88 (88.00)
Early stage (pT0-pT2)	12 (12.00)
Number of positive lymph nodes (pN stage) (n (%))	
1 node or more than 1 (positive) (pN1-pN2)	53 (53.00)
Not identified (negative) (pNX-pN0)	47 (47.00)

Table 7 Clinicopathological characteristics in 100 patient samples of CRC (Continue)

Clinicopathological characteristics	Value
Distant metastasis (pM stage) (n (%))	
Metastasized other organs (pM1)	33 (33.00)
Not identified (pM0)	67 (67.00)
Lymphovascular invasion (n (%))	
Presence	57 (57.00)
Absence	43 (43.00)
Lymph node metastasis (LNM) (n (%))	
Presence	53 (53.00)
Absence	47 (47.00)
Metastatic lymph node ratio (mLNR)	
Number of examined lymph nodes	
Range (mean±S.D.)	2-70 (16.78±9.46)
Median value	16.00
Number of positive lymph nodes	
Range (mean±S.D.)	0-24 (3.10±5.06)
Median value	1.00
Ratio range (mean±S.D.)	0.00-1.00 (0.21±0.31)
Median of mLNR	0.05
Comorbidities of patients (n (%))	
Presence	75 (75.00)
Absence or unknown	25 (25.00)

Abbreviation used: AJCC, American Joint Committee on Cancer; pT, tumor; pN, lymph node; pM, metastasis

Localization of ARID1A protein in normal large intestine tissues

The expression of ARID1A in normal large intestine tissues was investigated using the indirect method of IHC. The histopathological data demonstrated that ARID1A immunoreactivity was observed in various morphological structures of the normal large intestine (Figure 27A).

ARID1A is localized mainly in the nucleus of various cells in the normal large intestine. The nuclear ARID1A protein is localized mainly in the colonic epithelial cells that form intestinal glands or crypts of Lieberkühn. In the stroma, nuclear ARID1A protein expression was found in the intestinal immune cells such as granulocytes and lymphocytes, as well as the solitary lymphatic nodule in the lamina propria (Figure 27B–27C). Furthermore, ARID1A protein was expressed in the nuclei of the endothelial cells of blood vessels in the submucosal layer (Figure 27D), and in the nuclei of smooth muscle cells in both the inner circular (Figure 27E-27F) and outer longitudinal layers (Figure 27G) of the muscularis externa. The outermost layer of the large intestine, so called serosa or adventitia, was also found to express the ARID1A protein (Figure 27H).

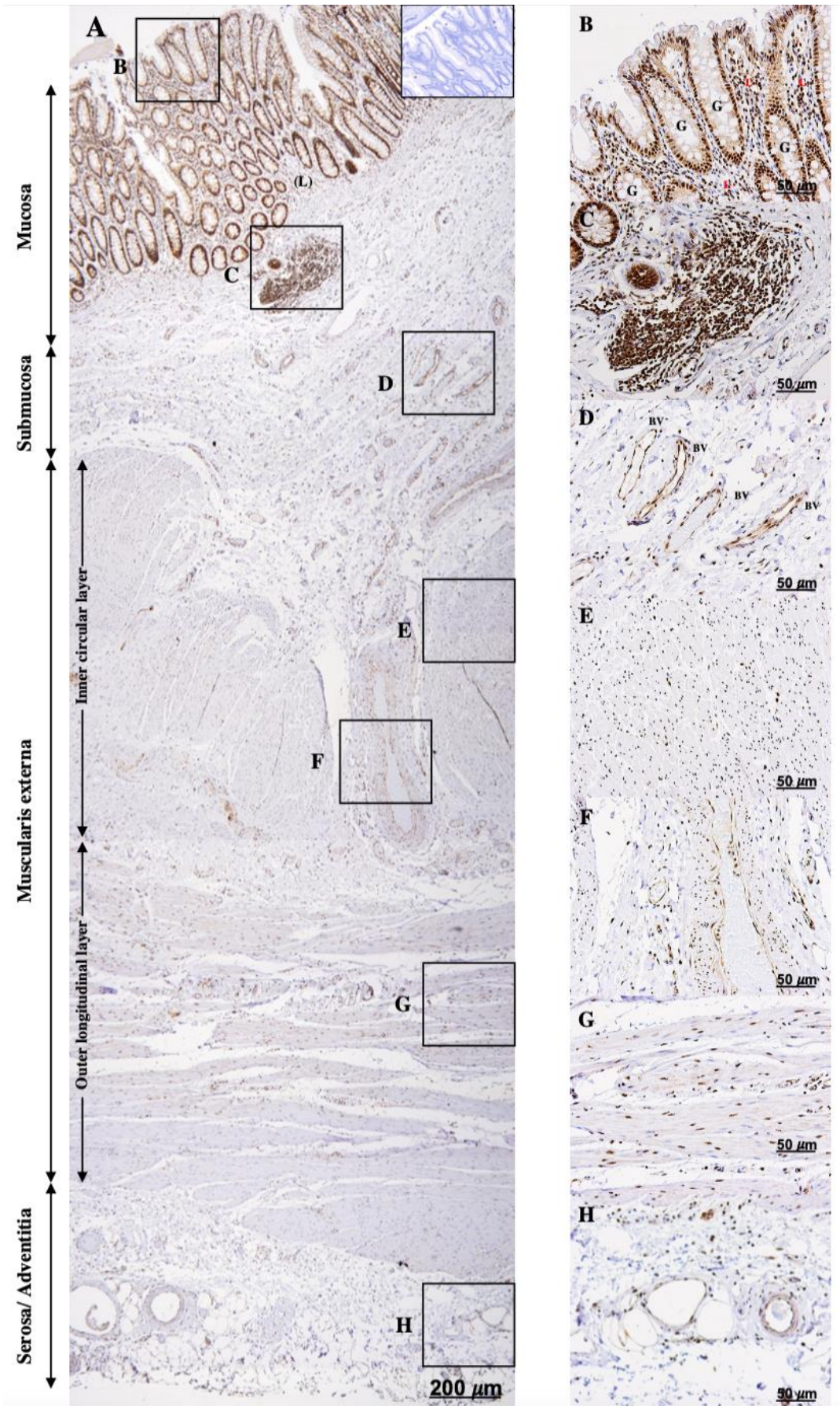

A
B
(L)
C
D
E
F
G
H
200 μm
Mucosa
Submucosa
Muscularis externa
Inner circular layer
Outer longitudinal layer
Serosa/ Adventitia
B
G
50 μm
C
D
BV
E
F
G
H

Figure 27 **ARID1A protein expression in normal large intestine tissues by IHC (A-H) The expression of nuclear ARID1A was observed in various structures of the normal large intestine tissues, including (B) intestinal gland at mucosal layer, (C) solitary lymphatic nodule, (D) blood vessel at submucosal layer, nuclei of smooth muscle cells in both inner circular (E-F) and outer longitudinal (G) layers of muscularis externa layer, and (H) serosa/adventitia layer, at low-power (50×)(A) and high-power (200×) (B-H) magnifications. Positive ARID1A staining is demonstrated in brown. The inset shows the negative control for ARID1A-IHC staining. Abbreviation used; BV, blood vessel; G, intestinal gland; L, lamina propria**

ARID1A immunoreactivity in cancerous *vs.* adjacent non-cancerous areas

An indirect method of IHC was conducted to investigate the ARID1A protein expression in CRC tissues in both cancerous and adjacent non-cancerous areas. In the adjacent non-cancerous area, ARID1A protein strongly appeared in the nuclei of intestinal epithelial cells. In contrast, decreased expression of ARID1A was noticeably observed in those cells in the cancerous area (Figure 28).

The expression of ARID1A protein was assessed and evaluated using a H-score. The H-score was evaluated from both the grading assessment of ARID1A stained intensity and the percentage of positive cells of ARID1A staining. The results showed that, in the adjacent non-cancerous areas, the intensity of staining in 61 samples was graded as strong, 34 samples as moderate, and 5 samples as weak staining. Negative staining was not observed in the adjacent non-cancerous area (Figure 29A). Meanwhile, the intensity of staining in the cancerous areas of 11 samples was graded as strong, 55 samples as moderate, 26 samples as weak, and 8 samples as negative staining (Figure 29A). In addition, the percentage of positively stained cells of ARID1A was significantly decreased in the cancerous area when compared with the adjacent non-cancerous area ($p<0.0001$) (Figure 29B). Similarly, the percentage of positively stained cells of ARID1A was significantly decreased in the cancerous area of all CRC pathological differentiation (Figure 29B). Consequently, the evaluation of the H-score revealed that the ARID1A H-score was significantly reduced in the cancerous area (mean value, 95.86 ± 5.57) compared to the adjacent non-cancerous area (mean value, 228.39 ± 5.44) ($p<0.0001$) (Figure 30A). Likewise, the ARID1A H-score was significantly decreased in the cancerous area of all CRC pathological differentiation (Figure 30A). Therefore, ARID1A protein expression was divided into a low (H-sore<150) or a high (H-score≥150) ARID1A expression group. The value of the H-score indicated that almost all the cancerous areas (84.00%) had low expression of ARID1A, whereas 16.00% remained high (Figure 30B). On the other hand, almost all the adjacent non-cancerous areas had high ARID1A expression (95.00%), although 5.00% exhibited a low expression of ARID1A (Figure 30B). Furthermore, the level of ARID1A protein was significantly decreased in the cancerous area when compared with the adjacent non-cancerous area ($p<0.0001$) (Figure 30). Similarly, the intensity of ARID1A protein expression was significantly decreased in the cancerous area of all CRC pathological differentiation (Figure 31).

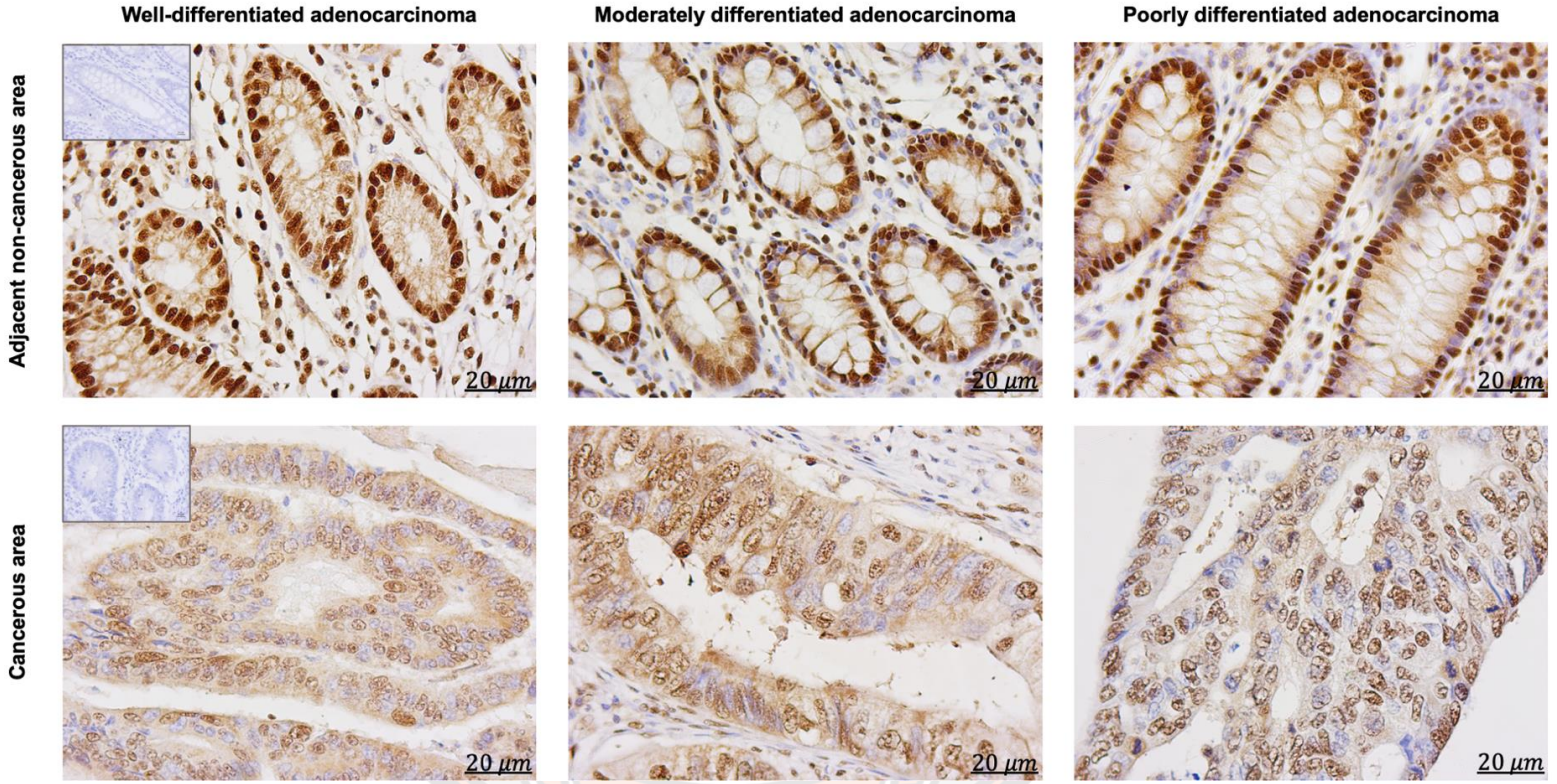


Figure 28 **Expression of ARID1A protein in CRC tissues**
(ARID1A IHC of the adjacent non-cancerous area (upper panel) in well, moderate, and poor differentiation of CRC, compared with the cancerous area (lower panel), respectively. Positive ARID1A nuclear staining is demonstrated in brown. The insets show the negative control for ARID1A-IHC staining. Original magnification power of 400× for all panels)

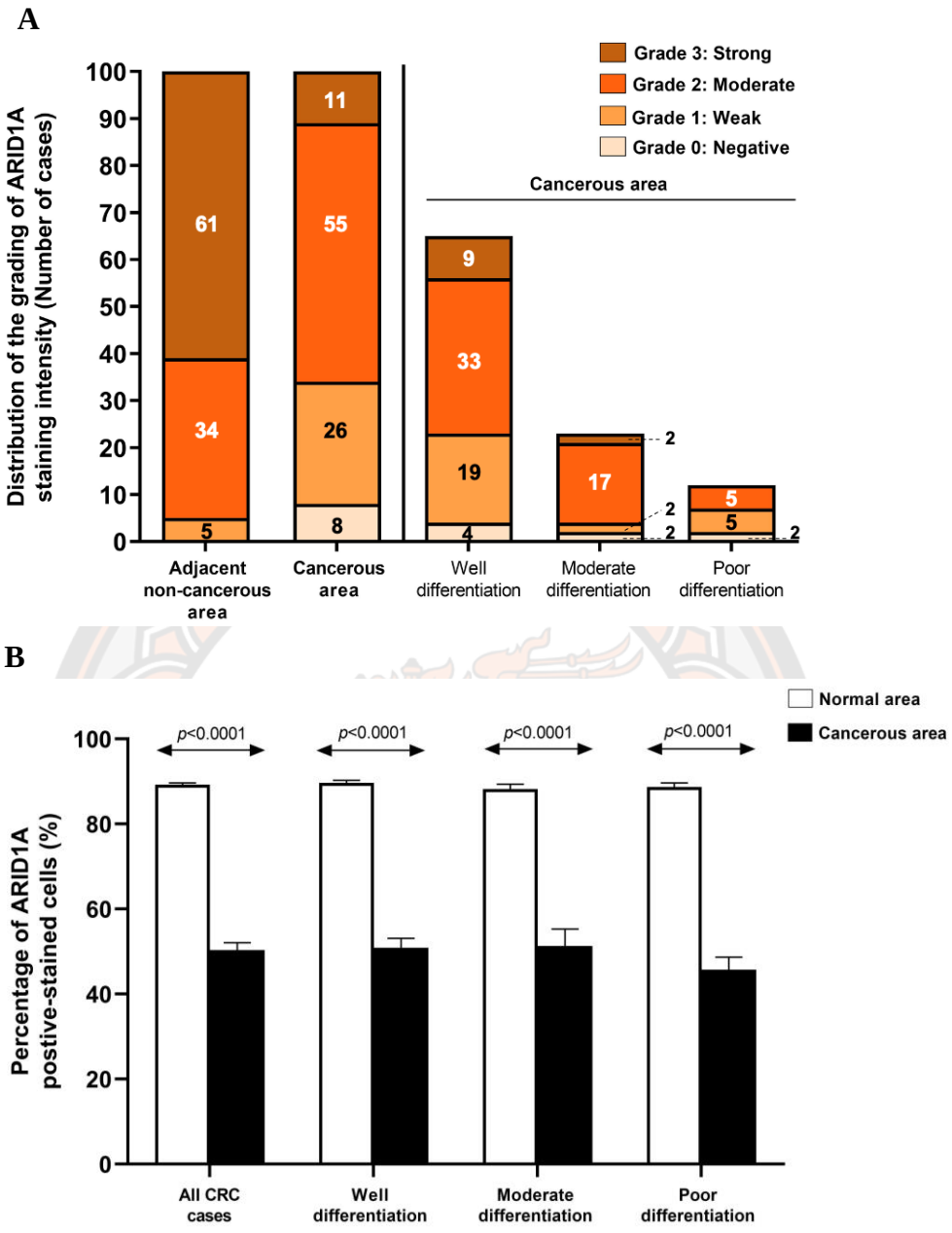


Figure 29 **Semi-quantitative analysis of the expression of ARID1A protein I (A) The distribution and number of cases with different gradings of ARID1A staining intensity in the adjacent non-cancerous and cancerous areas of CRC tissues B) The percentage of ARID1A positive-stained cells in the adjacent non-cancerous area (white bar) compared with the cancerous area (black bar) in each pathological differentiation of CRC. The quantitative data was presented as Mean±SEMs and analyzed by the Mann-Whitney U test)**

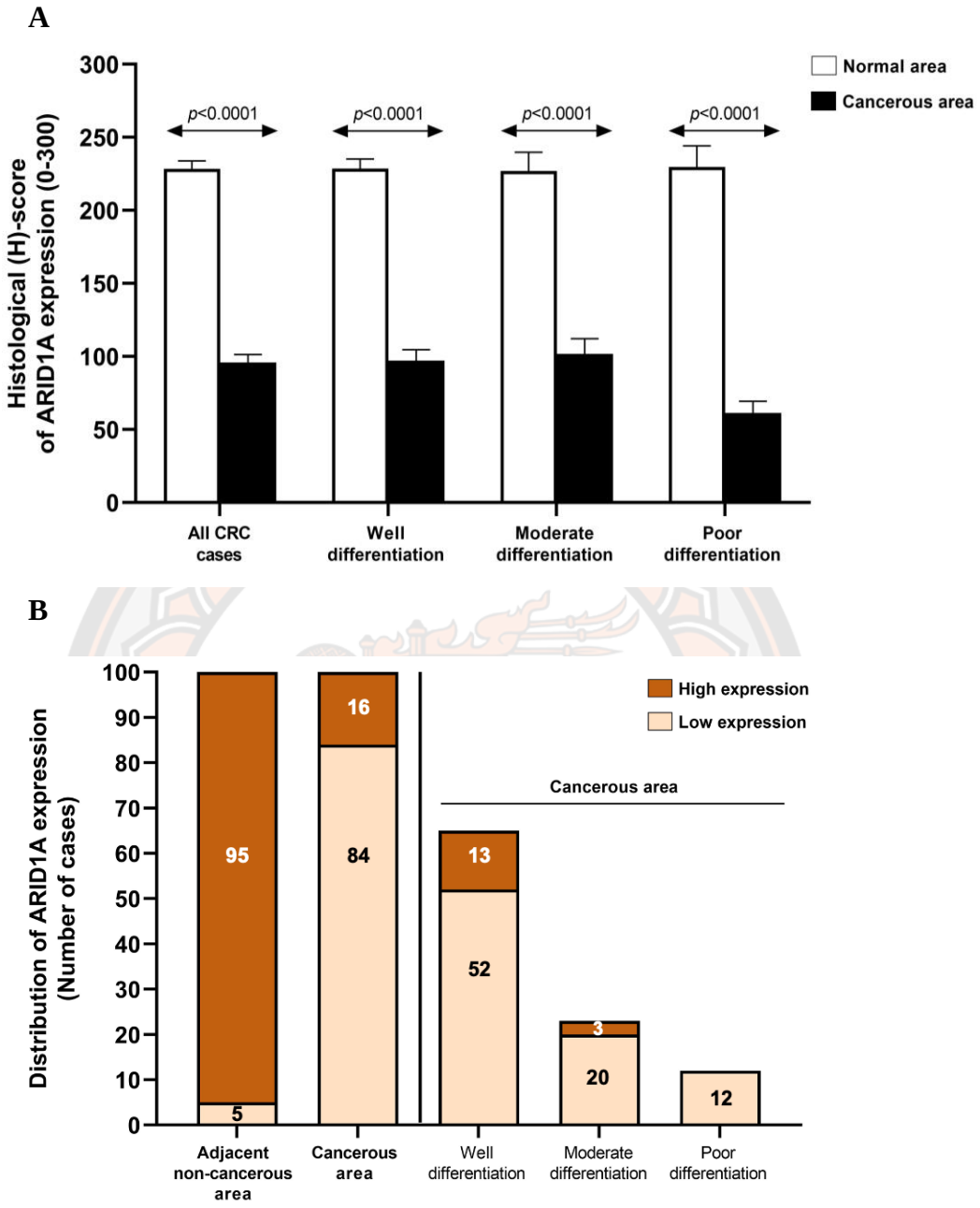


Figure 30 **Semi-quantitative analysis of the expression of ARID1A protein II (A) The H-score of the non-cancerous area (white bar) was compared to the cancerous area (black bar). B) The distribution of ARID1A expression, which is classified as low (light brown) or high (dark brown) expression based on the 50% cut-off value of H-score (150/300). The quantitative data was presented as Mean±SEMs and analyzed by the Mann-Whitney U test)**

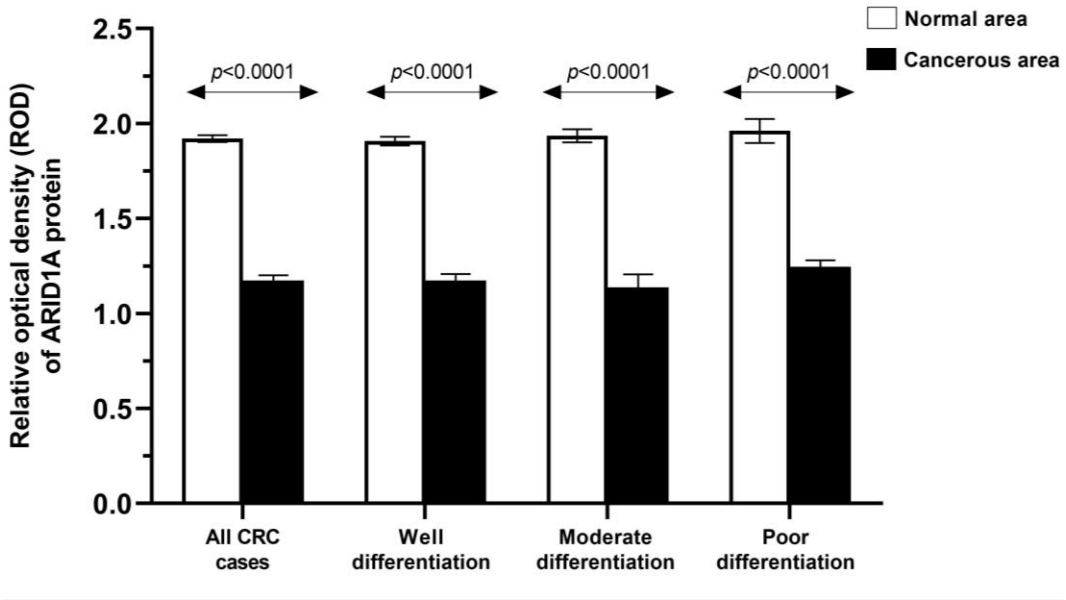


Figure 31 Quantitative analysis of the expression of ARID1A protein
(The relative optical density (ROD) of ARID1A protein was measured using ImageJ (Fiji) image analysis software and compared in the non-cancerous area (white bar) compared with the cancerous area (black bar). The quantitative data was presented as Mean±SEMs and analyzed by the Mann-Whitney U test)

The association of ARID1A protein expression with clinicopathology of CRC patients

The H-score of the cancerous areas was then applied to compare the clinicopathology of CRC patients. There were eighty-four cases with "low ARID1A expression" and sixteen cases with "high ARID1A expression". The association of ARID1A protein expression with the clinicopathological characteristics of 100 patients with CRC was demonstrated in Table 8.

The Fisher's exact analysis revealed that CRC patients with low ARID1A expression had a worse significant association with a greater number of positive lymph nodes (pN stage) (p=0.005), presence of lymphovascular invasion (p=0.006), LNM (p=0.005), a high ratio of metastatic lymph nodes (p=0.012), and presence of comorbidity, such as dyslipidemia, hypertension, and DM type II (p=0.010). Interestingly, the late stage of CRC was shown to possibly be associated with low ARID1A expression in the absence of explicit statistical significance (p=0.058). However, the other clinicopathological characteristics, including gender, elderly, tumor location, pathological differentiation, the greatest dimension of tumor, tumor invasion (pT stage), and distant metastasis (pM stage), were not associated with the ARID1A expression.

Impact of ARID1A expression on the progression-free survival of CRC patients

The Kaplan-Meier curve plotting and log-rank test analysis were conducted for analysis of the impact of ARID1A protein expression on 5-year PFS in patients with CRC. The analyses demonstrated that CRC patients with high ARID1A expression (62.50% of PFS rate) had a shorter PFS than those with low ARID1A expression (71.40% of PFS rate), although the log-rank test showed no significant difference between the two groups (p=0.531) (Table 9 and Figure 32).

Additionally, the univariate and multivariate analyses utilizing the Cox proportional hazards regression analysis were carried out to determine the relevance of prospective predictors of prognosis in the patients with CRC. Univariate analysis revealed that the late AJCC staging of CRC (p=0.021) and distant metastasis (p=0.006) were significantly correlated with a shorter PFS. However, low expression of ARID1A protein in CRC tissues was not correlated with a shorter PFS (p=0.543) (Table 10). A multivariate analysis was also performed that included all parameters having a p<0.05 in the univariate analysis and the ARID1A expression. However, a multivariate analysis revealed that all parameters were not associated with the short PFS of patients with CRC (Table 10).

Table 8 Association of ARID1A expression with clinicopathology of CRC patients (total n=100)

Clinicopathological characteristics	n (%)	ARID1A expression		*p*-value [a]
		Low expression [n (%)]	High expression [n (%)]	
Gender				**1.000**
Male	46 (46.00)	39 (39.00)	7 (7.00)	
Female	54 (54.00)	45 (45.00)	9 (9.00)	
Age				**0.739**
≥60 years old	79 (79.00)	67 (67.00)	12 (12.00)	
<60 years old	21 (21.00)	17 (17.00)	4 (4.00)	
Tumor location				**0.617**
Rectum/ Sigmoid colon	53 (53.00)	43 (43.00)	10 (10.00)	
Right side colon	36 (36.00)	32 (32.00)	4 (4.00)	
Left side colon	11 (11.00)	9 (9.00)	2 (2.00)	
Pathologic differentiation				**0.251**
Poor differentiation	12 (12.00)	12 (12.00)	0 (0.00)	
Moderate differentiation	23 (23.00)	20 (20.00)	3 (3.00)	
Well differentiation	65 (65.00)	52 (52.00)	13 (13.00)	
Tumor greatest dimension (cm)				**0.400**
≥4.50	62 (62.00)	54 (54.00)	8 (8.00)	
<4.50	38 (38.00)	30 (30.00)	8 (8.00)	
AJCC CRC stage				**0.058**
Stage IV	34 (34.00)	28 (28.00)	6 (6.00)	
Stage III	36 (36.00)	34 (34.00)	2 (2.00)	
Stage II	22 (22.00)	15 (15.00)	7 (7.00)	
Stage I	8 (8.00)	7 (7.00)	1 (1.00)	
pT stage				**1.000**
pT3 – pT4	88 (88.00)	74 (74.00)	14 (14.00)	
pT0 – pT2	12 (12.00)	10 (10.00)	2 (2.00)	

[a] *p*-value was analyzed using the Fisher's exact test.

* *p*-value <0.05 was considered to indicate statistical significance.

Table 8 Association of ARID1A expression with clinicopathology of CRC patients (total n=100) (continue)

Clinicopathological characteristics	n (%)	ARID1A expression		*p*-value [a]
		Low expression [n (%)]	High expression [n (%)]	
pN stage				**0.005***
pN1 – pN2	53 (53.00)	50 (50.00)	3 (3.00)	
pNX – pN0	47 (47.00)	34 (34.00)	13 (13.00)	
pM stage				**0.773**
pM1	33 (33.00)	27 (27.00)	6 (6.00)	
pM0	67 (67.00)	57 (57.00)	10 (10.00)	
Lymphovascular invasion				**0.006***
Present	57 (57.00)	53 (53.00)	4 (4.00)	
Not identified	43 (43.00)	31 (31.00)	12 (12.00)	
Lymph node metastasis (LNM)				**0.005***
Positive	53 (53.00)	50 (50.00)	3 (3.00)	
Negative	47 (47.00)	34 (34.00)	13 (13.00)	
Metastatic lymph node ratio (mLNR)				**0.012***
≥0.05	50 (50.00)	47 (47.00)	3 (3.00)	
<0.05	50 (50.00)	37 (37.00)	13 (13.00)	
Comorbidity				**0.010***
Presence	75 (75.00)	59 (59.00)	16 (16.00)	
Absence	25 (25.00)	25 (25.00)	0 (0.00)	

[a] *p*-value was analyzed using the Fisher's exact test.

* *p*-value <0.05 was considered to indicate statistical significance.

Abbreviation used: AJCC, American Joint Committee on Cancer; pT, tumor; pN, lymph node; pM, metastasis

Table 9 **The 5-year PFS of CRC patients with ARID1A protein expression**

ARID1A expression	Number of metastasis [event/total number (%)]	Progression-free survival (%)	*p*-value [b]
Low ARID1A	24/84 (28.57)	71.40	**0.531**
High ARID1A	6/16 (37.50)	62.50	
Overall	**30/100 (30.00)**	**70.00**	

[b] *p*-value was analyzed using the Log-Rank Test

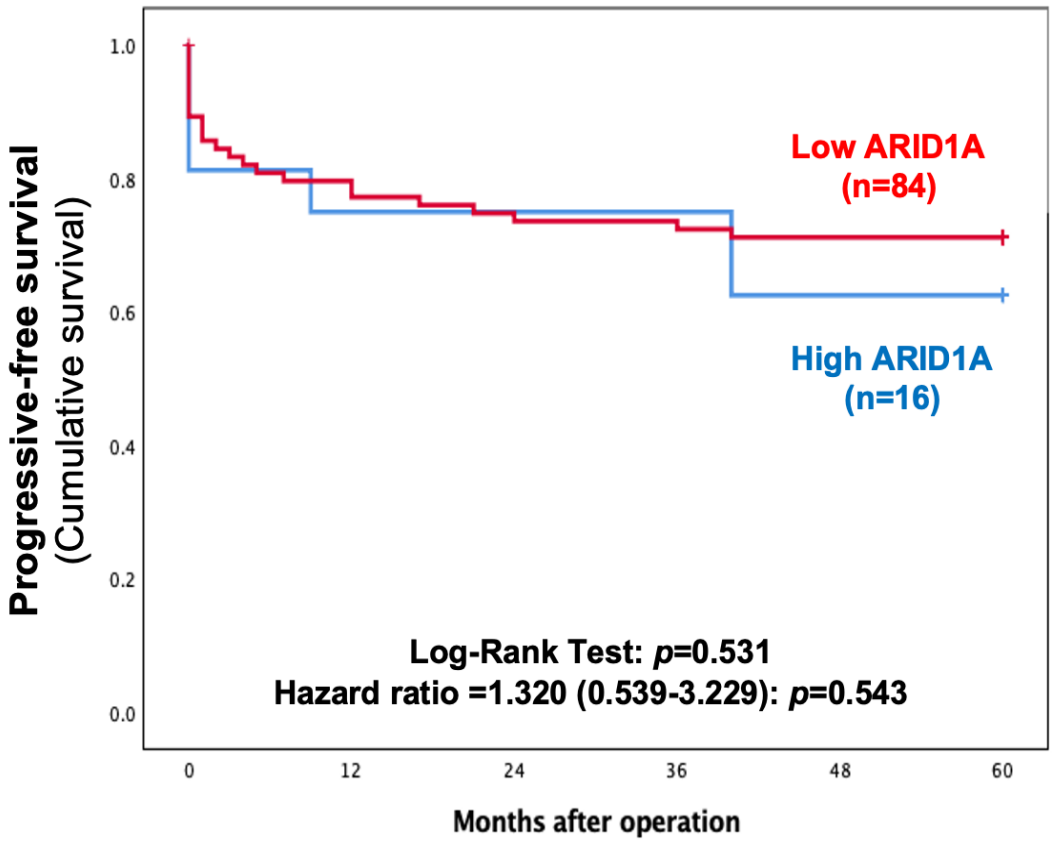


Figure 32 **The 5-year PFS of patients with CRC**

(The 5-year PFS of CRC patients with high ARID1A expression (blue line, n=16) demonstrated a tendency to have a worse prognosis than those with low ARID1A expression (red line, n=84). The Log-Rank Test showed no significant difference between groups (p=0.531, n=100))

Table 10 Univariate and multivariate analyses of clinicopathology in 100 patients with CRC using the Cox hazard regression analysis

Parameters	5-year Univariate analysis				5-year Multivariate analysis			
	HR	95% confidence intervals (CIs)		*p*-value [c]	HR	95% confidence intervals (CIs)		*p*-value [c]
		Lower	Upper			Lower	Upper	
ARID1A expression Low *vs* high ARID1A	1.320	0.539	3.229	**0.543**	0.997	0.404	2.459	**0.995**
Age (years old) ≥60 *vs* <60	1.655	0.758	3.615	**0.206**				
Gender Male *vs* female	1.265	0.609	2.627	**0.528**				
Tumor location Rectum/sigmoid *vs* right/left	0.848	0.412	1.746	**0.654**				
Tumor greatest dimension (cm) ≥4.50 *vs* <4.50	0.896	0.426	1.884	**0.772**				
Pathological differentiation Poor/Moderate *vs* well	1.260	0.577	2.752	**0.561**				
AJCC staging Late (Stage III-IV) *vs* early (Stage I-II) stage	0.025	0.001	0.568	**0.021***	1.002	0.000	8.791	**1.000**
Tumor invasion (pT stage) High (pT3-pT4) *vs* low (pT1-pT2)	0.462	0.110	1.941	**0.292**				
Positive lymph nodes (pN stage) Positive (pN1-pN2) *vs* negative (pN0)	0.700	0.337	1.454	**0.339**				

[c] *p*-value was analyzed using the Cox hazard regression analysis.

* *p*-value <0.05 was considered to indicate statistical significance.

Table 10 Univariate and multivariate analyses of clinicopathology in 100 patients with CRC using the Cox hazard regression analysis (continue)

Parameters	5-year Univariate analysis				5-year Multivariate analysis			
	HR	95% confidence intervals (CIs)		*p*-value [c]	HR	95% confidence intervals (CIs)		*p*-value [c]
		Lower	Upper			Lower	Upper	
Distant metastasis (pM stage) Presence (pM1) *vs* absence (pM0)	0.001	0.000	0.148	**0.006***	0.000	0.000	2.785	**0.847**
Lymphovascular invasion Presence *vs* absence	1.127	0.550	2.310	**0.744**				
Lymph node metastasis (LNM) Positive *vs* negative	0.700	0.337	1.454	**0.339**				
Metastatic lymph node ratio (mLNR) ≥0.05 *vs* <0.05	0.608	0.293	1.264	**0.183**				
Comorbidity Presence *vs* absence	1.142	0.508	2.565	**0.748**				

[c] *p*-value was analyzed using the Cox hazard regression analysis.

* *p*-value <0.05 was considered to indicate statistical significance.

Abbreviation used: AJCC, American Joint Committee on Cancer; pT, tumor; pN, lymph node; pM, metastasis

Expressions of EMT-related protein in cancerous *vs.* adjacent non-cancerous areas

CRC samples that had the ARID1A H-score of cancerous area less than 150, CRC patients who had distant metastasis, and positive for LNM were selected to investigate the alterations of EMT-related proteins using the indirect IHC method. Ten samples from each CRC pathological differentiation were taken for quantitative analysis using ImageJ (Fiji) image analysis software.

The IHC investigation demonstrated that expressions of epithelial proteins (E-cad and ZO-1) decreased, while expressions of mesenchymal proteins (vimentin and fibronectin) increased in the cancerous area compared to the adjacent non-cancerous area (Figure 33A-36A). As well, quantitative analysis showed that the means of the IHC intensity of the E-cad and of the ZO-1 proteins significantly decreased in the cancerous areas (0.09 ± 0.01, 0.05 ± 0.01) compared with those in the adjacent non-cancerous areas (0.28 ± 0.01, 0.19 ± 0.01) ($p<0.0001$) (Figure 33B and Figure 34B). In contrast, the means of the IHC intensity of vimentin and fibronectin proteins demonstrated a significant increase in the cancerous areas (0.25 ± 0.01, 0.25 ± 0.02) compared with those in the adjacent non-cancerous areas (0.07 ± 0.01, 0.04 ± 0.01), in all the pathological differentiations of CRC ($p<0.0001$) (Figure 35B and Figure 36B).

Based on the median IHC intensity in the cancerous area, individual EMT-related proteins were divided into low intensity, where the mean intensity was less than the median value, and high intensity, where the mean intensity was equal to or greater than the median value. The results indicated that the expression of E-cad protein was low in 19 of the 30 sample cases (66.30%), while 18 of the 30 cases (60.00%) had low ZO-1 protein expression. Also, expression of vimentin protein was high in 18 of the 30 cases (60.00%). Overall, 17 of the 30 sample cases (56.67%) had a high expression of fibronectin protein (Table 11). From these findings, the CRC samples were categorized into 4 groups: (i) low-epithelial proteins (both E-cad and ZO-1)/high-mesenchymal proteins (both vimentin and fibronectin) (6 patients, 20.00%), (ii) low-epithelial protein (E-cad or ZO-1)/high-mesenchymal protein (vimentin or fibronectin) (15 patients, 50.00%), (iii) either low-epithelial protein (E-cad and/or ZO-1) or high-mesenchymal proteins (vimentin and/or fibronectin) (7 patients, 23.33%), and (iv) high-epithelial proteins (both E-cad and ZO-1)/undetectable or low-mesenchymal proteins (both vimentin and fibronectin) (2 patients, 6.67%). The categorization of expressions of EMT-related protein was shown in Table 12.

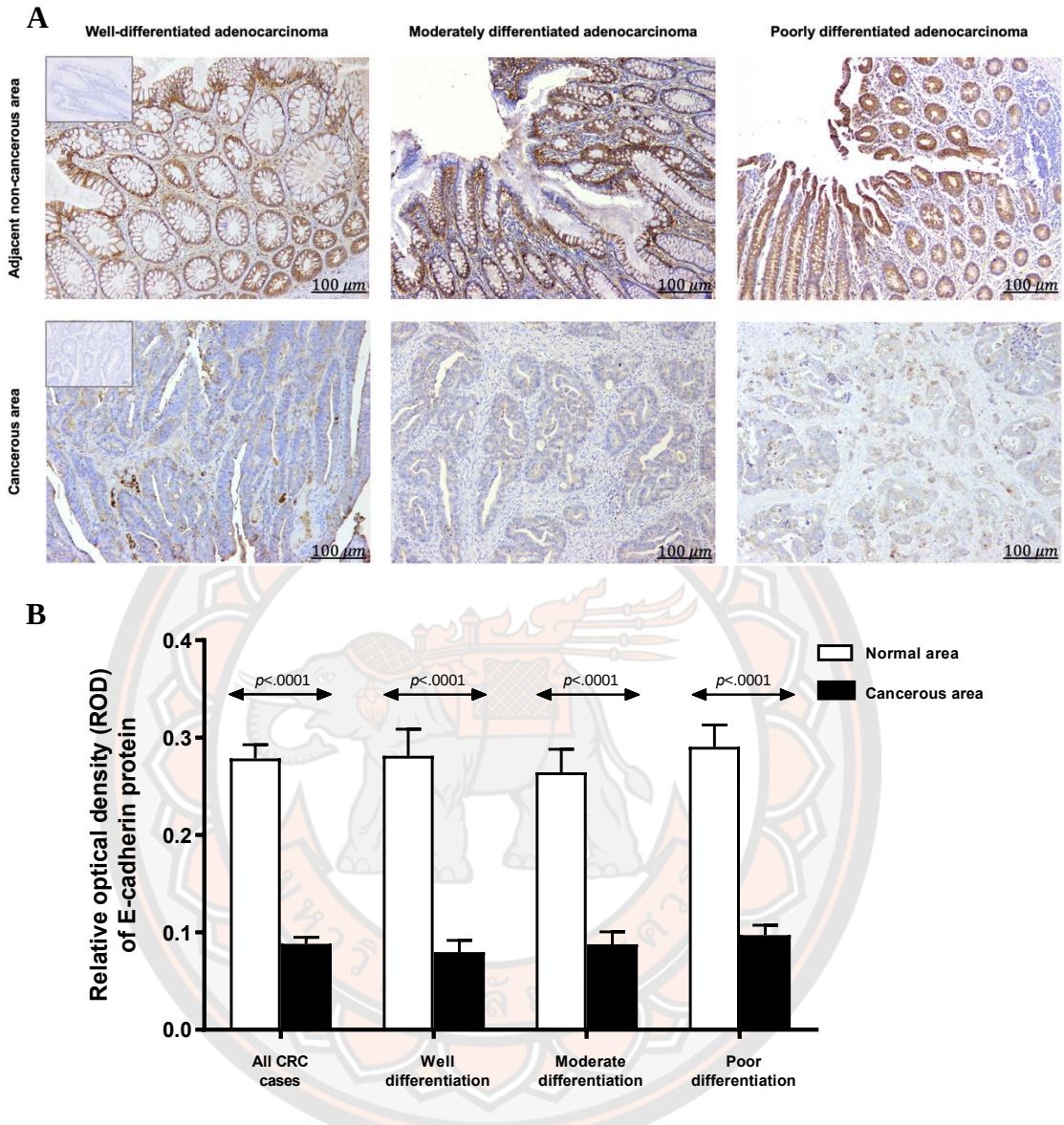


Figure 33 **Expression of E-cadherin protein in CRC tissues**

(A) E-cad IHC of the adjacent non-cancerous area (upper panel) in well, moderate, and poor differentiation of CRC, compared with the cancerous area (lower panel), respectively. The insets show the negative control for E-cad-IHC staining. Original magnification power of 100× for all panel B) The quantitative analysis of the IHC intensity of E-cad in the cancerous area (black bar) was significantly decreased compared to the adjacent non-cancerous area (white bar). The quantitative data was represented as Mean±SEMs and analyzed by the Mann-Whitney U test)

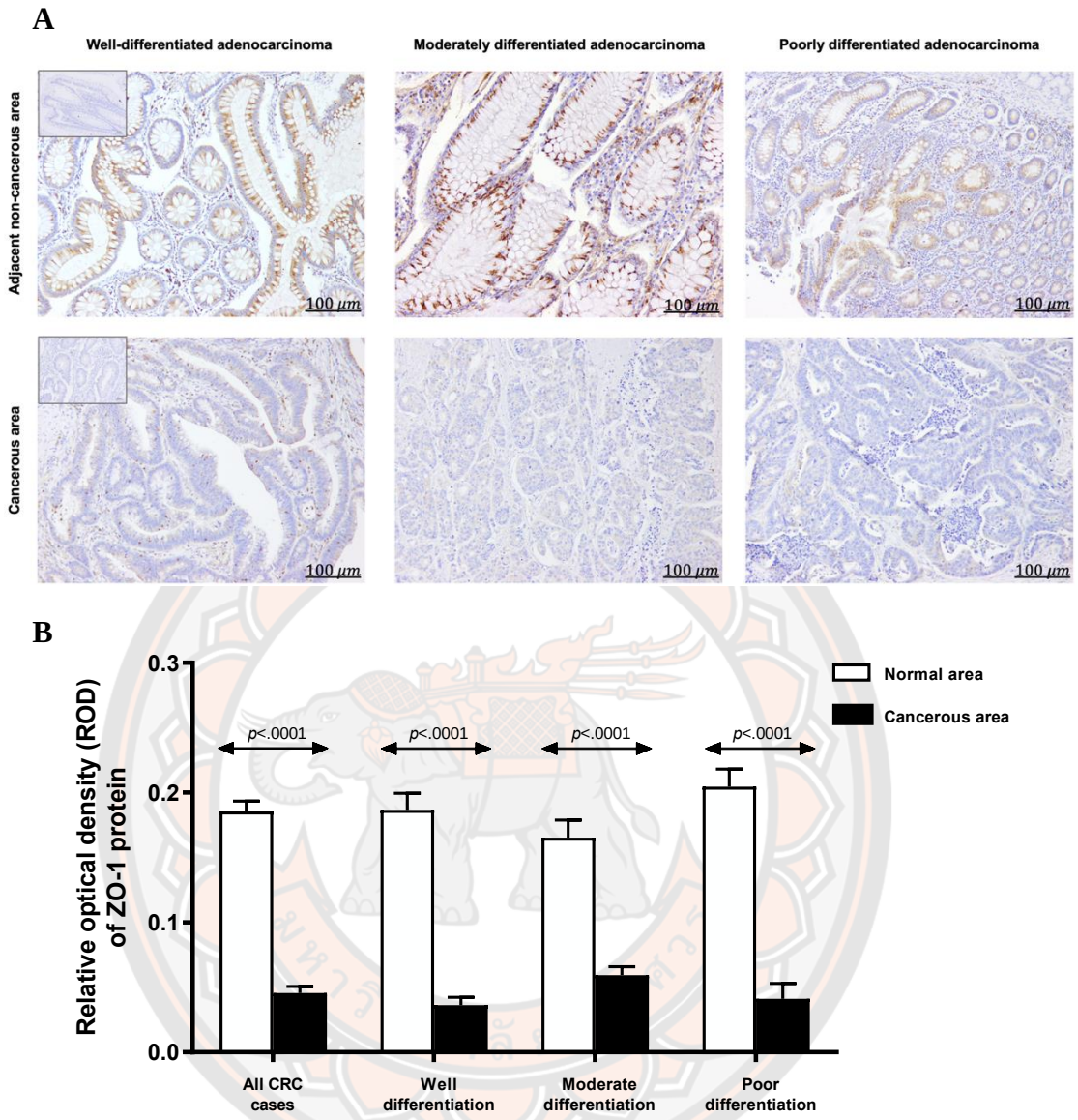


Figure 34 **Expression of zonula occludens-1 (ZO-1) protein in CRC tissues**

(A) ZO-1 IHC of the adjacent non-cancerous area (upper panel) in well, moderate, and poor differentiation of CRC, compared with the cancerous area (lower panel), respectively. The insets show the negative control for ZO-1-IHC staining. Original magnification power of 100× for all panel B) The quantitative analysis of the IHC intensity of ZO-1 in the cancerous area (black bar) was significantly decreased compared to the adjacent non-cancerous area (white bar). The quantitative data was represented as Mean±SEMs and analyzed by the Mann-Whitney U test)

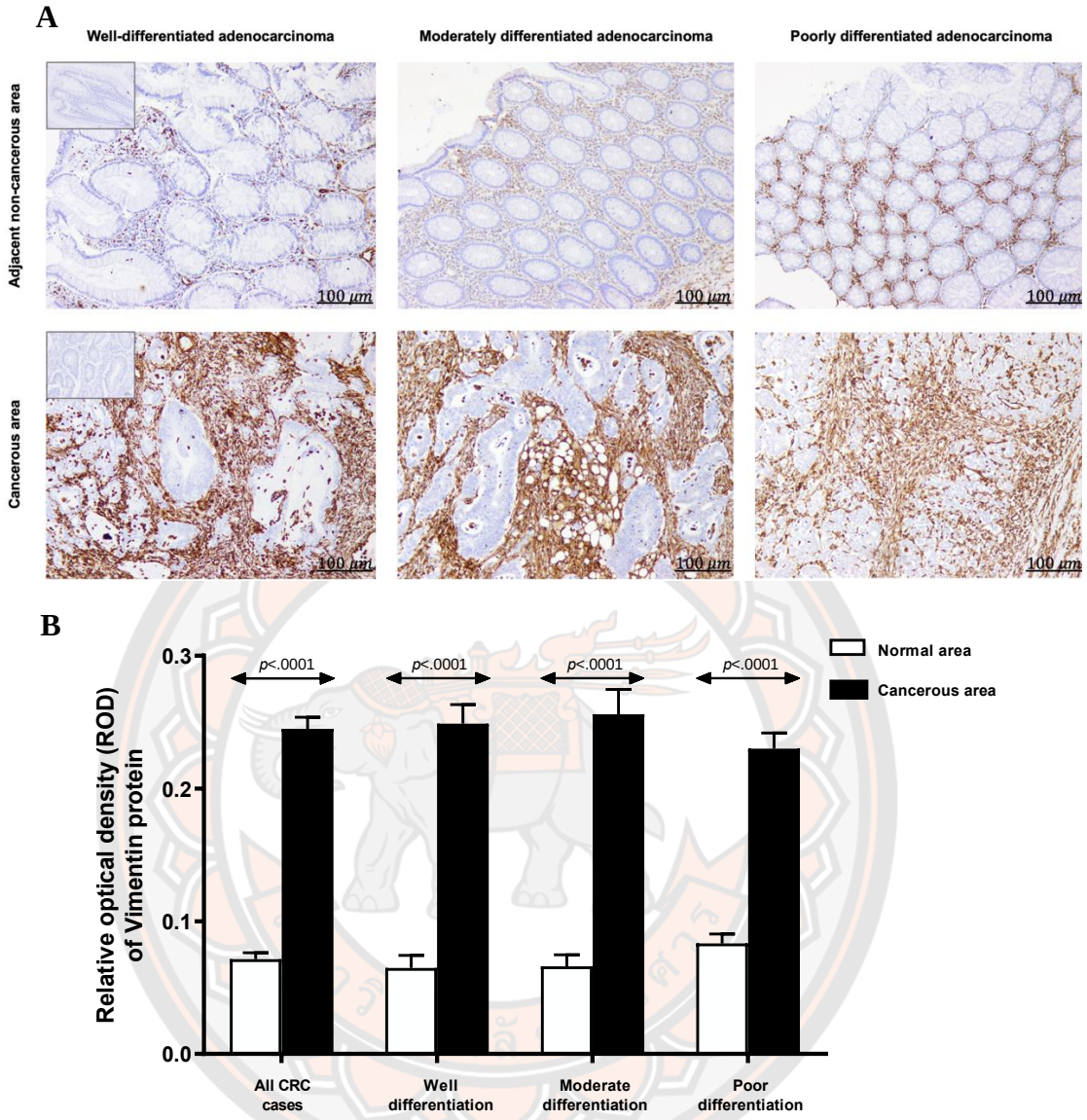


Figure 35 **Expression of vimentin protein in CRC tissues**

(A) Vimentin IHC of the adjacent non-cancerous area (upper panel) in well, moderate, and poor differentiation of CRC, compared with the cancerous area (lower panel), respectively. The insets show the negative control for vimentin-IHC staining. Original magnification power of 100× for all panel B) The quantitative analysis of the IHC intensity of vimentin in the cancerous area (black bar) was significantly increased compared to the adjacent non-cancerous area (white bar). The quantitative data was represented as Mean±SEMs and analyzed by the Mann-Whitney U test)

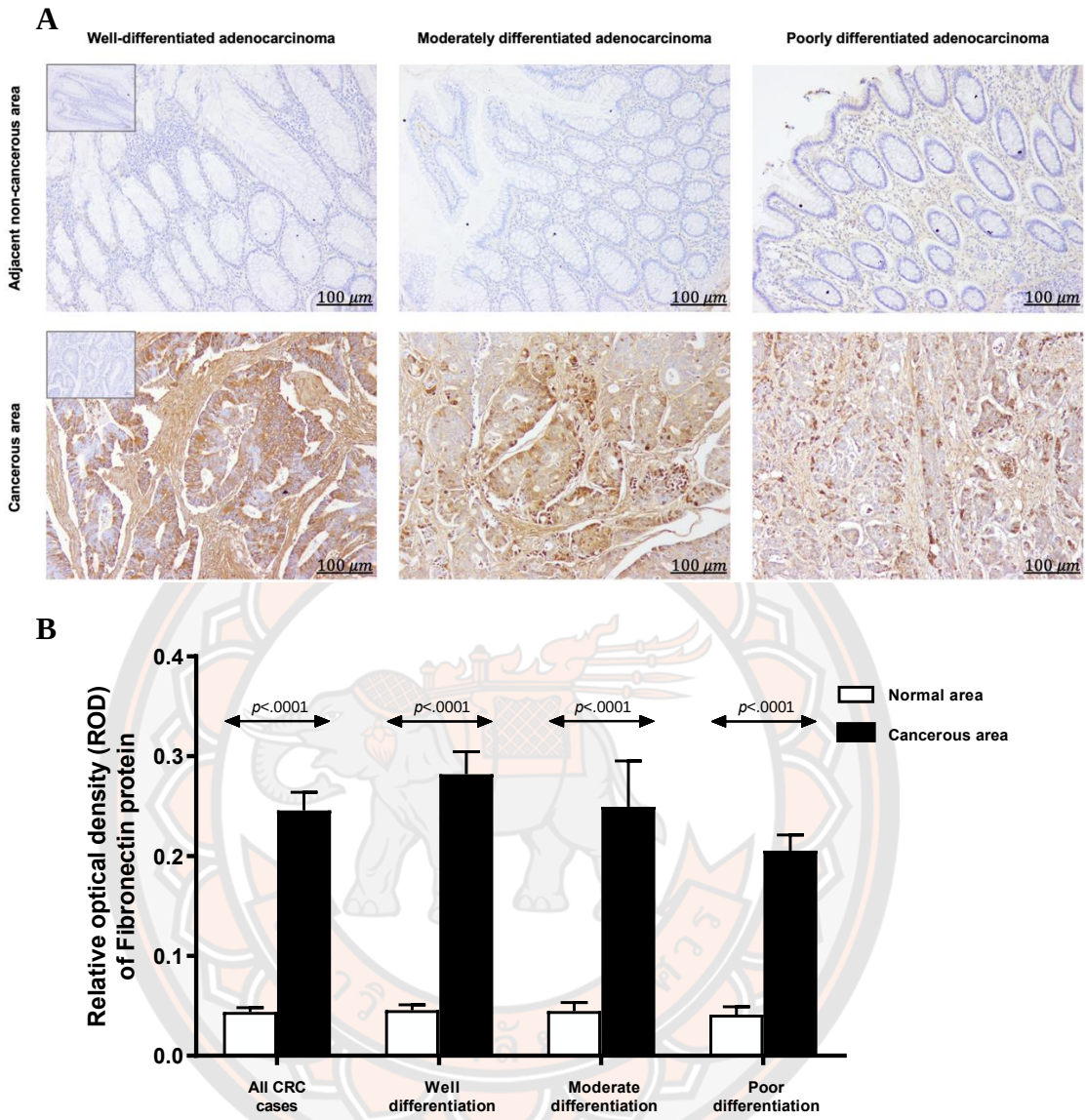


Figure 36 **Expression of fibronectin protein in CRC tissues**

(A) Fibronectin IHC of the adjacent non-cancerous area (upper panel) in well, moderate, and poor differentiation of CRC, compared with the cancerous area (lower panel), respectively. The insets show the negative control for fibronectin-IHC staining. Original magnification power of 100× for all panel B) The quantitative analysis of the IHC intensity of fibronectin in the cancerous area (black bar) was significantly increased compared to the adjacent non-cancerous area (white bar). The quantitative data was represented as Mean±SEMs and analyzed by the Mann-Whitney U test)

Table 11 Expressions of EMT-related protein in CRC tissues (n=30)

EMT-related protein	Median of intensity	Expressions of EMT-related protein	
		Low expression (n (%))	High expression (n (%))
Epithelial proteins			
E-cadherin (E-cad)	0.100	19 (63.33)	11 (36.67)
Zonula occludens-1 (ZO-1)	0.050	18 (60.00)	12 (40.00)
Mesenchymal proteins			
Vimentin	0.230	12 (40.00)	18 (60.00)
Fibronectin	0.220	13 (43.33)	17 (56.67)

Table 12 Categorization of the alterations of EMT-related protein expression in CRC tissues (n=30)

Alterations of EMT-related protein expression	Number of cases (n/total n (%))
Low-epithelial proteins (both E-cad and ZO-1)/High-mesenchymal proteins (both vimentin and fibronectin) (metastatic state)	6/30 (20.00)
Low-epithelial protein (E-cad or ZO-1)/High-mesenchymal protein (vimentin or fibronectin)	15/30 (50.00)
Either low-epithelial protein (E-cad and/or ZO-1) or high-mesenchymal proteins (vimentin and/or fibronectin)	7/30 (23.33)
High-epithelial proteins (both E-cad and ZO-1)/Undetectable or low-mesenchymal proteins (both vimentin and fibronectin) (normal state)	2/30 (6.67)

Abbreviation used: E-cad, E-cadherin; ZO-1, Zonula occludens-1

The association of low expression of ARID1A protein and alterations of EMT-related protein with clinicopathology of CRC patients

The association of low expression of ARID1A protein and alterations of EMT-related protein with the clinicopathological characteristics of 30 patients with CRC was illustrated in Table 13. The Fisher's exact analysis showed that patients with low ARID1A, decreased epithelial proteins (E-cad and ZO-1), and increased mesenchymal proteins (vimentin and fibronectin) expressions, had a worse significant association with a greater number of positive lymph nodes (pN stage) (p=0.030), the presence of LNM (p=0.030), and a high ratio of metastatic lymph nodes (p=0.019). However, the other parameters were not associated with the expressions of ARID1A and EMT-related protein.

Impact of low expression of ARID1A protein and alterations of EMT-related protein on the progression-free survival of CRC patients

The impact of low ARID1A protein and alterations of EMT-related protein expressions on 5-year PFS in patients with CRC was also analyzed using the Kaplan-Meier curve plotting and log-rank test analysis. The results demonstrated that CRC patients with low ARID1A, decreased epithelial proteins (E-cad and ZO-1), and increased mesenchymal proteins (vimentin and fibronectin) expressions had the worst prognosis among the other groups (16.70% of PFS rate). On the other hand, patients with low ARID1A without alteration of EMT-related protein had the best prognosis among the other groups (100.00% of PFS rate). However, the log-rank test showed no significant difference between groups (p=0.067) (Table 14 and Figure 37).

In addition, the relevance of the prospective predictors of prognosis in the patients was also examined using the Cox proportional hazards regression analysis. Univariate analysis revealed that low expression of ZO-1 (p=0.018), poor pathological differentiation (p=0.028), IV stage CRC (p=0.005), and distant metastasis (p=0.005) were significantly correlated with a shorter PFS **(**Table 15**)**. A multivariate analysis was also performed that included all parameters having a p<0.05 in the univariate analysis and EMT-related protein. Multivariate analysis showed that decreased expression of epithelial proteins (E-cad (p=0.030) and ZO-1 (p=0.033)), increased expression of vimentin (p=0.044), and IV stage CRC (p=0.001) were the independent prognostic factors related to CRC progression and then a shorter PFS (Table 15).

Table 13 Association of low expression of ARID1A protein and alterations of EMT-related protein with clinicopathology of CRC patients (total n=30)

Clinicopathological characteristics	n (%)	Low ARID1A protein and the alterations of EMT-related protein [n (%)]				*p*-value [d]
		↓↓ Epithelial with ↑↑ Mesenchymal	↓ Epithelial with ↑ Mesenchymal	↓ Epithelial or ↑ Mesenchymal	↑↑ Epithelial with ↓↓ Mesenchymal	
Gender						**0.475**
Male	16 (53.33)	5 (16.67)	7 (23.33)	3 (10.00)	1 (3.33)	
Female	14 (46.67)	1 (3.34)	8 (26.67)	4 (13.33)	1 (3.33)	
Age						**0.322**
≥60 years old	22 (73.33)	6 (20.00)	9 (29.99)	5 (16.67)	2 (6.67)	
<60 years old	8 (26.67)	0 (0.00)	6 (20.00)	2 (6.67)	0 (0.00)	
Tumor location						**0.852**
Rectum/ Sigmoid colon	17 (56.67)	3 (10.00)	8 (26.67)	5 (16.67)	1 (3.33)	
Right side colon	10 (33.33)	3 (10.00)	5 (16.67)	1 (3.33)	1 (3.33)	
Pathologic differentiation						**0.209**
Poor differentiation	10 (33.34)	2 (6.67)	3 (10.00)	4 (13.34)	1 (3.33)	
Moderate differentiation	10 (33.33)	1 (3.33)	5 (16.67)	3 (10.00)	1 (3.33)	
Well differentiation	10 (33.33)	3 (10.00)	7 (23.33)	0 (0.00)	0 (0.00)	

[d] *p*-value was analyzed using the Fisher's exact test. * *p*-value <0.05 was considered to indicate statistical significance.

Table 13 **Association of low expression of ARID1A protein and alterations of EMT-related protein with clinicopathology of CRC patients (total n=30) (continue)**

Clinicopathological characteristics	n (%)	Low ARID1A protein and the alterations of EMT-related protein [n (%)]				*p*-value [d]
		↓↓ Epithelial with ↑↑ Mesenchymal	↓ Epithelial with ↑ Mesenchymal	↓ Epithelial or ↑ Mesenchymal	↑↑ Epithelial with ↓↓ Mesenchymal	
Tumor greatest dimension (cm)						**0.244**
≥4.5	18 (60.00)	4 (13.33)	10 (33.33)	2 (6.67)	2 (6.67)	
<4.5	12 (40.00)	2 (6.67)	5 (16.67)	5 (16.66)	0 (0.00)	
AJCC CRC stage						**0.790**
Stage IV	19 (63.33)	3 (10.00)	10 (33.33)	4 (13.33)	2 (6.67)	
Stage III	11 (36.67)	3 (10.00)	5 (16.67)	3 (10.00)	0 (0.00)	
pT stage						**0.713**
pT4	7 (23.33)	1 (3.34)	4 (13.33)	1 (3.33)	1 (3.33)	
pT3	23 (76.67)	5 (16.67)	11 (36.67)	6 (20.00)	1 (3.33)	
pN stage						**0.030***
pN1 – pN2	28 (93.33)	5 (16.67)	15 (50.00)	7 (23.33)	1 (3.33)	
pNX – pN0	2 (6.67)	1 (3.34)	0 (0.00)	0 (0.00)	1 (3.33)	

[d] *p*-value was analyzed using the Fisher's exact test. * *p*-value <0.05 was considered to indicate statistical significance.

Table 13 **Association of low expression of ARID1A protein and alterations of EMT-related protein with clinicopathology of CRC patients (total n=30) (continue)**

Clinicopathological characteristics	n (%)	Low ARID1A protein and the alterations of EMT-related protein [n (%)]				*p*-value [d]
		↓↓ Epithelial with ↑↑ Mesenchymal	↓ Epithelial with ↑ Mesenchymal	↓ Epithelial or ↑ Mesenchymal	↑↑ Epithelial with ↓↓ Mesenchymal	
pM stage						**0.790**
pM1	19 (63.33)	3 (10.00)	10 (33.33)	4 (13.33)	2 (6.67)	
pM0	11 (36.67)	3 (10.00)	5 (16.67)	3 (10.00)	0 (0.00)	
Lymphovascular invasion						**0.916**
Present	23 (76.67)	4 (13.33)	12 (40.00)	5 (16.67)	2 (6.67)	
Not identified	7 (23.33)	2 (6.67)	3 (10.00)	2 (6.66)	0 (0.00)	
Lymph node metastasis (LNM)						**0.030***
Positive	28 (93.33)	5 (16.67)	15 (50.00)	7 (23.33)	1 (3.33)	
Negative	2 (6.67)	1 (3.34)	0 (0.00)	0 (0.00)	1 (3.33)	
Metastatic lymph node ratio (mLNR)						**0.019***
≥0.05	27 (90.00)	4 (13.33)	15 (50.00)	7 (23.33)	1 (3.33)	
<0.05	3 (10.00)	2 (6.67)	0 (0.00)	0 (0.00)	1 (3.33)	

[d] *p*-value was analyzed using the Fisher's exact test. * *p*-value <0.05 was considered to indicate statistical significance.

Table 13 Association of low expression of ARID1A protein and alterations of EMT-related protein with clinicopathology of CRC patients (total n=30) (continue)

Clinicopathological characteristics	n (%)	Low ARID1A protein and the alterations of EMT-related protein [n (%)]				*p*-value [d]
		↓↓ Epithelial with ↑↑ Mesenchymal	↓ Epithelial with ↑ Mesenchymal	↓ Epithelial or ↑ Mesenchymal	↑↑ Epithelial with ↓↓ Mesenchymal	
Comorbidity						**0.416**
Presence	20 (66.67)	4 (13.33)	8 (26.67)	6 (20.00)	2 (6.67)	
Absence	10 (33.33)	2 (6.67)	7 (23.33)	1 (3.33)	0 (0.00)	

[d] *p*-value was analyzed using the Fisher's exact test.

* *p*-value <0.05 was considered to indicate statistical significance.

Abbreviation used: AJCC, American Joint Committee on Cancer; pT, tumor; pN, lymph node; pM, metastasis

Table 14 **The 5-year PFS of CRC patients with low expression of ARID1A protein and alterations of EMT-related protein**

Low ARID1A and EMT expression	Number of metastasis [event/total number (%)]	Progression-free survival (%)	*p*-value [e]
↓↓ Epithelial with ↑↑ Mesenchymal	5/6 (83.33)	16.70	**0.067**
↓ Epithelial with ↑ Mesenchymal	10/15 (66.67)	33.30	
↓ Epithelial or ↑ Mesenchymal	4/7 (57.14)	42.90	
↑↑ Epithelial with ↓↓ Mesenchymal	0/2 (0.00)	100.00	
Overall	**19/30 (63.33)**	**36.70**	

[e] *p*-value was analyzed using the Log-Rank Test

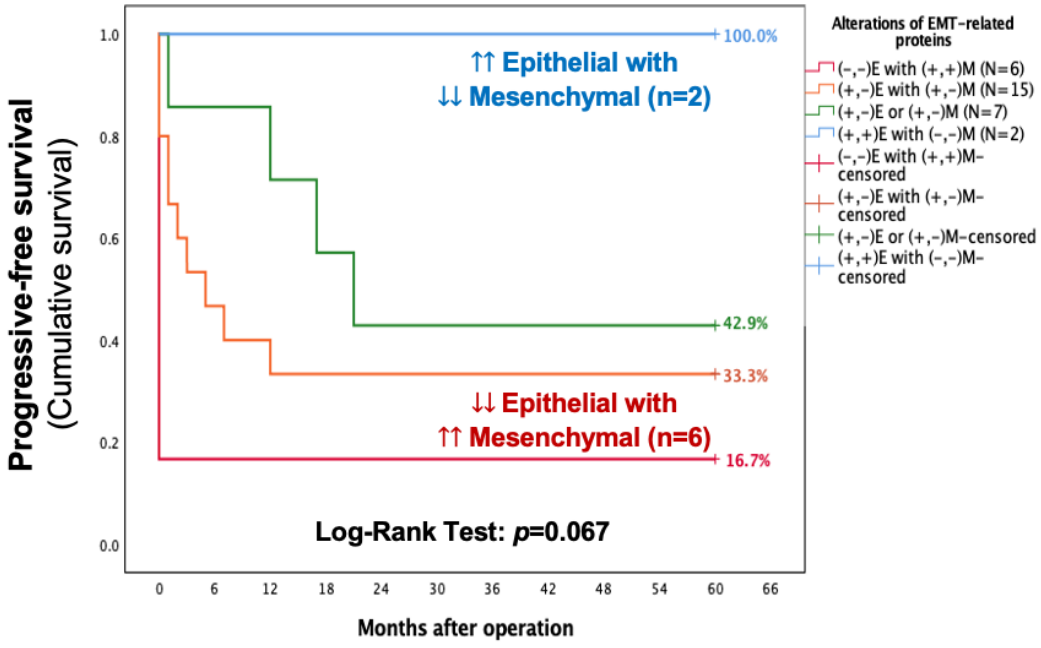


Figure 37 **The 5-year PFS of CRC patients with low expression of ARID1A protein and alterations of EMT-related protein**

(The 5-year PFS of CRC patients with the expression of low ARID1A, decreased epithelial markers, and increased mesenchymal markers (red line, n=6) demonstrated the worst prognosis among the other groups. The Log-Rank Test showed no significant difference between groups (p=0.067, n=30))

Table 15 **Univariate and multivariate analysis of clinicopathology in 30 patients with CRC using the Cox hazard regression analysis**

Parameters	5-year Univariate analysis				5-year Multivariate analysis			
	HR	95% confidence intervals (CIs)		*p*-value [f]	HR	95% confidence intervals (CIs)		*p*-value [f]
		Lower	Upper			Lower	Upper	
E-cadherin expression Low *vs* High	0.461	0.165	1.287	**0.139**	0.274	0.085	0.881	**0.030***
Zonula occludens-1 (ZO-1) expression Low *vs* High	0.258	0.084	0.791	**0.018***	0.242	0.066	0.894	**0.033***
Vimentin expression High *vs* Low	0.600	0.234	1.541	**0.288**	0.208	0.045	0.958	**0.044***
Fibronectin expression High *vs* Low	0.390	0.146	1.042	**0.060**	0.323	0.075	1.391	**0.129**
Age (years old) ≥ 60 *vs* < 60	1.247	0.471	3.297	**0.657**				
Gender Male *vs* Female	0.830	0.335	2.054	**0.686**				
Tumor location Rectum/sigmoid *vs* Right/left	1.423	0.576	3.515	**0.445**				
Tumor greatest dimension (cm) ≥ 4.5 *vs* < 4.5	0.730	0.286	1.864	**0.511**				
Pathological differentiation Poor/Moderate *vs* Well	2.858	1.123	7.274	**0.028***	0.556	0.150	2.058	**0.380**
AJCC staging IV *vs* III stage	0.121	0.027	0.536	**0.005***	0.032	0.004	0.252	**0.001***

[f] *p*-value was analyzed using the Cox hazard regression analysis.

* *p*-value <0.05 was considered to indicate statistical significance.

Table 15 **Univariate and multivariate analysis of clinicopathology in 30 patients with CRC using the Cox hazard regression analysis (continue)**

Parameters	5-year Univariate analysis				5-year Multivariate analysis			
	HR	95% confidence intervals (CIs)		*p*-value [f]	HR	95% confidence intervals (CIs)		*p*-value [f]
		Lower	Upper			Lower	Upper	
Depth of tumor invasion (pT stage) pT4 *vs* pT3	0.541	0.204	1.438	**0.218**				
Positive lymph nodes (pN stage) Positive (pN1 – pN2) *vs* Negative (pN0)	0.829	0.111	6.220	**0.855**				
Distant metastasis (pM stage) Presence (pM1) *vs* Absence (pM0)	0.121	0.027	0.536	**0.005***	N/A	N/A	N/A	**N/A**
Lymphovascular invasion Presence *vs* Absence	1.817	0.686	4.813	**0.229**				
Lymph node metastasis (LNM) Positive *vs* Negative	0.829	0.111	6.220	**0.855**				
Metastatic lymph node ratio (mLNR) ≥0.05 *vs* <0.05	1.418	0.325	6.177	**0.642**				
Comorbidity Presence *vs* Absence	1.528	0.597	3.912	**0.377**				

[f] *p*-value was analyzed using the Cox hazard regression analysis.

* *p*-value <0.05 was considered to indicate statistical significance.

Abbreviation used: AJCC, American Joint Committee on Cancer; pT, tumor; pN, lymph node; pM, metastasis; N/A, not applicable

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

CRC is one of the most common leading causes of cancer-related death, which is the second most common cancer diagnosed in women and the third most common incidence in men worldwide (Mármol et al., 2017; Sung et al., 2021). Currently, the worldwide burden of cancer prevalence and mortality rate from CRC have been rapidly increasing (Arnold et al., 2017; Bray et al., 2018). Depending on the extent of the localized and particularly metastatic tumor, CRC has a relatively poor prognosis. Patients with metastatic CRC had a shorter 5-year relative survival rate compared to patients with locally advanced CRC (Bendardaf et al., 2005).

In the present study, we aimed to investigate the prognostic significance of ARID1A in Thai CRC tissues. *ARID1A* is a critical component of the SWI/SNF chromatin remodeling complexes that has been identified as a novel tumor suppressor gene involved in cell cycle regulation, apoptosis promotion, and genomic instability inhibition (Wang et al., 2004; Wu et al., 2014). However, the *ARID1A* gene is the most frequently mutated subunit of the SWI/SNF chromatin remodeling complexes. *ARID1A* mutations have been found in a variety of cancer types (Wu et al., 2014), including ovarian clear cell carcinoma (46.22%) (Wiegand et al., 2010), endometrial carcinoma (40.00%) (Guan et al., 2011), gastric carcinoma (29.36%) (Wang et al., 2011), cholangiocarcinoma (15.31%) (Chan-On et al., 2013), and urothelial carcinoma of the bladder (15.15%) (Guo et al., 2013). Recently, Zhao and colleagues reported that *ARID1A* mutations have been found in 3.60-66.70% of CRC (Zhao et al., 2022). In this present study, the cBioPortal for cancer genomics database revealed that *ARID1A* mutations were found in 7.09% of CRC. A total of *ARID1A* mutations related to CRC were detected in 109 of 1510 queried samples, including truncating (63.30%), missense (33.94%), inframe (1.83%), and splice mutations (0.92%). These findings are consistent with several studies that have also reported that *ARID1A* mutations occur across the length of the gene, including truncating or frameshift (insertions and deletions) mutations

(Cancer Genome Atlas Network, 2012; Jones et al., 2012; Namjan et al., 2020). Mutations of *ARID1A* have been found to have a prognostic role, as loss of ARID1A shortens the time to cancer-specific mortality and cancer recurrence (Luchini et al., 2015; Mathur, 2018). Importantly, the *ARID1A* mutations were found to be significantly related to ARID1A protein expression loss or reduction (Guan et al., 2011; Wiegand et al., 2010). Our finding was consistent with those in previous studies demonstrating that the protein expression in the *ARID1A*-mutated group showed a tendency to be lower than in the *ARID1A* non-mutated group. Moreover, the genetic alterations, such as MMR deficiency and MSI, as well as the epigenetic alterations, such as promoter hypermethylation at the CpG island, are involved in ARID1A expression being lost or decreased in human CRC tissues and cell lines (Chou et al., 2014; Erfani et al., 2020). Therefore, our findings indicate that *ARID1A* mutations are involved in CRC carcinogenesis and affect the expression of ARID1A protein.

Decreasing ARID1A expression is associated with the PI3K/Akt signaling pathway activation in endometriosis and endometriosis-associated ovarian carcinomas and nasopharyngeal carcinoma (Samartzis et al., 2013; Yang et al., 2019). Multiple human malignancies have been found to have an interaction between ARID1A and the PI3K/Akt pathway (Sun et al., 2021). Through up-regulation of the Akt pathway, Xie et al. demonstrated that down-regulation of *ARID1A* might promote cell proliferation, increase chemoresistance, and prevent cell apoptosis in the SW620 CRC cell line, whereas the over-expressed *ARID1A* exhibits a reduction in cell proliferation (Xie et al., 2014). *ARID1A* deletion increased chromatin occupancy and decreased metastasis suppressors in liver cancer (Sun et al., 2017). Additionally, the Wnt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathways were down-regulated by *ARID1A* variations in colorectal adenocarcinoma. Furthermore, *ARID1A* is frequently co-mutated with some essential genes that are involved in CRC tumorigenesis (Zhao et al., 2022), such as *TP53* (Stein et al., 2020), *KRAS* (Fountzilas et al., 2018), and *APC* (Sen et al., 2019). Due to these findings, *ARID1A* may be an important gene that is involved in tumor initiation and progression of CRC.

Loss or reduction of the ARID1A protein expression has been increasingly found in various types of human malignancies, especially malignancies in the GI tract (Wang et al., 2021), such as gastric carcinoma (Abe et al., 2012; Inada et al., 2015), CRC (Erfani et

al., 2020; Kishida et al., 2019; Lee et al., 2016; Wei et al., 2014; Ye et al., 2014), hepatocellular carcinoma (He et al., 2015), cholangiocarcinoma (Namjan et al., 2020), and other gastrointestinal cancers. Our IHC findings demonstrated that ARID1A protein is strongly expressed in the nuclei of intestinal epithelial cells in the adjacent non-cancerous area. In contrast, decreased expression of ARID1A was noticeably observed in those cells in the cancerous area. The presence of nuclear ARID1A staining was then considered as positive immunostaining (Kishida et al., 2019; Wei et al., 2014). In our study, we found that the immunoreactivity signal of the ARID1A protein decreased in most of the cancerous areas of CRC samples (84.0%), whereas 16.0% remained high. Consistently, the semi-quantitative analysis demonstrated that ARID1A protein expression was significantly decreased in most cancerous areas of CRC samples compared to the adjacent non-cancerous areas in all pathological differentiation of CRC. Consistent with the recent findings, negative or decreasing ARID1A was found in 5.9% (Chou et al., 2014), 25.8% (Wei et al., 2014), 30.2% (Xie et al., 2014), and 66.5% (Erfani et al., 2020) of primary colorectal carcinomas, respectively. Our IHC results indicate that decreased ARID1A protein expression is commonly found in CRC tissues. Guan et al., demonstrated that signaling of ARID1A nuclear export was interrupted by in-frame insertions or deletions (indel) mutations. As a consequence, these *ARID1A* mutations may have an influence on the stability of ARID1A protein expression (Guan et al., 2012). According to Erfani et al., loss of ARID1A protein expression is involved in the oncogenic transformation of CRC (Erfani et al., 2020). Our results indicate that loss or reduction of ARID1A expression may play an essential role in promoting CRC tumorigenesis and progression.

Importantly, the alterations of ARID1A protein expression have been associated with the severity of clinicopathological characteristics and a poor prognosis of patients (Wu et al., 2014). Our results demonstrated that low expression of ARID1A protein in CRC was significantly associated with a greater number of positive lymph nodes, lymphovascular invasion, LNM, high mLNR, and comorbidity. Consistent with our findings, Lee et al. and Kishida et al. showed that lymphovascular invasion and LNM were significantly associated with loss or reduction of ARID1A protein expression in human CRC tissues (Kishida et al., 2019; Lee et al., 2016). Notably, our results showed that LNM was strongly associated with decreased ARID1A expression. LNM is one of

the prognostic indicators for predicting DFS and OS of CRC patients (Kim & Choi, 2019). Negative or reduced ARID1A protein expression has been correlated with LNM in various types of cancer, including primary breast cancer (Cho et al., 2015; Zhao et al., 2014), hepatocellular carcinoma (He et al., 2015), and nasopharyngeal carcinoma (Yang et al., 2019). Kishida et al. suggest that negative expression of ARID1A is a significant risk factor for LNM (Kishida et al., 2019). Consequently, ARID1A protein expression should be considered as a prognostic factor for estimating survival outcomes of CRC patients.

Moreover, there are a numerous number of reports on the relevance of ARID1A protein loss expression to the survival outcomes of patients. Reduction of OS, DFS, PFS, and RFS rates were significantly correlated with loss or decreased ARID1A protein expression in various types of cancer, such as ovarian clear cell carcinoma (Katagiri, Nakayama, Rahman, Rahman, Katagiri, Nakayama, et al., 2012), primary breast cancer (Cho et al., 2015; Zhang et al., 2012; Zhao et al., 2014), gastric cancer (Wang et al., 2012), hepatocellular carcinoma (He et al., 2015), and intrahepatic cholangiocarcinoma (Yang et al., 2016). The ARID1A expression could be used for guideline treatment and management for patients with cancer (Wang et al., 2012).

In our study, the association between ARID1A protein expression and PFS in CRC patients was then also investigated. Nonetheless, the results showed that the 5-year PFS of CRC patients with low ARID1A expression was not significantly different from those with high ARID1A expression. Thus, a recent study found that decreased ARID1A protein expression was not associated with survival outcomes of patients with CRC. Our findings are consistent with previous investigations in CRC (Chou et al., 2014; Erfani et al., 2020; Lee et al., 2016). The lack of this correlation was explained by Katagiri et al. They hypothesized that the loss of ARID1A expression occurs early in the development of carcinomas. Loss of ARID1A protein expression may not be as important for tumor progression as tumor initiation. For that reason, there is no difference in the clinical stage outcome patients with positive and negative ARID1A protein expression (Katagiri, Nakayama, Rahman, Rahman, Katagiri, Ishikawa, et al., 2012). Due to the limitations in this study, the small number of CRC samples may be insufficient to determine the prognostic significance of ARID1A expression. However, there is controversy regarding the prognostic significance of ARID1A expression in CRC. The first exploration of the impact of ARID1A expression on CRC survival reported that IV stage CRC patients with

positive ARID1A had worse OS compared to those with negative ARID1A (Wei et al., 2014). Then the prognostic significance of ARID1A expression in CRC remains ambiguous and needs to be clarified in further investigations

Furthermore, ARID1A expression is also implicated in EMT. The EMT is a cellular process that is involved in several biological processes, including normal embryonic development, tissue regeneration, organ fibrosis, and wound healing (Kalluri & Weinberg, 2009; Thiery, 2003). EMT is a highly dynamic process wherein normal cells lose their epithelial characteristics and acquire mesenchymal phenotypes that include enhanced migratory capacity, invasiveness, and resistance to apoptosis (De Craene & Berx, 2013; Kalluri & Neilson, 2003; Roche, 2018). Then, the EMT process has been associated with the initiation of oncogenesis, tumor progression, invasion, and metastasis (Pastushenko & Blanpain, 2019). Previous studies have reported that EMT plays a crucial role in the progression and aggressiveness of CRC (Barker & Clevers, 2001; Bates, 2005; Brabletz et al., 2005; Hur et al., 2013). These findings indicate that EMT may be an important molecular mechanism involved in CRC development.

In the present study, the alteration of EMT-related protein in human CRC tissues was examined. Our findings demonstrated that the expression of mesenchymal proteins (vimentin and fibronectin) increased, whereas epithelial proteins (E-cad and ZO-1) decreased in the cancerous area of human CRC tissues with low ARID1A expression. Consistently, previous studies showed that ARID1A knockdown exhibited the upregulated expression of mesenchymal markers (such as vimentin and fibronectin) and the downregulated expression of epithelial proteins (such as E-cad and ZO-1) in kidney and pancreatic cancers (Somsuan et al., 2019; Tomihara et al., 2021). Moreover, ARID1A silencing exhibited promotion of cancer cell proliferation, migration, invasion, and angiogenesis in various cancer cell lines, including gastric cancer, RCC, PDAC, breast cancer, and also in CRC (Erfani et al., 2021; Somsuan et al., 2019; Tomihara et al., 2021; Wang et al., 2020; Yan et al., 2014). These findings implicate that decreased ARID1A can induce the EMT process (Somsuan et al., 2019). To our knowledge, this is the first evidence showing that reduced ARID1A protein expression alters the expression of EMT-related protein in human CRC samples.

In an early EMT, the EMT-inducing transcription factors (EMT-TFs) were activated to regulate the EMT process, which then had a role in carcinogenesis (Vu &

Datta, 2017). There are three major groups of EMT-TFs, including the SNAIL family of zinc-finger transcription factors (SNAIL/SLUG), the zinc finger E-box binding homeobox (ZEB) family of transcription factors (ZEB1/ZEB2), and the TWIST family of basic helix-loop-helix (bHLH) transcription factors TWIST1/TWIST2 (Dongre & Weinberg, 2019; Vu & Datta, 2017). Activated EMT-TFs repress the expression of genes associated with the epithelial state, whereas they induce the expression of genes associated with the mesenchymal state (Cao et al., 2015; Dongre & Weinberg, 2019). Previous studies have reported that upregulated expressions of EMT-TFs, such as SNAIL1, SLUG, TWIST1, ZEB1, and ZEB2, are associated with downregulated expression of E-cad, the severity of clinicopathological characteristics, and poor prognosis of patients with CRC (Francí et al., 2009; Gomez et al., 2011; Kahlert et al., 2011; Shioiri et al., 2006; Zhang et al., 2013). Moreover, the progression of the EMT process involves several signaling pathways, including transforming growth factor β (TGF-β), Wnt, and growth factor receptor signaling (Cao et al., 2015; Polyak & Weinberg, 2009). TGF-β and Wnt signaling pathways are essential contributors to CRC progression and EMT drivers (Lampropoulos et al., 2012; Matsuzaki et al., 2006; Vincan & Barker, 2008). A recent study by Somsuan et al. revealed that down-regulated *ARID1A* increased the secretion of TGF-β then induced SNAIL1 expression in RCC (Somsuan et al., 2019). These findings could imply that *ARID1A* can trigger the EMT process through up-regulation of the related signaling pathways and EMT-TFs. However, due to research limitations, the expression of EMT-TFs using the IHC technique was not examined in the current study. Additional investigations are required to elucidate and provide more comprehensive mechanisms of the correlation between the expression of ARID1A, EMT-TFs, and EMT-related protein in human colorectal tissues.

Furthermore, our findings revealed that CRC patients who had aberrant expressions of ARID1A, epithelial (E-cad and ZO-1), and mesenchymal (vimentin and fibronectin) proteins demonstrated a strong association with aggressive lymph node involvement. Those patients also showed a tendency to have the shortest 5-year PFS. These findings are consistent with previous studies demonstrating that CRC patients with aberrant expression of epithelial and mesenchymal proteins had aggressive progression, metastasis, and poor pathological outcomes (Al-Maghrabi, 2020; Ngan et al., 2007; Rashed et al., 2017; Yi et al., 2016). Moreover, we found that low E-cad and ZO-1, high

vimentin, and IV stage are independent prognostic factors related to shorter PFS for CRC. Our results indicate that decreased ARID1A protein may promote progression and metastasis of CRC through the EMT process. To the best of our knowledge, this is the first study showing that aberrant expressions of ARID1A and EMT-related protein are associated with the severity of pathological outcomes in Thai CRC patients.

Conclusion

In summary, the results in this study demonstrated that *ARID1A* mutations are found in CRC. Mutations of *ARID1A* may lead to decreased ARID1A expression in CRC tissues. By immunohistochemistry, expression levels of ARID1A protein are significantly decreased in the cancerous area when compared to the adjacent non-cancerous area in all pathological differentiation of CRC. Moreover, the expression of mesenchymal proteins (vimentin and fibronectin) increased, whereas epithelial proteins (E-cad and ZO-1) decreased in the cancerous area of CRC tissues with low ARID1A expression. The low expression of ARID1A was associated with the severity of clinicopathological characteristics of patients, including a greater number of positive lymph nodes, lymphovascular invasion, LNM, high mLNR, and comorbidity. However, the 5-year PFS of CRC patients with low ARID1A expression was not significantly different from those with high ARID1A expression.

Altogether, our findings indicate that ARID1A possibly plays an essential role in CRC carcinogenesis and progression, as well as in the EMT process. The significantly decreased ARID1A expression is associated with several adverse clinicopathological features, except the PFS parameters. These findings will improve the clinicopathological assessment and prognostication of severity in CRC patients. Therefore, the ARID1A protein may be considered as a promising prognostic indicator for CRC prognosis and diagnosis.

APPENDIX

Materials and Instruments

1. 2-digits electronic analytical balance (Sartorius ED 822-CW, Sartorius AG, Germany)
2. 4-digits electronic analytical balance (Denver Instrument TP-214, Denver Instrument, NY, USA)
3. Autoclave (TOMY SX-500, Tomy Kogyo Co Ltd, Japan)
4. Beakers (Pyrex, NY, USA)
5. Centrifuge tubes size 15 and 50 ml (Kirgen Bioscience, China)
6. Coverslip size 2440 mm (Menzel-Glaser, Germany)
7. Cylinder (Pyrex, NY, USA)
8. Filter paper No.1 12.5 cm (Whatman, United Kingdom)
9. Fume hood (Purair P5-48-XT, Air science USA LLC, Fort Myers, FL, USA)
10. Glass slide size 25.476.2 mm (SAIL BRAND, China)
11. Hot air oven (Binder FED115, Binder GmbH, Germany)
12. Hot air oven UN55 (Memmert Co. Ltd., Shanghai, China)
13. Hot plate and stirrer (CB162, Stuart, Sigma-Aldrich, MO, USA)
14. Humidified chamber
15. ImmunoPen (Calbiochem, Millipore, Japan)
16. Inverted light microscope (Olympus CKX41, Olympus Co Ltd, Japan)
17. Low profile disposable microtome blades (Leica Biosystems, Germany)
18. Magnetic stirrers (Stuart CB162, Bibby scientific Ltd, United Kingdom)
19. Microcentrifuge tubes size 1.5 ml (Kirgen Bioscience, China)
20. Micropipette (Proline plus, Sartorius, Germany)
21. Micropipette tips: 10, 100, 1000 l (Kirgen Bioscience, China)
22. Microscope slide storage box (Thermo Fisher Scientific, MA, USA)
23. Olympus BX50 microscope (Olympus; Tokyo, Japan).
24. Parafilm PM996 (Sigma-Aldrich, MO, USA)
25. pH meter (Denver Instrument 215, Denver Instrument, NY, USA)
26. Rotary microtome (Shandom company, Thermo Scientific, MA, USA)
27. Vortex mixer (Scientific Industries, NY, USA)

Chemicals

1. 3-(Triethoxysilyl)-propylamine (Silane) ($C_9H_{23}NO_3Si$) (Merck, Germany)
2. 3, 3'-diaminobenzidine (DAB) substrate (Abcam, United Kingdom)
3. 35-40% Formaldehyde solution (LAB Scan, Thailand)
4. 95% ethanol (KTIS Group by KTBE, Thailand)
5. Absolute ethanol (RCI labscan, Thailand)
6. Acetone (RCI labscan, Thailand)
7. Antigen retrieval buffer (100X Citrate buffer pH 6.0) (Abcam, United Kingdom)
8. Biotinylated goat anti-rabbit IgG (H+L) secondary antibody (Rabbit specific HRP/DAB (ABC) Detection) (Abcam, United Kingdom)
9. Chromogen (Abcam, United Kingdom)
10. Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Merck, Germany)
11. Hematoxylin dye (C.V. Laboratories CO., LTD., Thailand)
12. Hydrochloric acid (HCl) (Merck, Germany)
13. Hydrogen peroxide (H_2O_2) (Abcam, United Kingdom)
14. Paraformaldehyde powder (Sigma-Aldrich, MO, USA)
15. Permount (Fisher Scientific, NH, USA)
16. Potassium chloride (KCl) (Merck, Germany)
17. Potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany)
18. Sodium azide (NaN_3) (Abcam, United Kingdom)
19. Sodium chloride (NaCl) (Merck, Germany)
20. Sodium hydroxide (NaOH) (Merck, Germany)
21. Sodium phosphate monobasic (NaH_2PO_4) (Merck, Germany)
22. Streptavidin Horseradish Peroxidase (HRP) conjugate with Biotinylated solution (Abcam, United Kingdom)
23. Xylene (RCI labscan, Thailand)

Reagents

Tissue fixatives for preserving colorectal tissues (Tissue fixation)

1. 10% Neutral buffered formalin (NBF)

1.1 NaH_2PO_4	4	g
1.2 Na_2HPO_4	6.5	g
1.3 35-40% Formaldehyde solution	100	ml
1.4 Add DW	900	ml

* Prepared 10% NBF in fume hood
* Mixed on a magnetic stirrer until completely dissolved
* Kept at room temperature

2. 10X Phosphate Buffered saline (PBS) pH 7.4: stock solution

2.1 DW	800	ml
2.2 Sodium chloride (NaCl)	80	g
2.3 Na_2HPO_4 (H2O)	14.4	g
2.4 KH_2PO_4	2	g

* Mixed on a magnetic stirrer until completely dissolved
* Adjusted the pH to 7.4 with HCl (lowering the pH) or NaOH (raising the pH)
* Added DW to 1,000 ml and then kept at room temperature

3. 1X Phosphate Buffered saline (PBS) pH 7.4: working solution

3.1 10X PBS	100	ml
3.2 DW	900	ml

* Kept the solution at room temperature after mixing it

4. 4% Paraformaldehyde (PFA): pH 6.9

4.1 Paraformaldehyde powder	40	g
4.2 1X PBS	800	ml

* Heated 1X PBS on a hot plate until 60C and then added PFA powder
* Mixed on a magnetic stirrer until completely dissolved
* Cool down solution at room temperature and then adjusted pH to 6.9
* Added 1X PBS to 1,000 ml and then kept at 4C
* 4% PFA can be stored for a maximum of one month from the date of preparation

Reagents for immunohistochemistry (IHC) technique

1. 10X Phosphate Buffered saline (PBS) pH 7.4: stock solution

1.1 DW	800	ml
1.2 Sodium chloride (NaCl)	80	g
1.3 Na_2HPO_4 (H2O)	14.4	g
1.4 KH_2PO_4	2	g

* Mixed on a magnetic stirrer until completely dissolved

* Adjusted the pH to 7.4 with HCl (lowering the pH) or NaOH (raising the pH)

* Added DW to 1,000 ml and sterilized by autoclave at 121°C for 15 min before being stored at room temperature

2. 1X Phosphate Buffered saline (PBS) pH 7.4: working solution

2.1 10X PBS	100	ml
2.2 DW	900	ml

* Kept the solution at 4C after mixing it

3. 1X Citrate buffer pH 6.0: working solution

3.1 100X Citrate buffer pH 6.0	10	ml
3.2 1X PBS	1000	ml

* Kept the solution at room temperature after mixing it

4. 3, 3'-diaminobenzidine (DAB): working solution

4.1 DAB chromogen	20	l
4.2 DAB substrate	1000	l

* Mixed by the vortex and then kept away from the light until detected

Reagent for coated glass slides

1. 2% 3-(Triethoxysilyl)-propylamine (Silane) in acetone

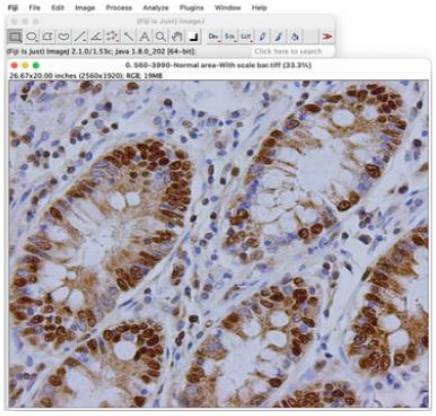
1.1 3-(Triethoxysilyl)-propylamine (Silane)	20	ml
1.2 Acetone	1000	ml

* Mixed on a magnetic stirrer and then kept at room temperature

Quantitative analysis of the intensity of ARID1A and EMT-related proteins expression using ImageJ (Fiji) image analysis software

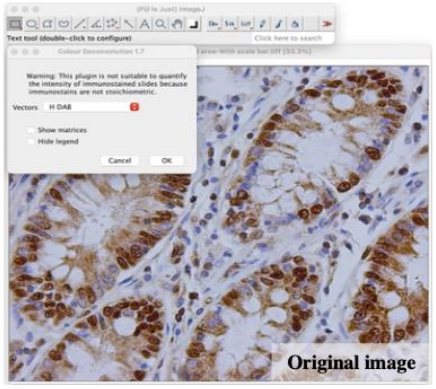
1. TIFF file format import

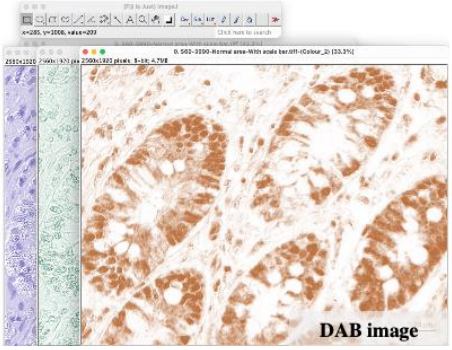
Open ImageJ (Fiji) program ➞ click File ➞ Open ➞ Select file ➞ Open



2. Converting of colour deconvolution

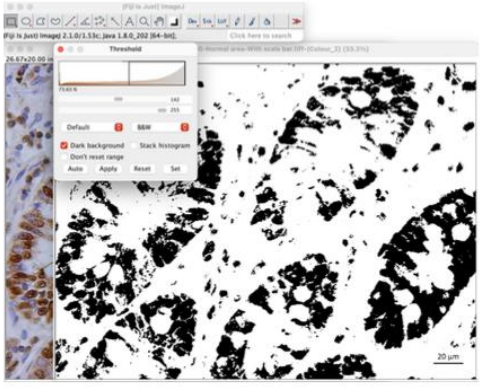
click Image ➞ Color ➞ Colour deconvolution ➞ choose H DAB ➞ Subtract images of DAB and hematoxylin will be automatically opened ➞ Choose only DAB image





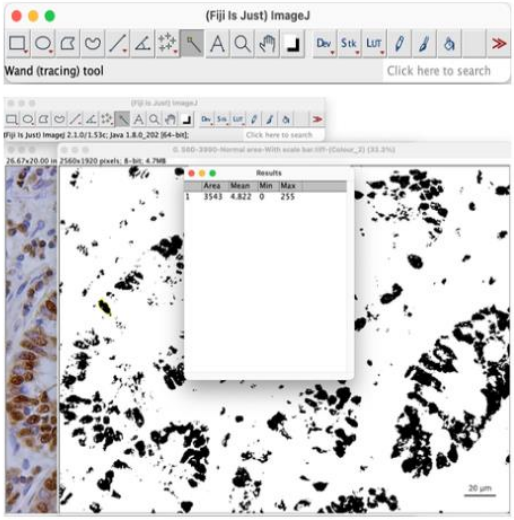
3. Colour threshold adjustment

click Image ➞ Adjust ➞ Threshold ➞ Adjust image threshold compared with the original image ➞ Apply



4. Mean grey value of ARID1A and EMT-related proteins expression measurement

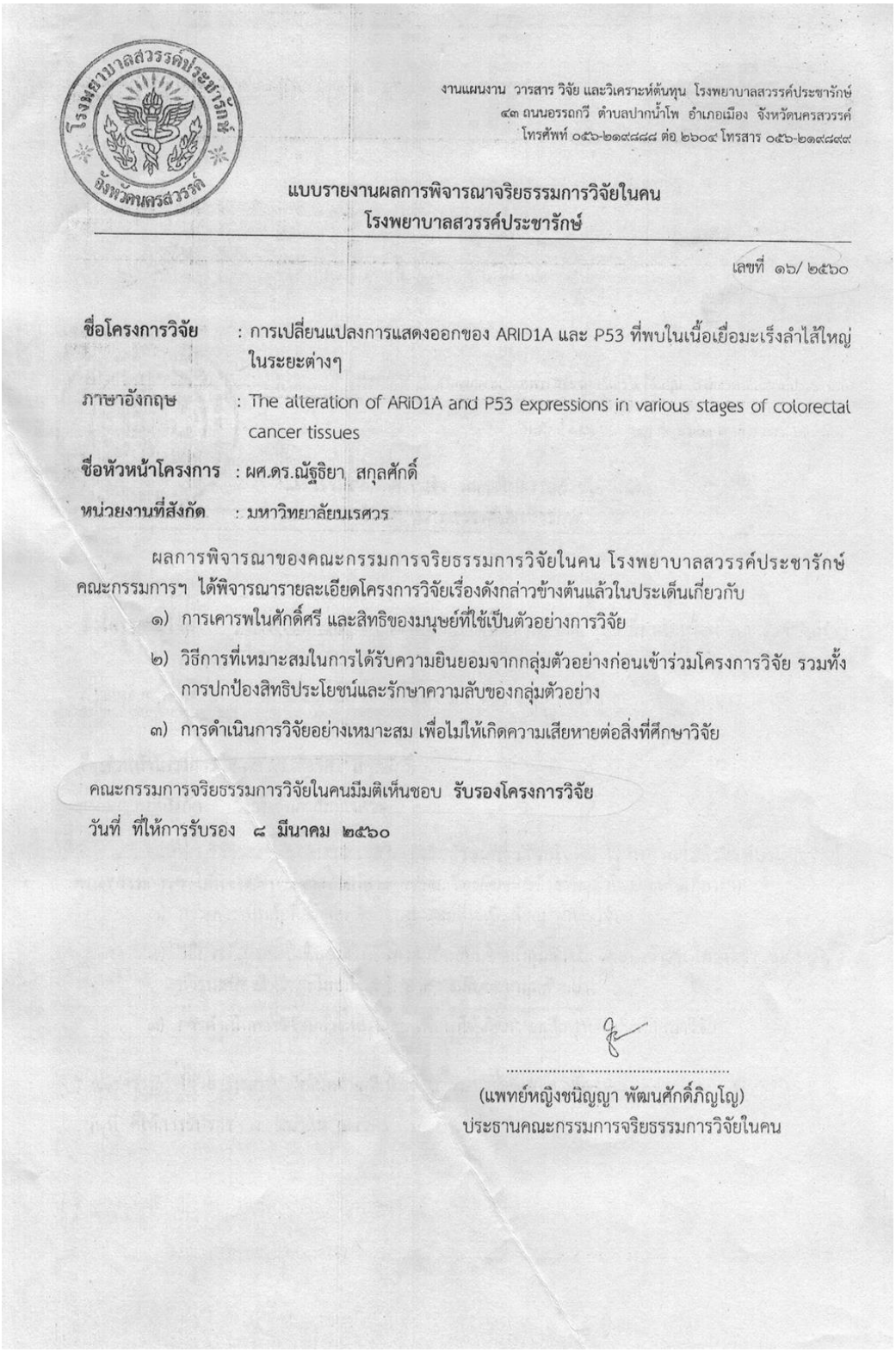
click Image ➞ Choose Wand (tracing) tool ➞ Selecting interesting cell ➞ Analyze ➞ Measure ➞ Max and mean intensities results



5. Relative optical density (O.D.) calculation

$$\text{O.D.} = \log 10 \left(\frac{\text{Max intensity}}{\text{Mean intensity}}\right)$$

Figure 1A **The schematic summary of quantitative analysis of the intensity of ARID1A and EMT-related proteins expression using ImageJ (Fiji) image analysis software**



งานแผนงาน วารสาร วิจัย และวิเคราะห์ต้นทุน โรงพยาบาลสวรรค์ประชารักษ์
๔๓ ถนนอรรถกวี ตำบลปากน้ำโพ อำเภอเมือง จังหวัดนครสวรรค์
โทรศัพท์ ๐๕๖-๒๑๙๘๘๘ ต่อ ๒๖๐๔ โทรสาร ๐๕๖-๒๑๙๘๙๙

แบบรายงานผลการพิจารณาจริยธรรมการวิจัยในคน
โรงพยาบาลสวรรค์ประชารักษ์

เลขที่ ๑๖/ ๒๕๖๐

ชื่อโครงการวิจัย : การเปลี่ยนแปลงการแสดงออกของ ARID1A และ P53 ที่พบในเนื้อเยื่อมะเร็งลำไส้ใหญ่ในระยะต่างๆ

ภาษาอังกฤษ : The alteration of ARID1A and P53 expressions in various stages of colorectal cancer tissues

ชื่อหัวหน้าโครงการ : ผศ.ดร.ณัฐธิยา สกุลศักดิ์

หน่วยงานที่สังกัด : มหาวิทยาลัยนเรศวร

ผลการพิจารณาของคณะกรรมการจริยธรรมการวิจัยในคน โรงพยาบาลสวรรค์ประชารักษ์ คณะกรรมการฯ ได้พิจารณารายละเอียดโครงการวิจัยเรื่องดังกล่าวข้างต้นแล้วในประเด็นเกี่ยวกับ

๑) การเคารพในศักดิ์ศรี และสิทธิของมนุษย์ที่ใช้เป็นตัวอย่างการวิจัย

๒) วิธีการที่เหมาะสมในการได้รับความยินยอมจากกลุ่มตัวอย่างก่อนเข้าร่วมโครงการวิจัย รวมทั้งการปกป้องสิทธิประโยชน์และรักษาความลับของกลุ่มตัวอย่าง

๓) การดำเนินการวิจัยอย่างเหมาะสม เพื่อไม่ให้เกิดความเสียหายต่อสิ่งที่ศึกษาวิจัย

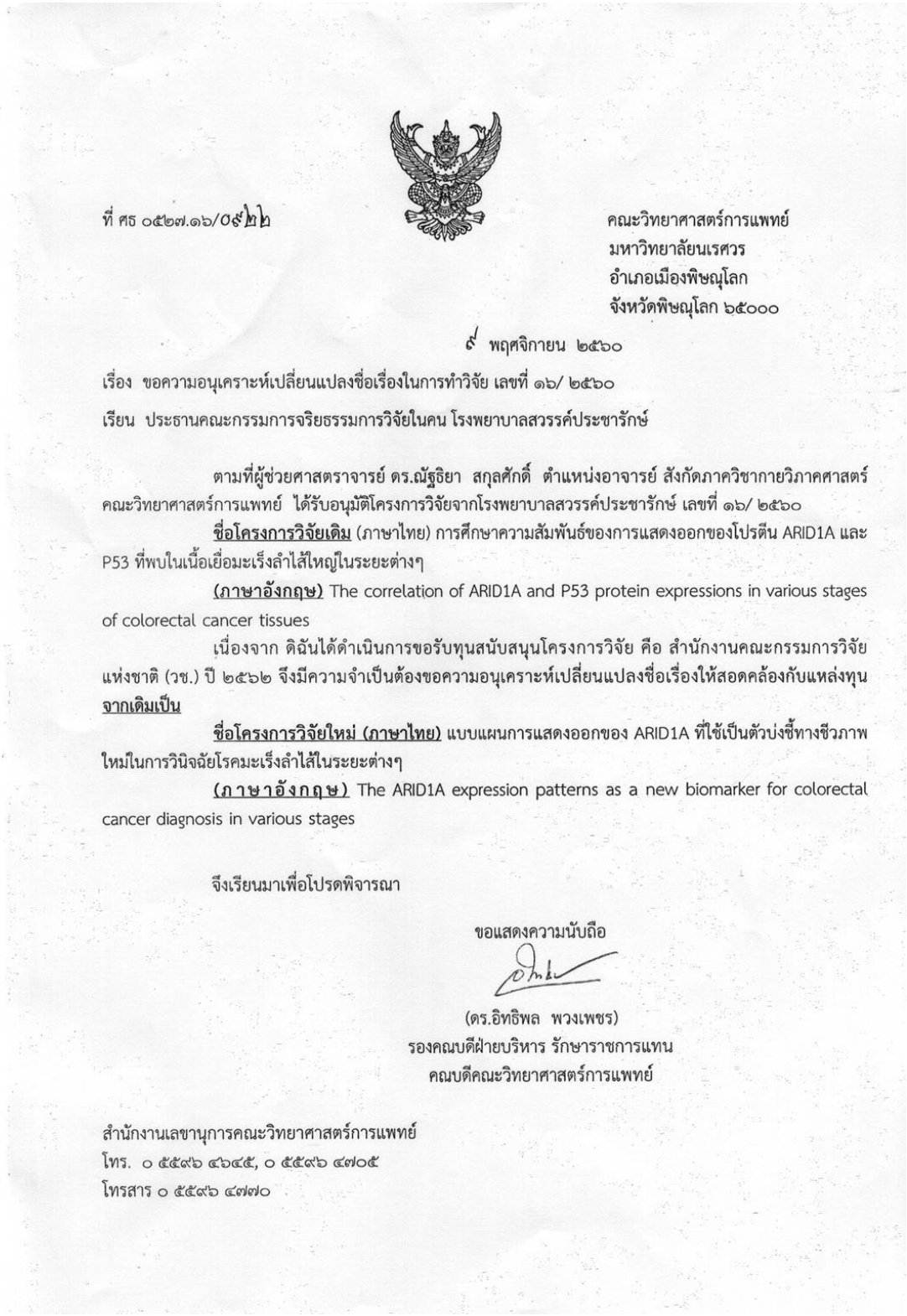
คณะกรรมการจริยธรรมการวิจัยในคนมีมติเห็นชอบ **รับรองโครงการวิจัย**

วันที่ ที่ให้การรับรอง ๘ มีนาคม ๒๕๖๐

.....

(แพทย์หญิงชนิญญา พัฒนศักดิ์ภิญโญ)
ประธานคณะกรรมการจริยธรรมการวิจัยในคน

Figure 2A Certificate of human ethical approval from Human Ethic Review Board of Sawan Pracharak Hospital, Nakhon Sawan, Thailand (approval no. 16/2560)



ที่ ศธ ๐๕๒๗.๑๖/๐๙๒๒

คณะวิทยาศาสตร์การแพทย์
มหาวิทยาลัยนเรศวร
อำเภอเมืองพิษณุโลก
จังหวัดพิษณุโลก ๖๕๐๐๐

๙ พฤศจิกายน ๒๕๖๐

เรื่อง ขอความอนุเคราะห์เปลี่ยนแปลงชื่อเรื่องในการทำวิจัย เลขที่ ๑๖/ ๒๕๖๐

เรียน ประธานคณะกรรมการจริยธรรมการวิจัยในคน โรงพยาบาลสวรรค์ประชารักษ์

ตามที่ผู้ช่วยศาสตราจารย์ ดร.ณัฐธิยา สกุลศักดิ์ ตำแหน่งอาจารย์ สังกัดภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์การแพทย์ ได้รับอนุมัติโครงการวิจัยจากโรงพยาบาลสวรรค์ประชารักษ์ เลขที่ ๑๖/ ๒๕๖๐

ชื่อโครงการวิจัยเดิม (ภาษาไทย) การศึกษาความสัมพันธ์ของการแสดงออกของโปรตีน ARID1A และ P53 ที่พบในเนื้อเยื่อมะเร็งลำไส้ใหญ่ในระยะต่างๆ

(ภาษาอังกฤษ) The correlation of ARID1A and P53 protein expressions in various stages of colorectal cancer tissues

เนื่องจาก ดิฉันได้ดำเนินการขอรับทุนสนับสนุนโครงการวิจัย คือ สำนักงานคณะกรรมการวิจัยแห่งชาติ (วช.) ปี ๒๕๖๒ จึงมีความจำเป็นต้องขอความอนุเคราะห์เปลี่ยนแปลงชื่อเรื่องให้สอดคล้องกับแหล่งทุน **จากเดิมเป็น**

ชื่อโครงการวิจัยใหม่ (ภาษาไทย) แบบแผนการแสดงออกของ ARID1A ที่ใช้เป็นตัวบ่งชี้ทางชีวภาพใหม่ในการวินิจฉัยโรคมะเร็งลำไส้ในระยะต่างๆ

(ภาษาอังกฤษ) The ARID1A expression patterns as a new biomarker for colorectal cancer diagnosis in various stages

จึงเรียนมาเพื่อโปรดพิจารณา

ขอแสดงความนับถือ

(ดร.อิทธิพล พวงเพชร)
รองคณบดีฝ่ายบริหาร รักษาราชการแทน
คณบดีคณะวิทยาศาสตร์การแพทย์

สำนักงานเลขานุการคณะวิทยาศาสตร์การแพทย์
โทร. ๐ ๕๕๙๖ ๔๖๔๕, ๐ ๕๕๙๖ ๔๗๐๕
โทรสาร ๐ ๕๕๙๖ ๔๗๗๐

Figure 3A Human ethical approval from Human Ethic Review Board of Sawan Pracharak Hospital, Nakhon Sawan, Thailand (approval no. 16/2560)

AF 08-09/5.0

COA No. 421/2021

IRB No. P10181/64

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร

99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596 8752

หนังสือรับรองโครงการวิจัยครั้งแรก

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ : ความสัมพันธ์ระหว่างการแสดงออกของโปรตีน ARID1A และโปรตีนที่เกี่ยวข้องในกระบวนการ epithelial-mesenchymal transition ในชิ้นเนื้อโรคมะเร็งลำไส้ใหญ่และลำไส้ตรงที่มีความแตกต่างทางจุลกายวิภาค

ผู้วิจัยหลัก : ผู้ช่วยศาสตราจารย์ ดร. ณัฐธิยา สกุลศักดิ์

สังกัดหน่วยงาน : คณะวิทยาศาสตร์การแพทย์

ผู้ร่วมวิจัย : นายภัทรพล สนธิ

วิธีทบทวน : แบบเร่งรัด

รายงานความก้าวหน้า : ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการเสร็จสิ้นก่อน 1 ปี

เอกสารรับรอง

1. AF 01-10 เวอร์ชั่น 1.0 วันที่ 15 กันยายน 2564
2. AF 02-10 เวอร์ชั่น 1.0 วันที่ 15 กันยายน 2564
3. AF 03-10 เวอร์ชั่น 1.0 วันที่ 15 กันยายน 2564
4. สรุปโครงการเพื่อการพิจารณาทางจริยธรรมการวิจัยในมนุษย์ เวอร์ชั่น 3.0 วันที่ 06 ตุลาคม 2564
5. โครงการวิจัยฉบับเต็ม เวอร์ชั่น 1.0 วันที่ 16 กันยายน 2564
6. ประวัติผู้วิจัย เวอร์ชั่น 1.0 วันที่ 16 กันยายน 2564
7. รายละเอียดเครื่องมือที่ใช้งานวิจัย 2.0 วันที่ 06 ตุลาคม 2564
8. หนังสือ ขอความร่วมมือการทำวิจัยและเก็บตัวอย่างชิ้นเนื้อผู้ป่วย
9. งบประมาณของโครงการวิจัย เวอร์ชั่น 1.0 วันที่ 15 กันยายน 2564

ลงนาม:.....

(นายแพทย์สมบูรณ์ ตันสุภสวัสดิกุล)

ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์

มหาวิทยาลัยนเรศวร

วันที่รับรอง : 07 ตุลาคม 2564

วันหมดอายุ : 07 ตุลาคม 2565

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

Figure 4A Certificate of human ethical approval from Naresuan University Ethical Committee for Human Research (NU-IRB) (approval no. P10181/64; COA no. 421/2021)

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