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碩士論文

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PCDH10 基因於大腸直腸癌之抑癌功能的鑑定

Identification of a putative tumor suppressor gene, *PCDH10*, in colorectal cancer

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摘要

大腸直腸癌高居國人癌症致死率第三位,已知致癌路徑主要有三個:造成基因不穩定的失異合性、微衛星不穩定，以及表觀基因修飾的調控。本實驗室先前分析大腸直腸癌檢體在第四號染色體上微衛星標記的缺失情形，進而發現 *PCDH10* (*protocadherin 10*) variant 1 mRNA 在大腸腫瘤檢體表現量顯著較正常黏膜組織低，並因受到 DNA 高度甲基化而降低基因表現，此初步結果支持 *PCDH10* 可能為大腸直腸癌相關之抑癌基因,因此選擇 *PCDH10* 基因為研究目標,了解 *PCDH10* 在大腸直腸癌致癌路徑中是否扮演抑癌基因的角色。*PCDH10* 有兩種 mRNA variants,本論文經由建構 *PCDH10* 表現載體於大腸直腸癌細胞株 HCT116 進行蛋白質表現,以研究此兩種基因產物之可能功能。結果顯示暫時性大量表現 PCDH10 variant 1 會抑制細胞生長及侵犯能力，而大量表現 PCDH10 variant 2 僅影響細胞生長能力。由於無法獲得會表現 PCDH10 蛋白的混合穩定株（mixed stable clones），我們利用兩株 *PCDH10* variant 1 單一穩定株（single stable clones）及一株 *PCDH10* variant 2 單一穩定株進行功能研究。結果顯示在 PCDH10 variant 1 蛋白表現量高時具有抑制細胞生長及群落形成能力，而 PCDH10 variant 2 的表現則不影響細胞的這些特性。此外，為確認 PCDH10 在細胞中表現位置，建構表現 PCDH10 和綠色螢光蛋白融合的載體，也以免疫螢光染色檢測 PCDH10 兩種蛋白在細胞中的位點。以共軛焦顯微鏡觀察發現 PCDH10 variant 1 和 variant 2 均表現在細胞膜上，並且兩者有共同存在（co-localization）的現象。由以上結果我們推測 PCDH10 於細胞膜上並進行蛋白功能，其中，PCDH10 variant 1 在大腸直腸癌細胞株 HCT116 可能具有抑癌基因功能。

關鍵字: **大腸直腸癌、抑癌基因、PCDH10**

Abstract

Colorectal cancer (CRC) is one of the most common malignancies and is the third leading cause of cancer death in Taiwan. Three major pathways, loss of heterozygosity, microsatellite instability and epigenetic regulation are demonstrated in CRC tumorigenesis. To identify putative tumor suppressor genes associated with CRC, deletion mapping on chromosome 4 was performed by loss of heterozygosity analysis with colorectal cancer paired tissues. Previous data figured out that expression of *PCDH10* variant 1 is significantly down-regulated in tumor tissues than their paired normal mucosa. On the other hand, the expression of *PCDH10* is regulated by epigenetic modification. These data support that *PCDH10* might be a putative tumor suppressor gene associated with in colorectal cancer. It has been reported that *PCDH10* gene is transcribed into 2 kinds of mRNA variants. In the present study, *PCDH10* mammalian expression vectors were constructed and over-expressed in colorectal cancer cell line, HCT116, and then the cells were analyzed by functional assays. The results showed transient over-expression of *PCDH10* variant 1 could suppress cell proliferation and invasion, while over-expression of *PCDH10* variant 2 only inhibited cell proliferation. Furthermore, we got two of *PCDH10* variant 1 single stable clones and one of *PCDH10* variant 2. *PCDH10* variant 1 single stable clones could repress cell growth and colony formation in protein expression level-dependent manner. There

is no same effect in *PCDH10* variant 2 stable expression clone. Moreover, through construction of PCDH10-GFP fusion protein vector and immunofluorecent stain of the PCDH10 variant 1 single stable clone, we identified the localization of PCDH10 protein in cells. Both *PCDH10* variant 1 and variant 2 were expressed on cell membrane and seemed to be co-localizated. In conclusion, PCDH10 variant 1 can suppress cell proliferation and invasion in HCT116, suggesting that PCDH10 variant 1 might be a putative tumor suppressor gene associated with colorectal tumorigenesis.

Keywords: colorectal cancer, tumor suppressor gene, PCDH10

Abbreviation

APC	Adenomatous polyposis coli
5-Aza-CdR	5-aza-2'-deoxycytidine
CIN	Chromosome instability
CRC	Colorectal cancer
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
cDNA	Complementary deoxynucleic acid
FAP	Familial adenomatous polyposis
GFP	Green fluorescence protein
HNPCC	Hereditary nonpolyposis colorectal cancer
LOH	Loss of heterozygosity
MMR	Mismatch repair
MSI	Microsatellite instability
MTT	(3-(4, 5-Dimethylthiazol-2-yl)-2,5-dipheyl tetrazolium bromide
PBS	Phosphate buffered saline
PCDH10	Protocadherin 10
PCR	Polymerase chain reaction
mRNA	Message ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase chain reaction

Content

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CHAPTER 1. INTRODUCTION

1.1. Colorectal cancer

Colorectal cancer refers to cancer occurring in the colon and the rectum. This cancer develops for as long as 15 years. Based on statistical analyses of epidemiology in 2008, by the Department of Health, Executive Yuan, R.O.C. (TAIWAN), the cancer is still the first leading cause of death in Taiwan, and colorectal cancer (CRC) is the third leading cause of cancer death for many years. It's the same thing in some developed countries, such as U.S. and Japan. Nowadays, colorectal cancer is often diagnosed by colonoscopy and treated with a combination of surgery and chemotherapy.

1.1.1. The colon

The colon is the last portion of the digestive system but without digestive ability. Total length of the colon is about 1.5 meters long, reabsorbing water and electrolytes (such as vitamins B and K) from remaining chyme, storing and eliminating of feces from the body are the main functions of the colon. The colon is located between small intestine and anus. The colon is classified into the right colon (also called proximal colon) and the left colon (also called distal colon). The right colon includes the cecum, ascending colon and transverse colon; and the left colon includes the descending colon, sigmoid

colon and rectum. Tumors developed in these regions are commonly called colorectal cancer. The mucosa of large intestine has scattered lymphocytes and lymphatic nodules, and is covered by columnar epithelial cells and muscusecrting goblet cells. The colon structure is similar to small intestine, but without villi.

1.1.2. Risk factors

1.1.2.1. Age and diet

For age, development of colorectal cancer often takes a long time with multiple genetic alterations and/or chromosome instability when time goes by. Diet is an important risk factor of the sporadic colorectal cancer, and the incidence of the colorectal cancer will increase with either high in red meat or low in fiber. Moreover, obesity and less exercise are also correlated to colorectal tumorigenesis [1, 2]. Thus, colorectal cancer might be a life style-related disease.

1.1.2.2. Polyps of the colon

Colorectal cancer often arises from the polyps in the colon, and two-thirds of the colon polyps are adenomas. Therefore, polyps which become to aberrant crypt foci (ACF), especially dysplastic ACF [3], are considered as biomarker of increasing the risk of colorectal cancer. When ACF expands to adenomatous polyps, tumor progression will accelerate to later stage. As adenomatous polyps become malignant polyps, cancer may be developed.

1.1.2.3 Hereditary

Based on the report of American Cancer Society, most colorectal cancer occurred in elderly people more than 50 years old and 75~85% of these cancers are sporadic [4], which the other 15~25% of colorectal cancer are hereditary. According to different gene alterations occurred, hereditary CRC is major classified as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC, or Lynch syndrome). FAP carries nearly 100% risk of colorectal cancer development by the age of 40 years old people without therapy. With tumor suppressor gene *APC* (adenomatosis polyposis coli) germline mutation, FAP patients have polyps in their early age. HNPCC patients do not have many polyps in their clinical phenotype and easily be diagnosed with a disaproportinate numbers of adenomas before 50 years old. Because of ineffectively repair non-coding repetitive sequence and insertions and deletions are accumulated in microsatellite [5] in HNPCC patients, DNA mismatch gene mutation and microsatellite instability often lead to MMR system dysfunction.

1.1.3. Staging

The colon are composed of four distinct layers, from inner to outer part of the colon are mucosa, submucosa, muscularis propria and serosa. According to tumor development and invasion to different layers of the colon, colorectal cancer staging is a very important index for diagnosis and statistics in epidemiology, also a predictor of

patient survival. Two systems are often used for diagnosis, therapy and research of colorectal cancer at present.

1.1.3.1. Dukes' system

The system was first proposed by Cuthbert Dukes in 1932, only separating as Stage A, B, and C. Later, stage D was added to describe tumor that has distant metastases. Until 1967, Aster and Coller described each stage in detail. Each stage was described as following:

Stage A: tumors only involver in mucosa

Stage B: tumors with invasion to other tissues or intestinal walls but no metastasis

Stage C: tumors with invasion to lymph node

Stage D: tumors with distant metastasis to other organ

1.1.3.2. The TNM staging system

The TNM (tumor, node and metastasis) classification was developed and maintained by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). This system can be applied to both clinical and pathological staging. There are general rules of the TMN system for all kinds of cancer (from AJCC Cancer Staging Atlas, 6th edition):

The TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M).

The T category describes the extent of the primary tumor.

TX: Primary tumor cannot be assessed

T0: No evidence of primary tumor

Tis: Carcinoma in situ

T1, T2, T3, T4: Increasing size and/or local extent of the primary tumor

The N category describes the absence or presence and extent of regional lymph node metastasis.

NX: Regional lymph nodes cannot be assessed

N0: No regional lymph node metastasis

N1, N2, N3: Increasing involvement of regional lymph nodes

The M category describes the absence or presence of distant metastasis.

MX: Distant metastasis cannot be assessed

M0: No distant metastasis

M1: Distant metastasis

The TNM classification for carcinomas of the colon and rectum provides more detailed than other staging system and is compatible with Dukes' system. The relationship of various stage grouping is compared in the next section.

1.1.3.3. AJCC/UICC stage groupings

Although several staging system have been developed, these staging system can be

compared to with another one as following table [4]:

AJCC/UICC Stage Groupings

	TNM			Modified Astiler-Coller	Dukes
Stage 0	Tis	N0	M0	N/A	N/A
Stage I	T1	N0	M0	Stage A	A
	T2	N0	M0	Stage B1	A
Stage IIA	T3	N0	M0	Stage B2	B
Stage IIB	T4	N0	M0	Stage B3	B
Stage IIIA	T1, T2	N1	M0	Stage C1	C
Stage IIIB	T3, T4	N1	M0	Stage C2, C3	C
Stage IIIC	Any T	N2	M0	Stage C1, C2, C3	C
Stage IV	Any T	Any N	M1	Stage D	N/A

1.1.4. Pathways involved in colorectal tumorigenesis

1.1.4.1 Adenoma-carcinoma sequence

Cancer development needs multiple gene alterations, and is involved in loss of function of tumor suppressor genes or activation of oncogenes. In 1969, Ashley first proposed the concept that carcinogenesis in different cancers are “two-hit” or “multiple hit” [6]. Later, Kundson utilized the results of an observation of statistical analysis data of retinoblastoma to build up the “two-hit hypothesis” [7]. A first hit will occur on activation of an oncogene but the cancer will not occur certainly unless a second hit arise from inactivation of a tumor suppressor gene. Therefore, many oncogenes and tumor suppressor genes are involved in cancer tumorigenesis. According to the investigations of sporadic and hereditary colorectal cancer patient tissues, the carcinogenesis mechanism of colorectal cancer has been understood approximately. “Adenoma-carcinoma sequence” is a well-known theory in colorectal cancer first

proposed in 1990 by Fearson and Volgestein, to validate colorectal cancer is a multistep carcinogenesis, in which malignant tumor is formed after 4 to 5 gene alterations [8]. In 2002, genomic instability and gene alterations which were dependent on different genes participated in colorectal cancer tumorigenesis had been described in detail in adenoma-carcinoma sequence pathway [9-12].

1.1.4.2. Genomic instability: loss of heterzygosity and microsatellite instability

Genomic instability includes loss of heterzygosity (LOH, also called chromosome instability) and microsatellite instability (MSI). Chromosome instability is caused by hyperdiploidy and allelic losses. Deletions are often found in chromosome 17p [13], 18q, 5q and 22q, and some important genes such as *p53, DCC, DPC4, SMAD2, APC* [14] and *MCC*. Among these genes, *p53* and *APC* have 70% high somatic mutation. Tumors with these gene mutations are called as LOH positive tumors, and 80% of colorectal carcinoma is included. Among them, 80% of LOH positive tumors occur on distal colon. LOH can be detected by using polymorphic markers. Microsatellites are short sequence repeat unit of 1~ 6 nucleotides. Germline mutation of mismatch repair (MMR) genes, such as *hMSH2, hMLH1, hPMS2, hMLH3*, and *hMLH6*, occurs in HNPCC [5]. If more than one MMR genes were mutated or defected, errors during DNA replication will not be repaired. Some genes, such as *TGFβ-RII* and *Bax*, which have tri-nucleotide repeat sequences, would easily have gene insertion or deletion. On

the other hand, about 15% of colorectal cancer belong to MSI positive tumors and are rarely found on distal colon. Among them, 50% to 70% of HNPCC patients are MSI positive tumors and easily develop to colorectal cancer or other epithelial tumors, such as endometrial, ovarian or gastric adenomas. Only 10% to 15% of sporadic CRC belong to MSI positive tumors. National Cancer Institute has recommended 5 microsatellite markers (BAT25, BAT26, D5S236, D2S123 and D17S250) to define the level of MSI: MSI-H (MSI-high frequency) means 2 or more markers are unstable; MSI-L (MSI-low frequency) means one marker is unstable; MSS (microsatellite stable) means no marker is unstable.

1.1.4.3 Gene alterations

Colorectal cancer tumorigenesis is most initiated from *APC* gene mutation and 60 to 80% of sporadic colorectal cancer has *APC* gene mutation and germline *APC* mutation also arise in FAP [14, 15]. Most somatic *APC* mutate in MCR sequence (mutation cluster region, in codon 1286~1513) and easily become mis-sense mutation and a truncated protein is produced. *APC* normally binds with GSK-3β/axin complex to phosphorylate and degrade β-catenin via ubiquitilation. When a mitotic signal is induced, the WNT/Wingless pathway will be activated and GSK-3β is antagonized as a result, β-catenin cannot be degraded, and then accumulated in the nucleus from lateral cell membrane and interact with other transcription factors, such as LEF

(lymphocyte-enhancing factor) and Tcf-4 (T cell factor), to activate *c-Myc* or some other target genes consequently, cell proliferation is promoted. When early adenoma becomes to advanced adenoma, *k-ras* gene become to be mutated. Carcinoma formation might involve in mutations of p53 or SMAD2/4. SMAD2/4 gene are transcription factors in TGF β pathway [9, 10, 16].

1.1.4.4. Epigenetic regulation

In addition to genomic instability, epigenetic modification in tumor suppressor genes is a popular issue [17-19]. Dysfuntion of important tumor suppressor genes with aberrant promoter hypermethylation will cause tumor development. Serrated neoplasia pathway is another pathway observed in CRC progression with *BRAF* gene mutation and CpG island hypermethylation [20, 21].

1.1.5. LOH study on chromosome 4

Deletion of chromosome 4 is involved in various types of cancer, such as mesothelioma, small cell lung carcinoma [22], breast cancer [23, 24] and CRC [25]. Colorectal cancer has been reported that gene deletion on chromosome 4, 8, 17 and 18 will cause higher incidence of sporadic colorectal cancer. Allelic losses are often seen in CRC, consequently, LOH study to identify the deletion regions on chromosomes is one of the most useful methods to search putative tumor suppressor genes involved in CRC. Our previous study focused on the deletion mapping of chromosome 4 via LOH

study to identify putative tumor suppressor genes which have not been proposed. Through analysis of 106 CRC tissue pairs with 22 microsatellite markers on chromosome 4, three high LOH regions were defined and microsatellite marker D4S402 had the highest LOH frequency of 32.9%. To narrow down the region that may contain tumor suppressor gene candidates, the senior, Sheng-Tai Tzeng, continued to analyze the LOH frequency with 10 microsatellite markers, which surround D4S402 and have average intervals of 1~2 cM length, in 70 CRC tissue pairs. Results revealed that LOH frequencies of D4S2394 and D4S1575 are 23.1% and 28.0%, respectively. After literature review and focused on the 4.54 Mb region between these two microsatellite markers, *PCDH10* was chosen as the putative tumor suppressor gene associated with CRC in the present study.

1.2. Protocadherin 10

Protocadherin 10 (abbreviated as *PCDH10*) is a member of protocadherin family that belongs to cadherin superfamily. Although structure has not been resolved, PCDH10 is considered as an adhesion molecule similar to cadherin superfamily, but its interaction is not as strong as cadherin superfamily.

1.2.1. Protocadherin family

Cadherins are Ca^{2+}-dependent cell adhesion molecules [26] involving in biological processing, such as cell death, neuronal connection, cell recognition and signal transduction [27-29]. Protocadherin family is the largest subfamily of the cadherin superfamily, which contains more then 70 members that are dominantly expressed in the nervous system. In 1993, Sano et al. defined a large number of cDNA of which amino acid sequences are similar to cadherin superfamily. However, their c-terminal structure is quite different to cadherins [30], and these proteins were named protocadherin family. Protocadherin family is highly expressed in brain and might be involved in species development. It has 6 or 7 extracellular cadherin domains (EC domains), a single transmembrane domain and a cytoplasmic domain that is similar to cadherins. The most differences between cadherins and protocadherins in protein structure are: (1) W (Tryptophan) in EC1 domain is highly conserved and (2) Hydrophilic pockets in cadherins [31]. According to genomic structure, it has been

divided into clustered protocadherins (containing more than 50 members, such as *Pcdhα, Pcdhβ and Pcdhγ*), and nonclustered protocadherins [32].

1.2.2. Structure of *PCDH10*

PCDH10 is located at chromosome 4q 28.3 and belongs to protocadherin family. *PCDH10* has two mRNA variants, which may encode two protein products that are considered to be single-pass type I membrane proteins and locate on cell membrane. PCDH10 contains six extracellular cadherin domains, one transmembrane domain and one cytoplasmic tail domain. *PCDH10* mRNA variant 1 encodes a cadherin-related neuronal receptor that is considered to involved in development and neuronal formation. PCDH10 structure has not been resolved until now. According to UniProt software in EXPASY website, PCDH10 variant 1 structure is predicted as following: signal peptide (amino acid 1~18), extracellular domain (amino acid 19~715), transmembrane domain (716~736) and cytoplasmic domain (amino acid 737~1040). Glycosylation modification may occur at amino acids 273 and 557. PCDH10 variant 2 has identical structure except for the cytoplasmic domain (amino acid 878~896). In this study, we try to explore the function of both PCDH10 variants.

1.2.3. Functional study of *PCDH10*

Mouse *Pcdh10*, also called *OL-PCDH10*, was first identified and is highly expressed in olfactory bulb, limbic system and axons [33, 34]. In 2000, human *PCDH10* was first

cloned from mouse brain cDNA [35], named as KIAA1400. The official name of human *PCDH10* was defined in 2001 and its expression was detected in brain, kidney, smooth muscle, breast, heart, lung and trachea by RT-PCR [36]. Nowadays, studies indicate *PCDH10* is involved in neural development in different species, including mouse [34, 37], chicken [38] and zebrafish [39]. OL-pcdh10 can also interact with Nap1 protein to promote cell migration in morphogenesis [40], and with hBex1 protein to inhibit apoptosis of Bcr/Abl^{+} leukemic cells [41]. *mPcdh10* functions as a homophilic cell-cell adhesion molecule and localizes between the cell-cell junction [33].

1.2.4. *PCDH10* as a novel tumor suppressor gene in various cancers

Although the functions of *PCDH10* are not clear yet, *PCDH10* was identified as a novel tumor suppressor gene in breast cancer [42], nasopharyngeal and esophageal carcinomas [43], hematologic malignancies [43, 44], and gastric cancer [45]. Ying et al. utilize MS-RDA (methylation-sensitive-representational difference analysis) to search out that *PCDH10* was frequently silenced in various carcinomas, which included colorectal, nasopharyngeal, esophageal, hepatocellular, breast, cervical, lung, hematologic and gastric cancers [43-45]. They also constructed *PCDH10* variant 1 mammalian expression vector to perform functional assays in different kinds of cancer cell lines. In 2006, they confirmed that over-expression of *PCDH10* can suppress cell

proliferation, migration and colony formation ability of nasopharyngeal and esophageal cancer cell lines [43]. Recently, they further demonstrated *PCDH10* variant 1 inhibits not only invasion and colony formation ability of gastric cancer cell line but also tumor growth in nude mice. In addition, methylation of *PCDH10* in gastric normal mucosa had poor survival than non-methylation in the Kaplan-Meier survival curves [45]. However, no related study of *PCDH10* variant 2 is published yet.

1.2.5. Previous study at our lab

1.2.5.1. *PCDH10* expression is regulated by epigenetic modification in CRC

Analysis of 70 pairs of colorectal cancer patient tissues through methylation-specific PCR (MSP) reveals that promoter hypermethylation regulates *PCDH10* gene expression in tumor tissue but with no significant difference in patient age, gender, tumor location, and Dukes' stage. In addition, by treatment with a differences demethylation drug, 5-aza-2'-deoxycytidine (5-Aza-CdR), *PCDH10* gene expression could be restored in 5 CRC cell lines (These studies were performed by Chia-Yun Chang).

1.2.5.2. *PCDH10* down-regulated in CRC tumor tissues

Analysis of mRNA expression of PCDH10 variant 1 via RT-PCR and Real-time PCR, *PCDH10* showed higher expression in normal mucosa than in tumor tissues with statistical significance. On the other hand, the mRNA expression of *PCDH10* variant 2

was detected in some of the tissues that expressed *PCDH10* variant 1 (These studies were performed by Chao-Hua Fu)

1.3. Objectives in the thesis

Many cadherins (E-cadherin [46] and VE-cadherin [47]) and protocadherins (PCDH8 [48], PCDH11 [49] and PCDH20 [50]) have been reported that may involve in cancer progression [51]. Moreover, recent studies of *PCDH10* in multiple carcinoma support that *PCDH10* may be a tumor suppressor gene. We found out *PCDH10* as a tumor suppressor gene candidate involved in colorectal cancer by using loss of heterozygosity on chromosome 4. Previous study in our lab, demonstrated that *PCDH10* is more expressed in normal mucosa compared with tumor tissues from CRC patients and is regulated by epigenetic modification, suggesting that *PCDH10* might be a putative tumor suppressor gene in colorectal cancer. In the thesis, both *PCDH10* variant 1 and variant 2 were studied. To continue the prior study of *PCDH10*, we focused on both *PCDH10* variant 1 and variant 2 in this study and identified these two gene function in CRC. Furthermore, mouse *PCDH10* protein localization was confirmed on cell membrane with homophilic interaction [33], we also demonstrated human *PCDH10* protein localization in human cell line.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell lines

Colorectal cancer cell lines, HCT116, HCT15, HT29, KM12, were gifts from Dr. SL Yu's lab, cultured with RPMI-1640 medium containing 10 % fetal bovine serum and incubated at 37℃ with 5 % CO_2 environment. Colorectal cancer cell lines, SW620 and SW480, were purchased from FIRDI, cultured with Leibovitz's L-15 medium containing 10 % fetal bovine serum and incubated at 37℃ without 5 % CO_2 environment. HEK293T cell line was gift from Dr. SW Lin's lab, cultured with DMEM containing 10 % fetal bovine serum and incubated at 37℃ with 5 % CO_2 environment.

2.2. Vector construction

2.2.1. PCDH10 mammalian expression vector

To study gene function of *PCDH10*, mammalian expression vectors were constructed, using pcDNA 3.1-V5-His-TOPO (Invitrogen) as the targeting vector. Two variants were included in *PCDH10*, and *PCDH10* variant 1 construction was done as following description. First of all, the open reading frame of *PCDH10* variant 1 were amplified by PCR with paired primers which contained BamHI and XbaI cutting sites without

stop codon (PCDH10-1f: GGATCCATGATTGTGCTATTATTGT and PCDH10-1r: TCTAGAGAGCATATCCTTTTCCGT), MCF7 cDNA were used as a template. The PCR reaction was performed with KOD polymerase (TOYOBO) and the program began with pre-denaturing at 94 ^{0}C 3 min first, 10 cycles of 94 ^{0}C for 30 sec, 58 ^{0}C for 45 sec, 72 ^{0}C for 3 min and 30 sec, and 10 cycles of 94 ^{0}C for 30 sec, 56 ^{0}C for 45 sec, 72 ^{0}C for 3 min and 30 sec, 15 cycles of 94 ^{0}C for 30 sec, 54 ^{0}C for 45 sec, 72 ^{0}C for 3 min and 30 sec, finally extension at 72 ^{0}C for 5 min and paused at 4^{0}C. As the PCR reaction was terminated, 2 units BerTag polymerase (Bertec) was added and extension at 72 ^{0}C for 30 min for A-tailing procedure. The *PCDH10* variant 1 PCR fragments were checked by restriction enzyme digestion and purified by Gel-M^{TM} Gel Extraction System (Viogene) from 1 % agarose gel. The gel extraction products were ligated to pGEM-T Easy vector (Promega) via TA-cloning and transformed into DH5α competent cells. Plasmids were extracted from Mini-plus^{TM} Plasmid DNA Extraction System (Viogene). Plasmids sequences were checked by The Second Core Laboratory in NTU and analyze data by Chromas and DNAman software. Two mutations were found and corrected as the reference sequence in NCBI by using site-directed mutagenesis with primer PCDH10-s1f: GGAAATCTCTGAGAGCGCCACGCCAGG CAC and PCDH10-s2f: GTCAGCTGGTATGGATCTCTTCTCCAATTGCACTG. Site-directed mutagenesis reaction was performed with KOD polymerase and started

from pre-denaturing at 95 ^{0}C 3 min first, 18 cycles of 95 ^{0}C for 30 sec, 56 ^{0}C for 1 min, 68 ^{0}C for 6 min and 30 sec, finally extension at 72 ^{0}C for 7 min and paused at 4^{0}C. (refer to Stratagene-Quick change site direct mutagenesis Kit and [52]). Then PCR products were digested by DpnI (New England Biolabs) at 37^{0}C for 3 hours then transformed into DH5α competent cell, and DNA plasmids were extracted for sequencing. After sequencing, the corrected *PCDH10* fragments were cut and re-ligated to targeting expression vectors, pcDNA3.1/V5-His-TOPO vector, and re-check the sequencing data were the same with reference sequence in NCBI. *PCDH10* variant 2 mammalian expression vectors were constructed by Chao-Ha Fu with the same cloning strategy as variant 1. These constructs were named to pcDNA3.1/PCDH10.v1 and pcDNA3.1/PCDH10.v2. The constructs were named to PCDH10.v1 and PCDH10.v2 in this thesis.

2.2.2. PCDH10-GFP mammalian expression vector

To study the *PCDH10* localization, *PCDH10* variant 1 and 2 fusion with GFP constructs were made. Using PCDH10 variant 1 and variant 2 constructed in pcDNA3.1/V5-His-TOPO vector as template, PCR reaction was performed with primers N3-PCDH10-f: CCGGAATTCATGATTGTGCTATTATTGT, both for PCDH10 two variants, N3-PCDH10-1r: CGCGGATCCGCATATCCTTTTCCGTGTC was for *PCDH10* variant 1 and N3-PCDH10-2r: CGCGGATCCGAGGAGGCTTTCT

GATGG was for variant 2. The PCR condition was performed with KOD polymerase (TOYOBO) and the program began with pre-denaturing at 94 ^{0}C 3 min first, 40 cycles of 94 ^{0}C for 30 sec, 55 ^{0}C for 45 sec, 72 ^{0}C for 3 min and 30 sec, finally extension at 72 ^{0}C for 5 min and paused at 4^{0}C. PCR products were purified and cut with BamHI and XbaI then ligated to pEGFP-N3 vector (Clontech). Ligation products were transformed into DH5α competent cell, and DNA plasmids were extracted for sequencing. Plasmids sequences were checked by The Second Core Laboratory in NTU and analyze data by Chromas and DNAman software. The constructs were named to PCDH10.v1-GFP and PCDH10.v2-GFP to represent a GFP protein was fused in the N-terminal of target protein.

2.2.3. Gel purification

The protocol based on Gel-M^{TM} Gel Extraction System (Viogene). In the beginning, slice desired agarose gel and collects in an eppendorf with appropriate volume of GEX buffer and reacts at 60 ^{0}C until the agarose gel has dissolved. Solution was added to Gel-M column with collection tube and centrifuged at a full-speed, 60 sec. 500 μL WF buffer and 700 μL WS buffer were added separately to wash DNA pellet and centrifuge at a full-speed, discard flow-through. After that, centrifuge at a full-speed for 3 mins, and change Gel-M column to a new eppendorf. Dry ethanol for 3 mins and 30 μL ddH_2O (pre-warm at 65 ^{0}C) was added onto membrane stay for 5 mins, centrifuge at a

full-speed for 2 mins, and stored at -20^{0}C.

2.2.4. Preparation of competent cells

DH5α single colony was selected from LB plate and incubated in 3 mL LB broth without antibiotics at 37 ^{0}C overnight. The next day, transfer bacteria liquid to 100 mL LB (diluted to 100X) broth and incubate at 37 ^{0}C for 2~3 hours until the OD600 = 0.5~0.7. The flask was on ice for 30 min and separated in tube, centrifuge at 6000 rpm, 4 ^{0}C for 10 min. The supernatant was discarded and 0.1 M $CaCl_2$ of half volume of tube was added and mix thoroughly by gently vortexing and on ice for 30 min. Then centrifuge at 3000 rpm, 4 ^{0}C for 10 mins and discard supernatant. 10 % glycerol with 0.1 M $CaCl_2$ of 1/10 volume of the tube was added and mixed gently. After on ice and put it at 4 ^{0}C overnight, separate 100 μL / tube for aliquot and stored at -80 ^{0}C.

2.2.5. Transformation

DNA and 100 μL DH5α competent cell were mixed well and on ice for 30 min. After heat-shock the competent cell at 42 ^{0}C for 90 sec and on ice for 2 min, 900 μL LB broth was added to recovery cells and incubate at 37 ^{0}C for 1 hr. Centrifuging at 6000 rpm for 3 min and discard supernatants, cell pellets were suspended in 200 μL LB broth and spread onto LB plate with Ampicillin (50 mg / mL) and incubate at 37 ^{0}C overnight (about 16 ~ 18 hours).

2.2.6. Mini-preparation of plasmid DNA

The protocol based on Mini-Plus^{TM} Plasmid DNA Extraction System (Viogene). In the beginning, inoculate single colony to 5 mL LB broth at 37 ^{0}C for 16 hrs then collect the pellet in an eppendorf. 200 μL MX-1 solutions were added and vortex until the pellet has been dissolved. 250 μL MX-2 solutions were added and mixed gently (DO NOT VORTEX!) and stay at room-temperature for 5 min. 250 μL MX-3 solutions were added and mixed gently then stay at room-temperature for 5 min. All solutions were added to Mini-Plus column with collection tube and centrifuge at a full-speed for 30 sec. 500 μL WF buffer and 700 μL WS buffer were added and centrifuge at a full-speed, discard flow-through separately. Then re-centrifuge at a full-speed for 3 mins and change column to a new eppendorf. Dry ethanol for 3 min and 30 μL ddH_2O (pre-warm at 65 ^{0}C) was added onto membrane and stay for 5 min, then centrifuge at a full-speed for 2 min. Samples were stored at -20 ^{0}C.

2.3. Cell transfection

For transient expression, 5x10^{5} HCT116 cells were seeded before the day of transfection. 2 μg plasmid DNA (purified by QIAGEN Plasmid Midi Kit) and 5μl Lipofectamine™ 2000 (Invitrogen) were transfected into cells. After transfection for 48 hours, cells were collected and lysed by RIPA and protein expression was confirmed by western blot. For stable clone selection, 1200μg/mL G418 (Geneticin,

GIBCO) was added after transfection for 48 hours at least for 2~3 weeks

2.4. Immunofluorescent stain

Cells were seeding and attached on the chamber slide or coverslip. After the cells were attached to the slide, fixed with 4% paraformaldehyde and permeabilize with 0.1% Triton X-100 in 1X PBS. Anti-V5 antibodies (Invitrogen) were used as primary antibody (1:200) and FITC antibodies (Jackson) were used as secondary antibody (1:100). Cell nuclei were stained by DAPI (1:1000). The slide was mounted with Fluorescence mounting medium (Dako) and visualized under fluorescence and confocal microscope.

2.5. Cell proliferation assay

This assay was performed by colorimetric MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-dipheyltetrazolium bromide, a tetrazole) assay, MTT is reduced to formazan by mitochondrial reductase in living cells. Purple formazan was dissolved by DMSO (dimethyl sulfoxide) to measure the absorbance of 570nm. Cells were collected and seeded in 96-well plate (for stable clones, 2500 cells were seeding in each well; for transient transfection, 1×10^4 cells were seeding in each well), 50μg MTT was added to each well and cultured for 90 min then formazan was dissolved by DMSO. The value of 570nm was measured after platting cell for 24, 48, 72, 96 and 120 hours. This experiment was performed triplicate.

2.6. Colony formation assay

Cells were counted (for stable clones, 2500 cells were seeding in each well; for transient transfection, 1×10^4 cells were seeding in each well) and mixed with half of 7% low melting point agarose (Sigma) to 6-well plate and incubated at 37^0C with 5 % CO_2 environment. After cultured for 21 days, the colonies can be visualized by naked eye, colonies were stained with 5% crystal violent (Sigma) and cell number were counted. The experiment was performed triplicate.

2.7. Matrigel invasion assay

The assay was performed in Transwell Permeable Supports (Corning) with 8.0 μm pore size. 30 μg matrigel (R&D) was coated on each transwell insert and incubated at 37^0C more than one hour. $2x10^5$ cells were harvested and resuspended in serum-free medium to each transwell, complete medium was added to the lower chamber. After incubation at 37^0C for 24 hours, invaded cells in the upper chamber were fixed with 100% methanol and stained with Giemsa (Sigma). Cell number was counted under 200X microscope field. The experiment was performed triplicate.

CHAPTER 3. RESULTS

3.1. Construction of PCDH10 mammalian expression vectors

According to NCBI RefSeqs, the coding sequence of *PCDH10* variant 1 and variant 2 are 3123bp (NM_032961) and 2691bp (NM_020815), respectively. The difference between these two variants is the C-terminal portion of the cytoplasmic domain (Figure 1). In our previous data, two mRNA variants of *PCDH10* were only highly expressed in MCF7 cell line, and weak expressed in HCT116 and HT29,but were not detected in other CRC cell lines, including HCT15, KM12, SW620, SW480, SW48, COLO205, HCC2998, CaCo2, LoVo and T84. To obtain an effective template for cloning, MCF7 cDNA was chosen as PCR template. For TA-cloning, primers were designed with BamHI and XbaI restriction enzyme cutting sites, the stop codon was deleted and the whole coding sequence was in-frame within mammalian expression vector, pcDNA3.1/V5-His-TOPO, to produce a protein fused with V5 epitope and His tag (Figures 2 and 3). Two point mutations (G401A, T2807C) were found after sequencing during *PCDH10* variant 1 vector construction; therefore, site-directed mutagenesis was performed to correct the mutations as *PCDH10* RefSeqs in NCBI. Construction of *PCDH10* variant 2 was performed by Chao-Hua Fu with similar cloning strategy. PCDH10.v1 and PCDH10.v2 were used for PCDH10 variant 1 and variant 2 constructed into pcDNA3.1/V5-His-TOPO.

3.2. PCDH10 expression in HEK293T

To confirm whether two mRNA variants of *PCDH10* can express successfully in human mammalian cell lines, an easy-transfected cell line, HEK293T, was used. PCDH10.v1, PCDH10.v2 and pcDNA3.1/V5-His-TOPO plasmids were transfected into HEK293T. After transfection for 48 hours, transfected cells were collected and lysed by RIPA buffer. Protein expression was detected by western blotting. Due to PCDH10 protein was fused with V5 epitope and His tag, the protein expression was detectable by either mouse anti-His antibody (Zymed) or mouse anti-V5 antibody (Invitrogen). Result of western blotting showed both *PCDH10* variant 1 and variant 2 were successfully expressed in HEK293 cell line (Figure 4).

3.3. HCT116 as a model cell line

For investigating the functions of *PCDH10* in colorectal cancer, an appropriate cell model was established in advance. A high invasive cell line was searched to identified the putative tumor suppressor gene, *PCDH10*. The matrigel invasion assay was performed with 6 CRC cell line, including HCT116, HCT15, HT29, KM12, SW620, and SW480. The results of the invasion assay showed HCT116 is the highest invading cell followed by SW480 and SW620 cells, and the others almost do not invade (Figure 5). Eventually, HCT116 is chosen as our model cell line in this study.

3.4. PCDH10 expression in HCT116

PCDH10.v1, PCDH10.v2 and pcDNA3.1/V5-His-TOPO plasmids were transfected into HCT116. After transfection for 48 hours, transfected cells were collected and the protein expression was checked by western blotting. Results of western blotting revealed that both *PCDH10* variant 1 and variant 2 were successfully expressed in HCT116 cells (Figure 6A).

3.5. Inhibition of cell invasion ability by transient over-expression of *PCDH10* variant 1

To verify the effect of *PCDH10* on HCT116, matrigel invasion ability was performed. The invading cell numbers of *PCDH10* variant 1 transfected cells were inhibited about 30% that of mock cells. Whereas, the invading cell numbers of *PCDH10* variant 2 transfected cells were no difference from that of mock cells (Figures 6B and 6C).

3.6. Suppression of cell proliferation by transient over-expression of *PCDH10*

Cell proliferation was measured by MTT assay and performed after transfection for 48 hours. Mock, PCDH10 variant 1 and variant 2 transfected cells were suspended in RPMI-1640 medium with 800μg/mL G418 and harvested for MTT assay after cell seeding for 24, 48, 72, 96 and 120 hours. The growth of cells transfected with *PCDH10* variant 1 or variant 2 were inhibited since cell seeding for 48 hours (Figure

7).

3.7. Stable expression clones of PCDH10 in HCT116

For investigating the effects of *PCDH10* variant 1 and variant 2 in CRC, PCDH10.v1, PCDH10.v2 and pcDNA3.1/V5-His-TOPO were transfected into HCT116. After transfection for 48 hours, 1200μg/mL of G418 was added to select PCDH10-expressing stable clones at least for 2-3 weeks. As for mixed stable clone only mRNA expression, but not protein, could be detected in the cells. Therefore, we further selected PCDH10 expressing single stable clones. For single stable clone seletion, transfected cells were collected after transfection for 48 hours, diluted to 1:10, and then spreaded on 100 mm dish with 1200 μg/mL of G418. When single colony was big enough to pick up, it was transferred into 24 well plate. Protein expression was performed with adequate cell numbers. As a result, one out of 88 clones of PCDH10.v1, and one out of 53 of PCDH10.v2 were obtained and protein expression was confirmed by western blotting. In addition to one PCDH10.v1 stable clone picked up by Tzu-Ming Jao, we totally have two of PCDH10.v1 single stable clones, one of PCDH10.v2 single stable clones, and three of PCDH10.v1v2 single stable clones. Finally, two of PCDH10.v1 single stable clones and one of PCDH10.v2 single stable clone were used to perform gene functional assays.

3.8. Suppression of cell proliferation and colony formation ability in one

PCDH10 variant 1 stable clone

MTT assay and colony formation assay were performed with PCDH10.v1-1, PCDH10.v1-2 and PCDH10.v2-1 single stable clones. Protein expression of three stable clones was detectable by western blotting (Figure 8). In cell proliferation assay (MTT assay), growth rates of PCDH10.v1-1 and PCDH10.v2-1 have no significant difference with mock cells. However, PCDH10.v1-2 has lower growth rate compared to mock cells (Figure 9). In colony formation assay, colony numbers of PCDH10.v1-1 and PCDH10.v2-1 were similar to that of mock cells; while PCDH10.v1-2 had few colony number compared to mock cell (Figure 10). As a result, cell proliferation and colony formation were reduced in PCDH10.v1-2 single stable clones.

3.9. PCDH10 variant 1 and variant 2 expressed on cell membrane

We used two methods to determine PCDH10 protein localization in cells. One was construction of PCDH10 protein fused with GFP at the C-terminal portion, by which protein distribution can be observed directly by fluorescent microscope. PCDH10.v1-GFP and PCDH10.v2-GFP vectors were constructed by direct-cloning (Figure 11). PCDH10-GFP constructs were transfected into HEK293 and HCT116 cells. After transfection for 48 hours, protein localization in both of HEK293T and HCT116 cells, GFP protein only distributed in nucleus and cytoplasm, however, PCDH10 variant 1 and variant 2 were expressed on cell membrane exclusively were

observed by confocal microscope (Figure 12, 13, 14). The nucleus of HEK293T is large and had less cytoplasm is less than HCT116, therefore, identification of PCDH10 protein distributed on cell membrane is a little bit difficult. Nevertheless, because of the higher percentage of cytoplasm in HCT116, the protein distribution can be visualized more easily. In addition, we also performed immunofluorecent stain with PCDH10.v1-2 single stable clone to detect protein localization by using anti-V5 antibody as primary antibody. PCDH10 variant 1 protein were distributed on cell membrane and few perinuclear regions of PCDH10.v1-1 single stable clone (Figure 15).

3.10. PCDH10 variant 1 and variant 2 are co-localized with homophilic interaction

To observe the co-localization of PCDH10 variant 1 and variant 2, PCDH10.V1 and PCDH10.V2-GFP constructs were co-trasnfected into HCT116, in which PCDH10.V1 can be detected via immunofluorecent stain with Cy3-labeled antibody, and PCDH10 variant 2 protein fused with GFP can be observed directly. Additionally, three controls were set up as following: (1) pcDNA3.1/V5-His-TOPO and pEGFP-N3 vectors; (2) PCDH10.V1 and pEGFP-N3; (3) pcDNA3.1/V5-His-TOPO and PCDH10.V2-GFP. In control (1), only GFP protein was detectable in whole cell. In control (2), PCDH10 variant 1 protein was shown by red fluorescence that distributed in cytoplasm and on

cell membrane, while GFP protein still expressed in whole cell. In control (3), PCDH10 variant 2 fused with GFP was observed on cell membrane. When PCDH10.v1 and PCDH10.V2-GFP were co-transfected into HCT116 cells, both PCDH10 variant 1 and 2 proteins were in cytoplasm and on cell membrane, suggesting these two proteins co-localized in the cells (Figure 16).

CHAPTER4. DISCUSSION

Most *PCDH10* studies indicated its important role in species development [33, 34, 37-39, 53]. Although there is little knowledge about the *PCDH10* gene function in human. Recently, *PCDH10* variant 1 was reported as a candidate tumor suppressor gene in hematologic, esophageal, nasopharyl and gastric cancers [43-45]. According to the preliminary data of LOH study in colorectal cancer tissue pairs at our lab, *PCDH10* might be also involved in CRC carcinogenesis. *PCDH10* expression is silenced by DNA methylation in CRC primary tissues and cell lines, in which gene expression could be restored by treatment of a demethylation drug, 5-aza-2'deoxycytidine. Moreover, *PCDH10* variant 1 showed higher expression in normal mucosa than in tumors from CRC patients via RT-PCR and real-time PCR analysis.

Almost no endogenous mRNA expression of PCDH10 variants were detected in 12 CRC cell lines at our lab, we cannot silence PCDH10 gene expression but instead over-expression of this gene at the beginning. Following the studies of *PCDH10* at our lab, this thesis is focused on *PCDH10* function in CRC cell line, HCT116. At first, *PCDH10* mammalian expression vectors were constructed, and then transfected into HCT116 for gene over-expression. In transient transfection, effects of *PCDH10* variant 1 expression decreased cell invasion ability and cell proliferation. On the other hand, *PCDH10* variant 2 over-expression suppressed only cell proliferation but not invasion

ability. For single stable clones, two PCDH10 variant 1 single stable clones revealed different results in MTT and colony formation assays. PCDH10.v1-2 expressing higher *PCDH10* variant 1 protein suppressed cell proliferation and cell colony formation ability. However, PCDH10.v1-1 clone with low *PCDH10* variant 1 protein expression did not inhibit cell growth ability. We suppose that the influence of *PCDH10* variant 1 protein depends on its protein expression level. PCDH10.v1-1 clone might reduce PCDH10 protein expression after several passages, and then maintain PCDH10 variant 1 protein with low expression for many generations. Actually, we performed MTT assay for PCDH10.v1-1 at its earlier passage. The protein expression of PCDH10.v1-1 was quite high and the cell proliferation seemed to be inhibited. However, after 2-3 more passages, MTT assay and colony formation assay were performed as shown in Figure 9 and 10, PCDH10.v1-1 had no different phenotype compared with mock cells. As a result, we suggest that *PCDH10* variant 1 might suppress cell proliferation, which is correlated with protein expression level.

After screening all cell lines at our lab by RT-PCR, only MCF7 showed strong mRNA expression of *PCDH10* variant 1 and variant 2. Almost no *PCDH10* variant 1 expression could be detected in CRC cell lines held in our lab. Therefore, MCF7 cDNA was chosen as template for construction of *PCDH10* mammalian expression vector. Two point mutations were found after full-length sequencing, three possibilities

could be speculated. First, two point mutations may originate from MCF7 genomic DNA. Second, the mutations occurs during reverse transcription because reverse transcriptase has no proofreading function and induces some bases error. Last, they may be the PCR artifacts. KOD polymerase, which we used in PCR has proofreading ability, however, some mis-incorporations would occured occasionally.

HEK293T (human embryonic kidney 293 cell with the SV40 large T antigen) is a good cell model to perform gene transfection. To establish the transfection protocol at our lab, HEK293T was transfected with *PCDH10* mammalian expression vectors at first. The predicted molecular weights is about 116 kDa for *PCDH10*.v1, and is about 95 kDa for *PCDH10*.v2. Analysis by western blotting, the transfected cell lysates showed the molecular weight is about 140kDa for PCDH10.v1, and is about 115 kDa for PCDH10.v2. The results confirm the hypothesis that glycosylation is involved in PCDH10 post-translation modification.

In literature reviews, HCT116 is one of the most common cell lines used in CRC studies because of its high invasion ability. Invasion assay of 6 CRC cell lines have the same result. One more advantage of HCT116 is its high proliferation rate, to avoid experiments were restricted with cell growth for a long period of time. Even by treatment with different concentration of G418 (800, 1200 and 1600μg/mL), selection of mixed *PCDH10* stable clones still failed in protein detection by western blotting,

although mRNA expression was detectable. Several studies for *PCDH10* in nasophary, esophageal and gastric cancer, only showed PCDH10 mRNA expression in stable clones [43, 45]. Until the single stable clones were established, the *PCDH10* protein expression could be detected and the protein levels were quite low compared to transient protein expression. However, we only obtained two PCDH10.v1 stable clones, one of PCDH10.v2 stable clones and three of PCDH10.v1v2 stable clones after screening for at least 50 clones for each transfection. Some single stable clones lost its PCDH10 protein expression at early generation or after cell passaging for several generations. Our preliminary study demonstrated that *PCDH10* was regulated by promoter hyper-methylation and some epigenetic events were reported in CRC [17], suppose that *PCDH10* stable clones are difficult to be maintained because high expression of PCDH10 hinders cell growth. Consequently, the results of transient expression experiments showed that over-expression of *PCDH10* caused cell death or apoptosis and as a negative control, HCT116 cells almost died on the third day after G418 treatment, suggesting the G418 concentration is appropriate for positive selection of transfected cells (Data not shown). Taken together, it might be reasonable that only few single stable clones with low protein expression were obtained in the study. In addition, stable clones transfected with PCDH10.v1 and v2 were also selected. Only three out of 61 clones of PCDH10.v1v2 were selected and analyzed by western

blot, only PCDH10 variant 1 protein, but not variant 2 can be detected in PCDH10.v1v2 stable clones.

Localization of PCDH10 was determined directly and indirectly in this study. Both of PCDH10 variant 1 and variant 2 were shown on cell membrane and might co-localize. Furthermore, the homophilic interaction of PCDH10 variant 1 was shown in PCDH10.v1-2 cells, supporting the report of mouse *Pcdh10* [33].

Protocadherin family is a member of cadherin superfamily. Cadherins are well-known cell adhesion molecules with strong interaction between cells. Protocadherins have difference cytoplasmic domain with cadherins, and shown weaker interaction in adhesion ability, suggesting that they may have other important functions other than cell-cell adhesion. Recently, more protocadherins were reported and might be involved in tumorigenesis [48, 50, 54-59]. *PCDH10* variant 1 also is a new identified tumor suppressor gene in breast cancer [42], nasopharyngeal, esophageal carcinomas [43], hematologic malignancies [43, 44] and gastric cancer [45]. Combined with the results for our study that over-expression of *PCDH10* variant 1 would suppress cell invasion and proliferation ability depending on the protein expression level. *PCDH10* variant 1 might be the tumor suppressor gene associated with CRC.

PCDH10 variant 1 and variant 2 mRNA transcripts may share the same promoter because they only have a short different region in cytoplasmic domain. We suppose

that the difference between the cytoplamic domain may deliver different signals in cells. In our previous data, mRNA expression of *PCDH10* variant 1 was down-regulated in CRC tumor tissues and *PCDH10* variant 2 mRNA expression was only detected in certain samples which have high PCDH10 variant 1 expression. The results in this study also reveal that over-expression PCDH10 variant 2 of seems not to influence cell phenotypes, suggesting that PCDH10 variant 2 might be a by-product during the transcription of PCDH10 variant 1.

In conclusion, *PCDH10* variant 1 might be a tumor suppressor gene in colorectal tumorigenesis and also expressed on cell membrane to carry out its function. On the other hand, *PCDH10* variant 2 is also expressed on cell membrane and co-localizaed with *PCDH10* variant 1. However, more experiments need to be designed to identify whether these two variants have any cross-interaction in cells.

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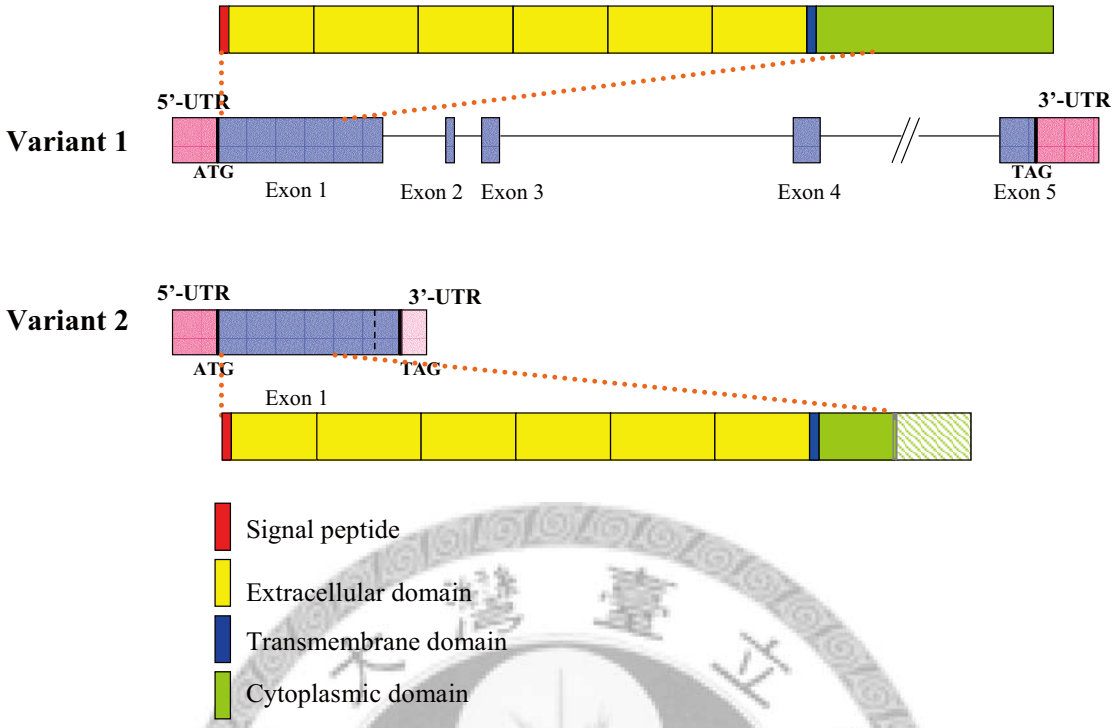


Figure 1. Two mRNA transcripts of *PCDH10*

According to NCBI, *PCDH10* has 2 variants named for variant 1 and variant 2. *PCDH10* gene has 5 exons and variant 1 mRNA is composed of all of the 5 exons. While variant 2 transcripts read through Exon 1 and stop at intron between Exon 1 and Exon 2 which contains a poly-A like sequence. As for protein structure, both of variant 1 and variant 2 have one signal peptide, six extracellular cadherin domains, one transmembrane domain and one cytoplasmic domain. The difference between these two proteins is the C-terminal parts of cytoplasmic domains.

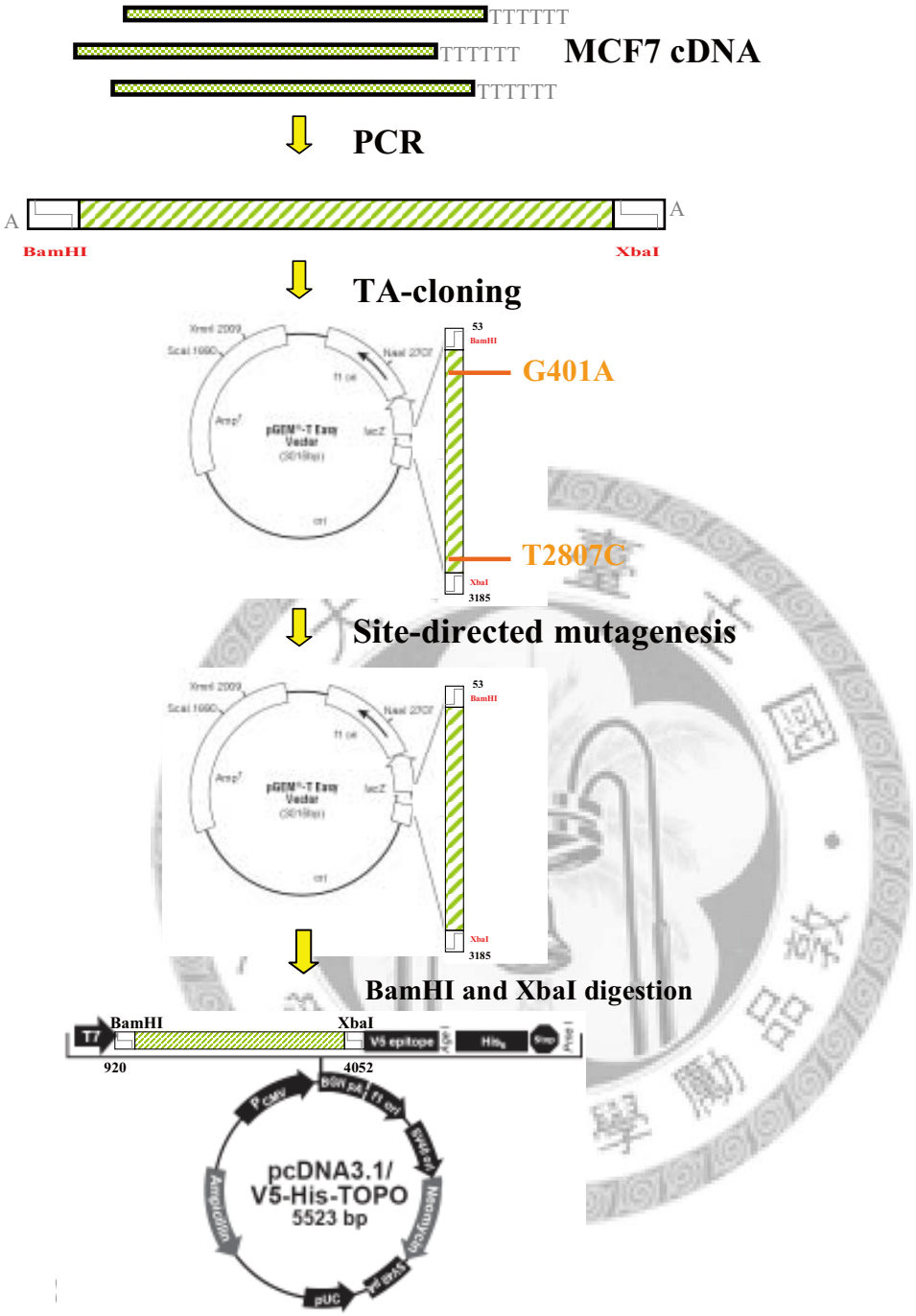


Figure 2. Cloning strategy of *PCDH10* variant 1 mammalian expression vector. *PCDH10* variant 1 cDNA fragments were synthesized by using MCF7 mRNA as template and were finally cloned into the pcDNA3.1/V5-His-TOPO vector. Two point mutations in MCF7 cDNA were corrected by site-directed mutagenesis. The construct was named as PCDH10.v1 in this thesis. [Construction of *PCDH10* variant 2 expression vector, named PCDH10.v2, was achieved by Chao-Hua Fu.]

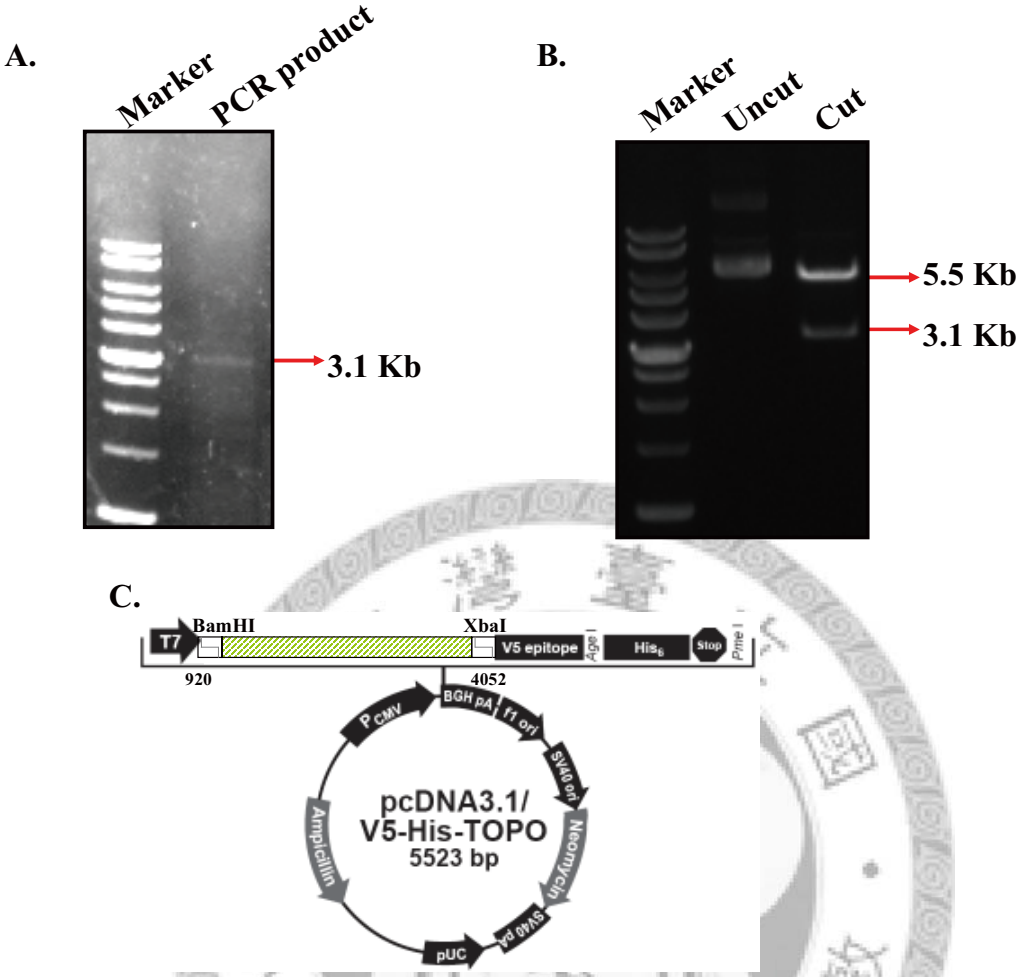


Figure 3. PCDH10 variant 1 construction.

(A) Full-length cDNA fragments of *PCDH10* variant 1 were checked by 1% agarose gel after PCR reaction by using MCF7 cDNA as template. (B) After TA-cloning, *PCDH10* variant 1 fragments were digested by BamHI and XbaI, extracted and ligated into pcDNA3.1/V5-His-TOPO vector. The PCDH10.v1 expression vector (Uncut) was checked by both restriction enzymes, BamHI and XbaI, digestion (Cut). (C) Characteristics of *PCDH10* variant 1 in pcDNA3.1/V5-His-TOPO vector. PCDH10 variant 1 was inserted into BamHI and XbaI cloning sites. PCDH10 protein is fused with V5 epitope and His tag for protein detection by western blotting. A mammalian antibiotic gene, neomycin, is used for stable clone selection.

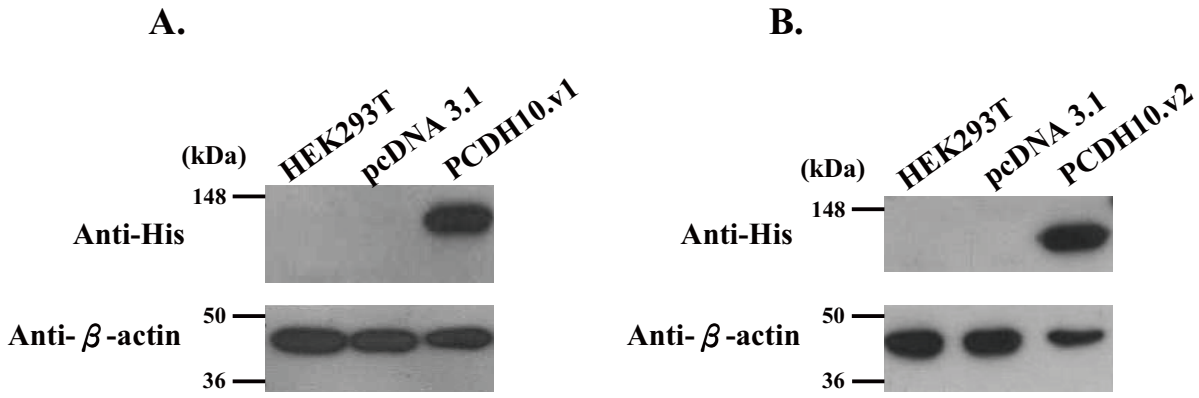


Figure 4. PCDH10 expression in HEK293T cells

Protein expression of PCDH10 in HEK293T cell was confirmed by western blotting. After transfection for 48 hours, transfected cells were collected and lysed by RIPA buffer. 50 μg for each protein lysate was loaded onto 8% SDS-PAGE for western blot analysis. The first antibodies are mouse anti-His antibody (1:2000, Zymed) and mouse anti-β-actin antibody (1:10000, Sigma, for protein loading control), and the second antibody is goat anti-mouse HRP antibody (1:8000). Both PCDH10 variant 1 and variant 2 proteins are successfully expressed in HEK293T. The predicted molecular weights of PCDH10 variant 1 and variant 2 are 116kDa and 95kDa ,respectively, which the detected molecular weights are about 140kDa and 120kDa for PCDH10 variant 1 (A) and variant 2 (B), suggesting that the proteins contain post-translation glycosylation.

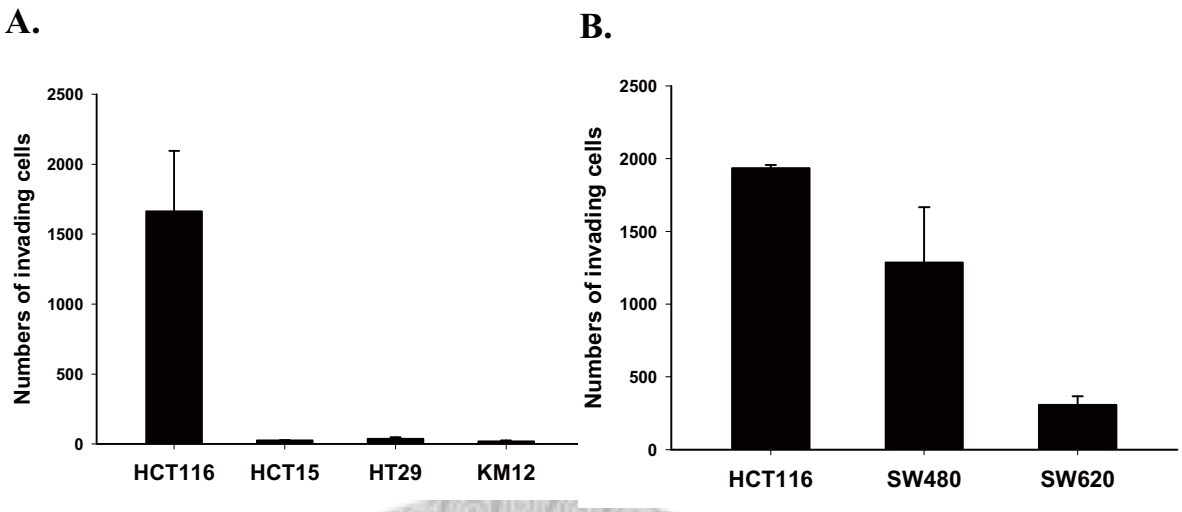


Figure 5. Invasive abilities of six CRC cell lines in matrigel invasion assay.

Matrigel invasion assay was performed with 6 colon cancer cell lines, HCT116, HCT15, HT29, KM12, SW480 and SW620. $2x10^5$ cells were seeded for each transwell and incubated at 37℃ for 24 hours. HCT116, SW480 and SW620 are more invasive than the other 3 cell lines (HCT15, HT29 and KM12). Four repeats were set for each cell line in the assay and data were shown by mean ± SD.

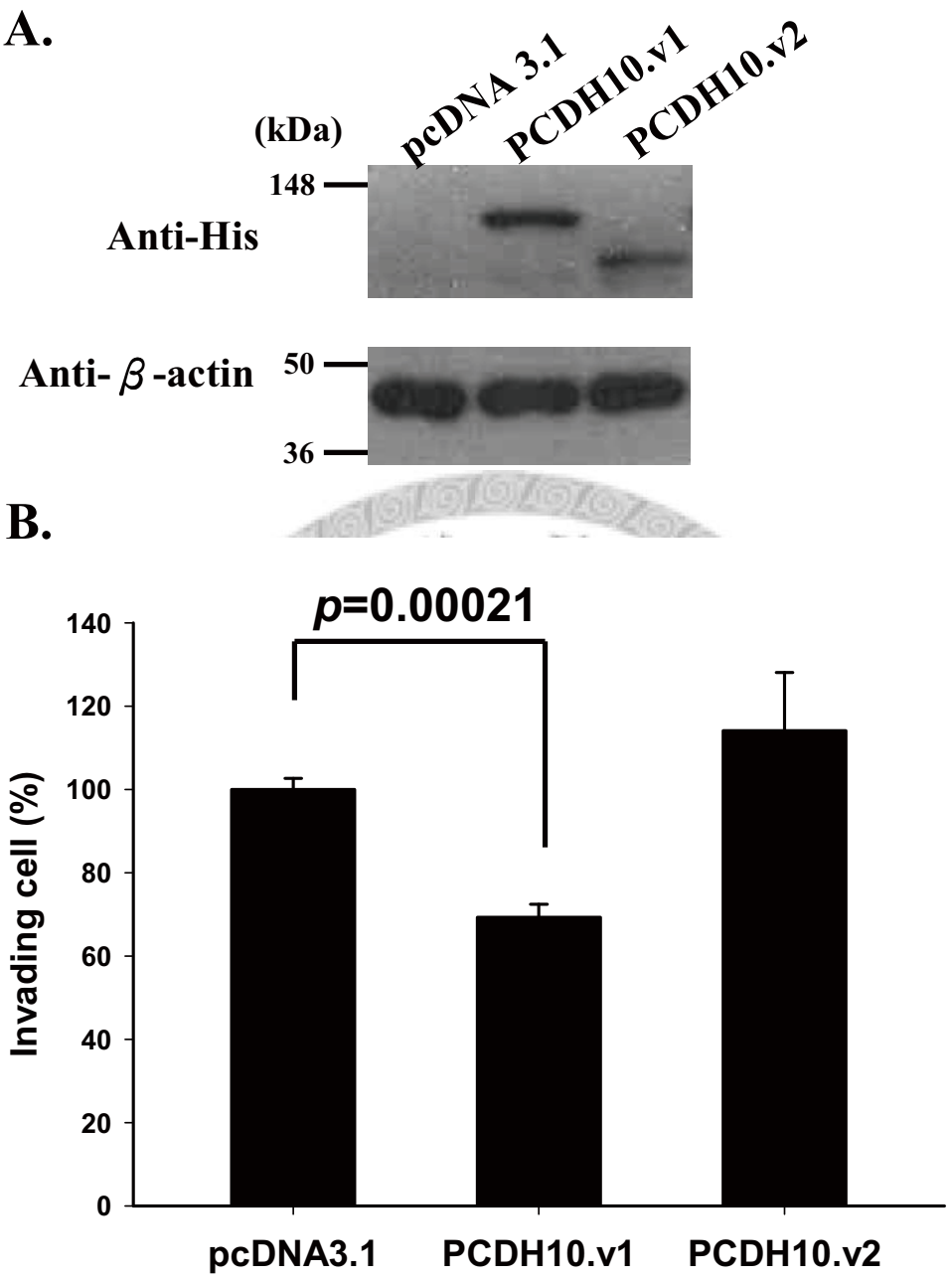


Figure 6. Suppression of cell invasion ability by transient over-expression of PCDH10 variant 1.

After transfection for 48 hours, protein expression of PCDH10 in HCT116 cells was confirmed by western blot analysis (A), and matrigel invasion assay was performed with PCDH10 transfectants (B). Cell invasion ability was reduced by over-expression of PCDH10 variant 1, but not by variant 2. Three repeats were set for each cell line in the assay and data were shown by mean ± SD of three repeats. Statistical analysis was performed by student's *t*-test.

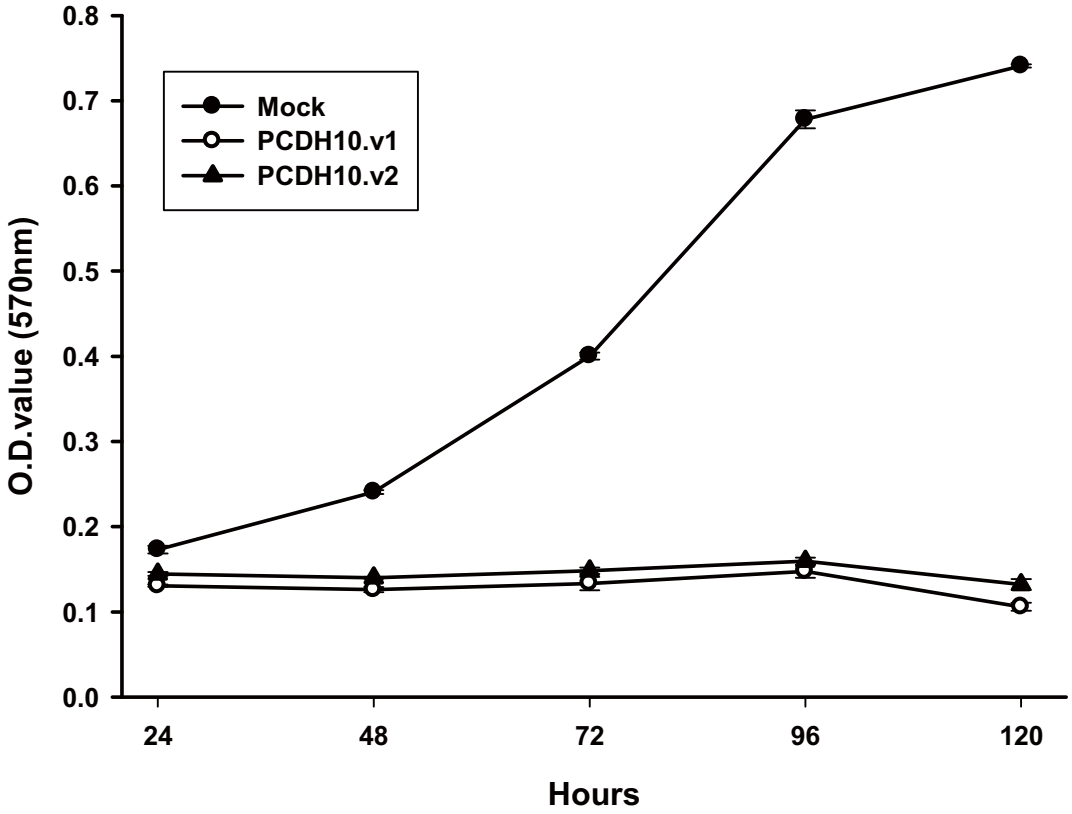


Figure 7. Inhibition of cell proliferation by transient over-expression of PCDH10 variant 1 and variant 2.

Cell growth was measured by MTT assay. After transfection for 48 hours, cells were seeded in 96-well plates and cultured in RPMI-1640 medium with 800 μg/mL of G418. After seeding for 24, 48, 72, 96 and 120 hours, MTT assay was performed. Cell growth was suppressed both in over-expression of PCDH10 varaiant 1 and variant 2 compared to cells which were transfected with pcDNA3.1/V5-His-TOPO (mock). Data were shown by mean ± SD of three repeats of each cell line. Statistical analysis was performed by student's *t*-test, and $p<0.0001$ was found for PCDH10 variant 1 or variant 2 versus mock at 48, 72, 96 and 120 hours.

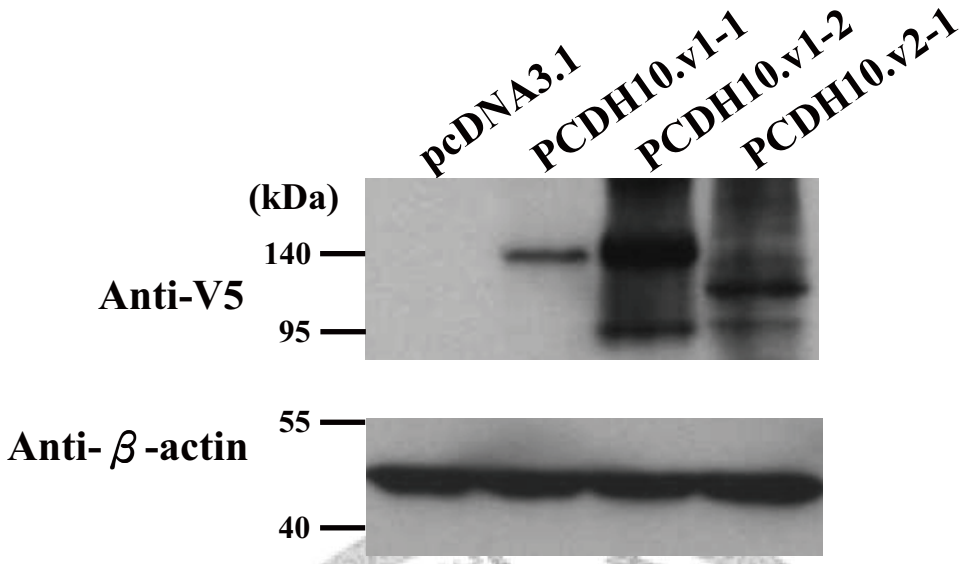


Figure 8. Protein expression of PCDH10 single stable clones.

Protein expression of PCDH10 single stable clones of HCT116 was confirmed by western blot analysis. Cell pellets of three single stable clones were collected and lysed by RIPA buffer. 50 μg of protein lysate for each sample were loaded onto 8% SDS-PAGE for western blot analysis. The first antibodies are mouse anti-V5 antibody (1:5000) and mouse anti β-actin antibody (1:10000), and the second antibody is goat anti-mouse HRP antibody (1:8000). Both PCDH10 variant 1 and variant 2 proteins are successfully expressed in HCT116. The predicted molecular weights of PCDH10 variant 1 and variant 2 are 116kDa and 95kDa ,respectively, which the detected molecular weights are about 140kDa and 120kDa for PCDH10 variant 1 (A) and variant 2 (B), suggesting that the proteins contain post-translation glycosylation..

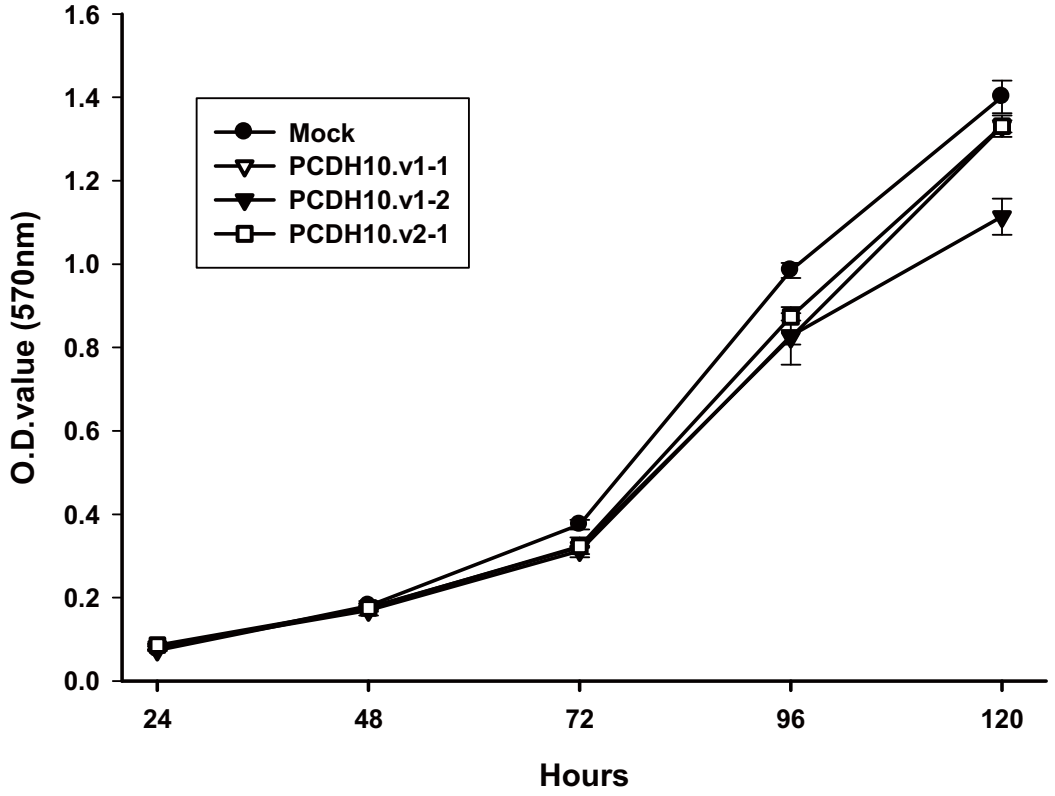


Figure 9. Suppression of cell proliferation in PCDH10.v1-2 single stable transfectant.

Cell growth was measured by MTT assay. Cell growth was suppressed only in PCDH10.v1-2 transfectant, but not in PCDH10.v1-1 and PCHD10.v2-1 transfectants compared to mock cells. Data was shown by mean ± SD of three repeats. Statistical analysis was performed by student's *t*-test, and $p<0.0001$ was found for PCDH10.v1-2 versus mock at 120 hours

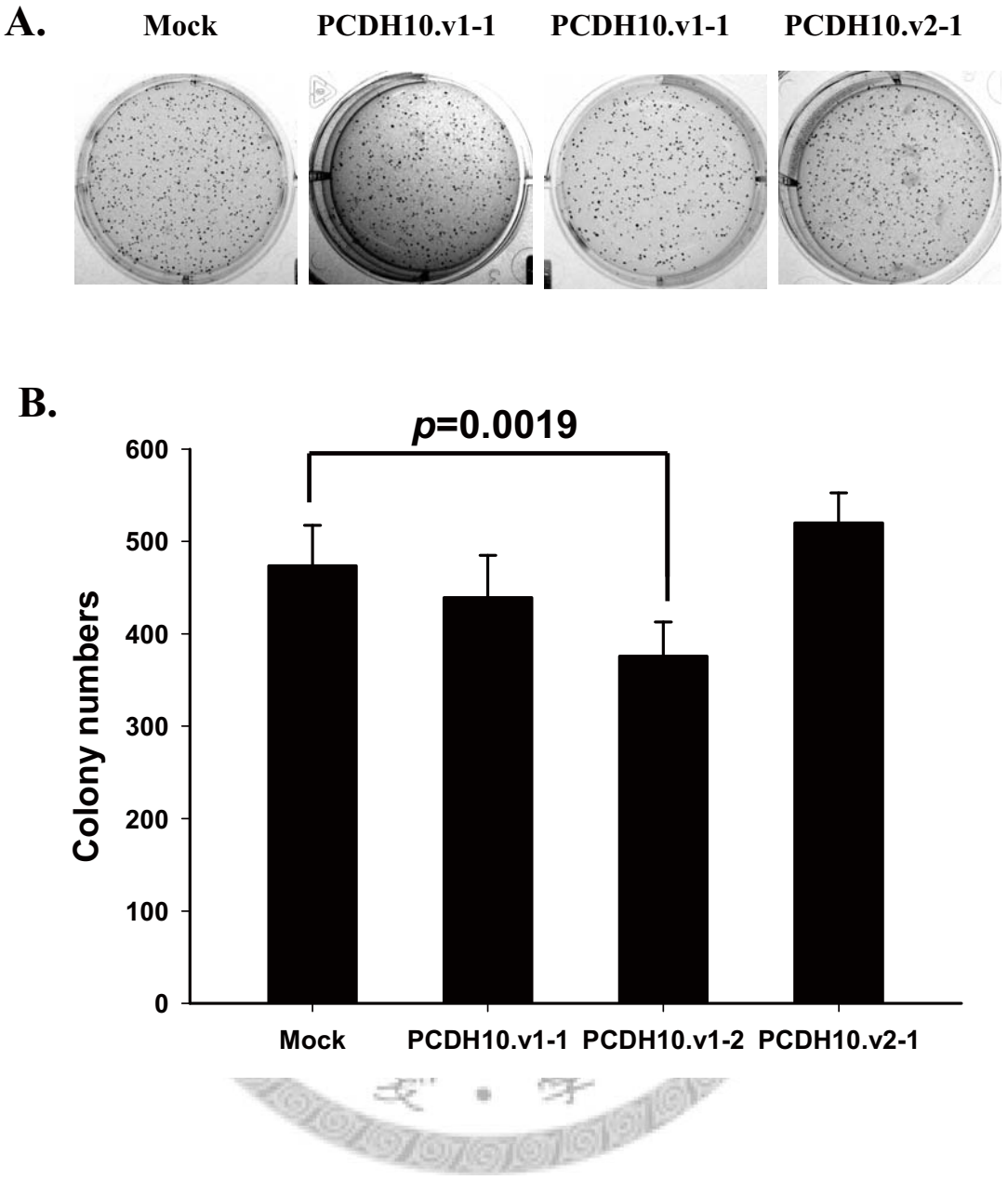


Figure 10. Suppression of colony formation ability in PCDH10.v1-2 single stable clone.

Colony formation assay was performed with PCDH10 stable clones. 2000 cells was seeded in six-well plates for 21 days. Colonies were stained by crystal violet and photographed for each transfectants (A) and the colony numbers were counted and compared (B). Colony numbers were shown by mean ± SD. Statistical analysis was performed via student's *t*-test, and p=0.0019 was found for PCDH10.v1-2 compared with cells transfected with pcDNA3.1/V5-His-TOPO (Mock). Six repeats were set up for each cell in the assay.

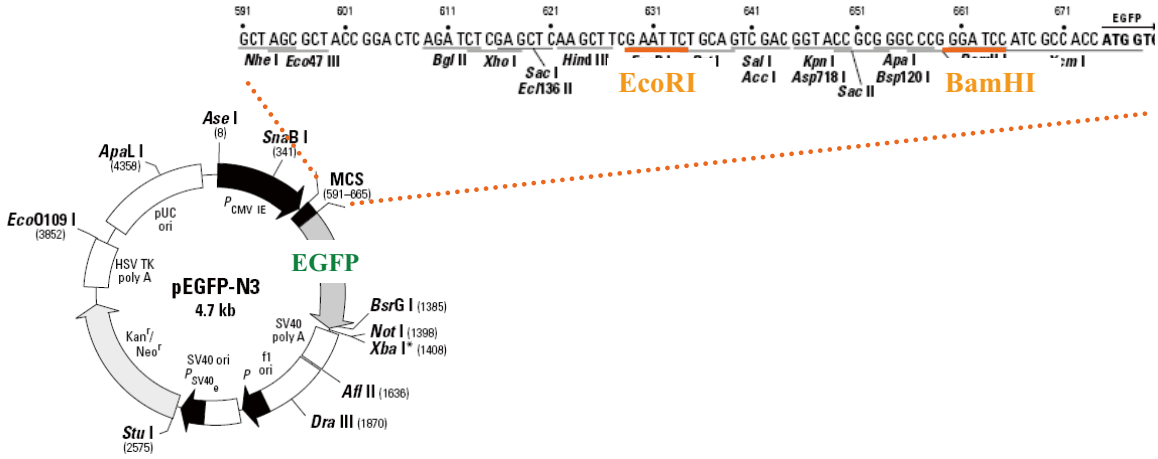


Figure 11. Construction of PCDH10-GFP mammalian expression vector.

To verify localization of PCDH10 proteins directly, PCDH10 variant 1 and variant 2 cDNA were cloned into pEGFP-N3 vector to express PCDH10 fused with GFP protein.

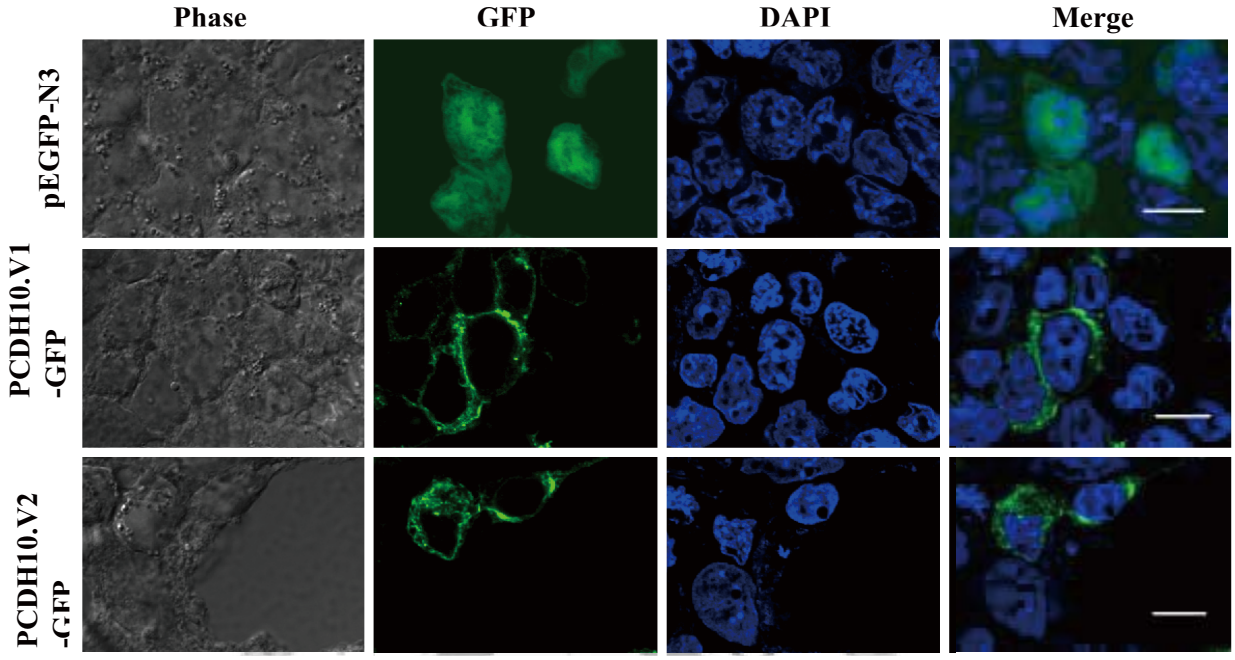


Figure 12. PCDH10 distribution in HEK293T cells.

Subcellular localization of PCDH10 was directly determined via GFP fusion proteins. PCDH10.V1-GFP, PCDH10.V2-GFP and pEGFP-N3 plasmids were transfected into HEK293T. After transfection for 48 hours, cells were stained with DAPI. GFP protein was present in cell nucleus and cytoplasm, while PCDH10 variant 1 and variant 2 GFP fusion proteins were present on cell membrane and in cytoplasm. Images were photographed by using confocal microscopy. Bar= 10μm.

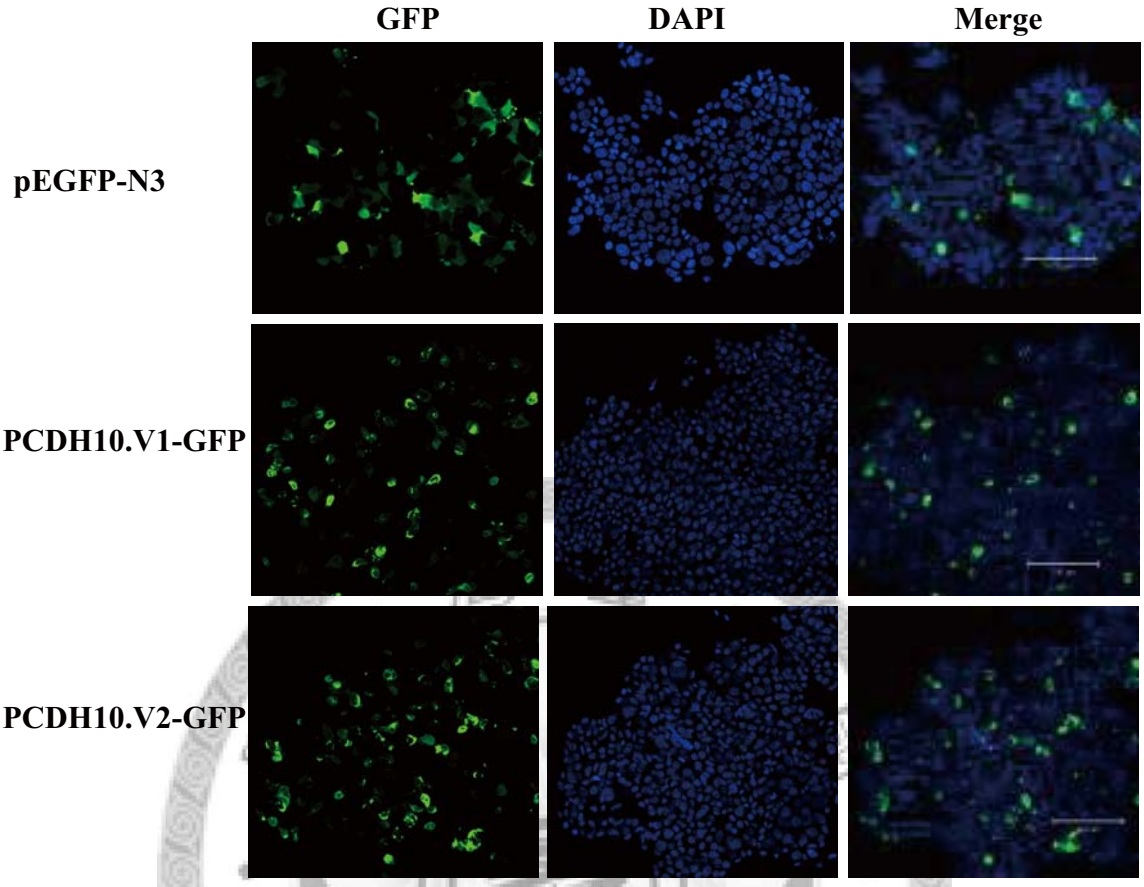


Figure 13. PCDH10 distribution in HCT116 cells.

Subcellular localization of PCDH10 was directly determined via GFP fusion proteins. PCDH10.V1-GFP, PCDH10.V2-GFP and pEGFP-N3 plasmids were transfected into HCT116. After transfection for 48 hours, cells were stained with DAPI. GFP protein was present in cell nucleus and cytoplasm, while PCDH10 variant 1 and variant 2 GFP fusion proteins were present on cell membrane and in cytoplasm. Images were photographed by using confocal microscopy. Bar= 100μm.

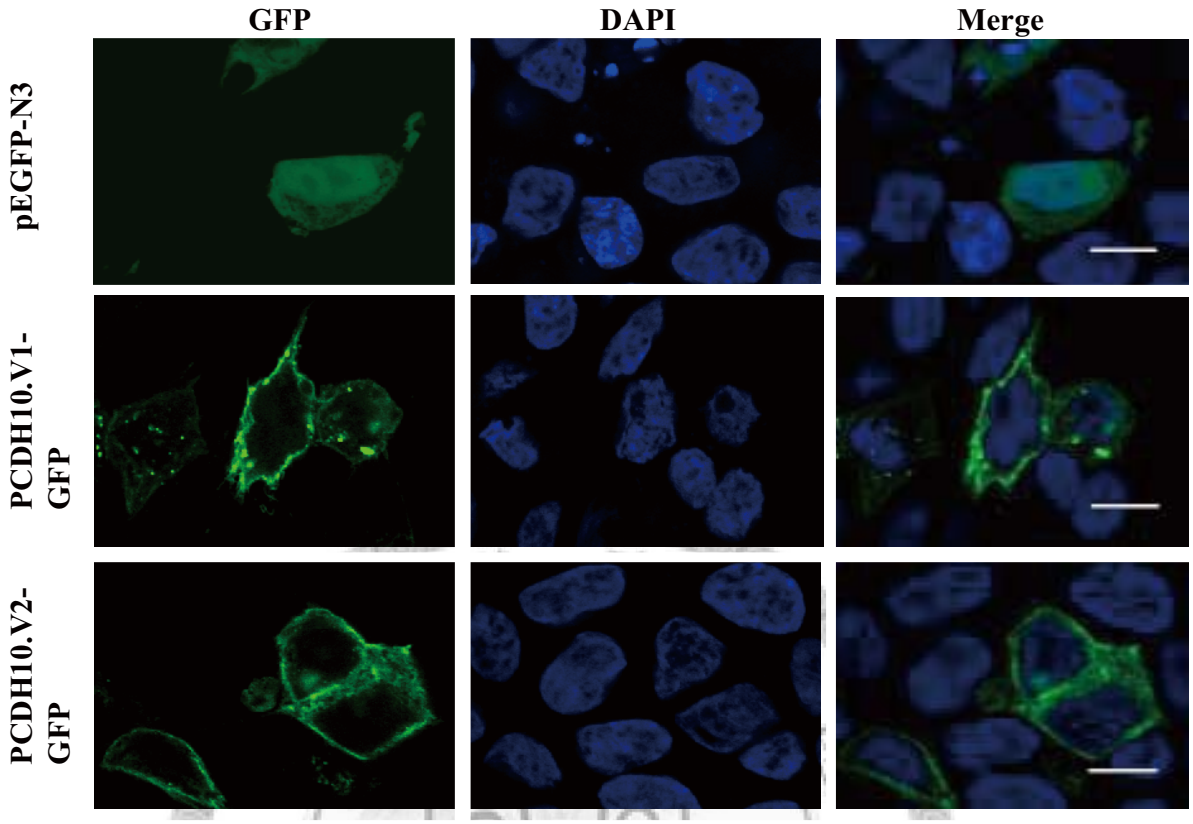


Figure 14. PCDH10 distribution in HCT116 cells.

Subcellular localization of PCDH10 was directly determined via GFP fusion proteins. PCDH10.V1-GFP, PCDH10.V2-GFP and pEGFP-N3 plasmids were transfected into HCT116. After transfection for 48 hours, cells were stained with DAPI. GFP protein was present in cell nucleus and cytoplasm, while PCDH10 variant 1 and variant 2 GFP fusion proteins were present on cell membrane and in cytoplasm. Images were photographed by using confocal microscopy. Bar= 10μm.

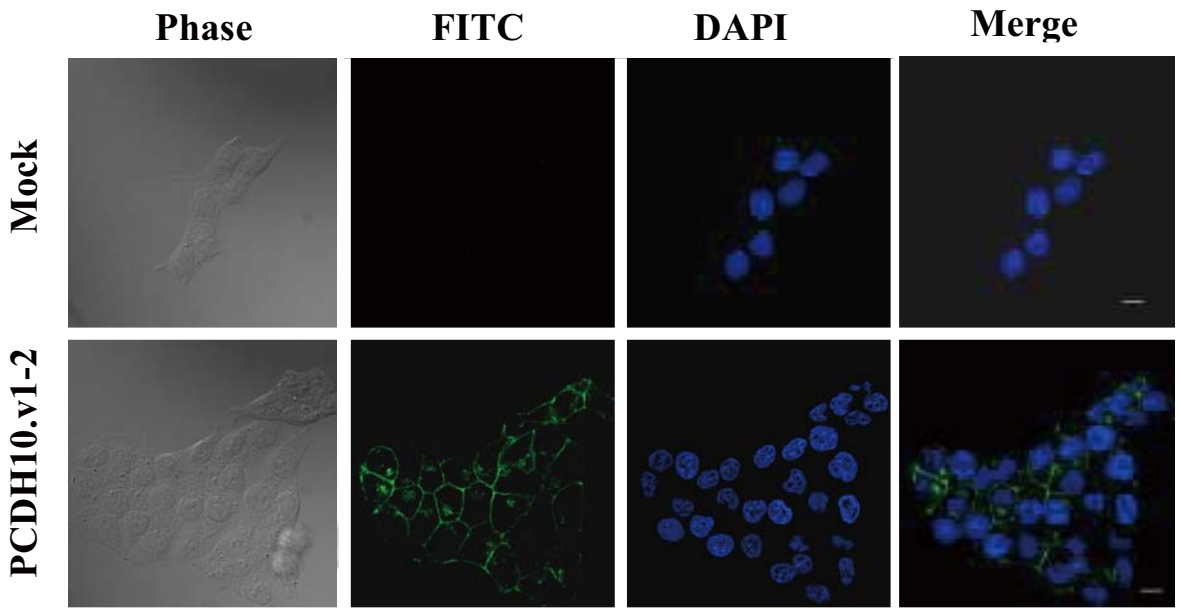


Figure 15. PCDH10 variant 1 protein distribution in PCDH10.v1-2 stable clone. Subcellular localization of PCDH10 variant 1 was determined by immunofluorescent stain with mouse anti-V5 antibody and the secondary antibody labeled with FITC. PCDH10 variant 1 was present on cell membrane and in some Golgi apparatus. Cell nuclei were stained with DAPI. Images were photographed by using confocal microscopy. Bar= 10μm. (630X)

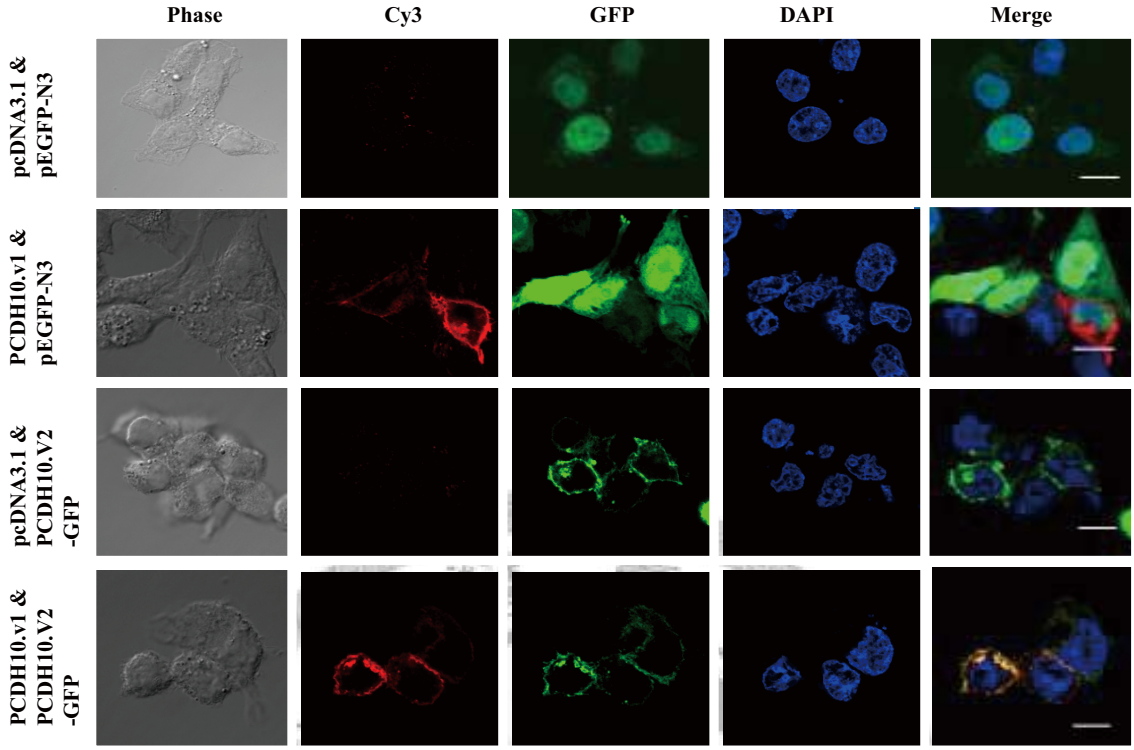
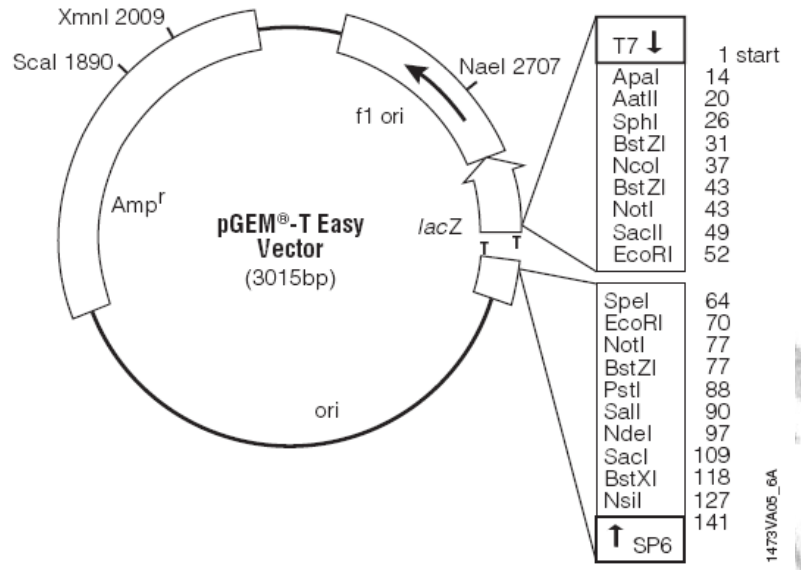


Figure 16. Co-localization of PCDH10 variant 1 and variant 2 in HCT116 cells.
Subcellular co-localization of PCDH10 variant 1 and variant 2 was determined by co-transfected PCDH10.v1 and PCDH10.V2-GFP plasmids into HCT116 cells. After transfection for 48 hours, cells were stained with anti-V5 antibody and followed with the secondary antibody labeled with FITC for PCDH10 variant 1 protein. Cell nuclei were stained with DAPI. Both PCDH10 variant 1 and variant 2 proteins both localized on cell membrane and perinuclear region. Images were photographed by using confocal microscopy. Bar= 10μm. (630X)

APPENDIX



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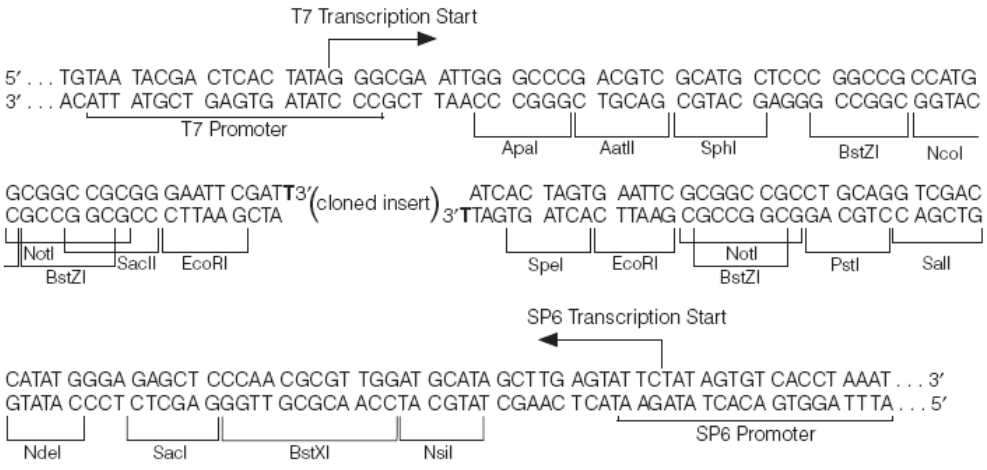
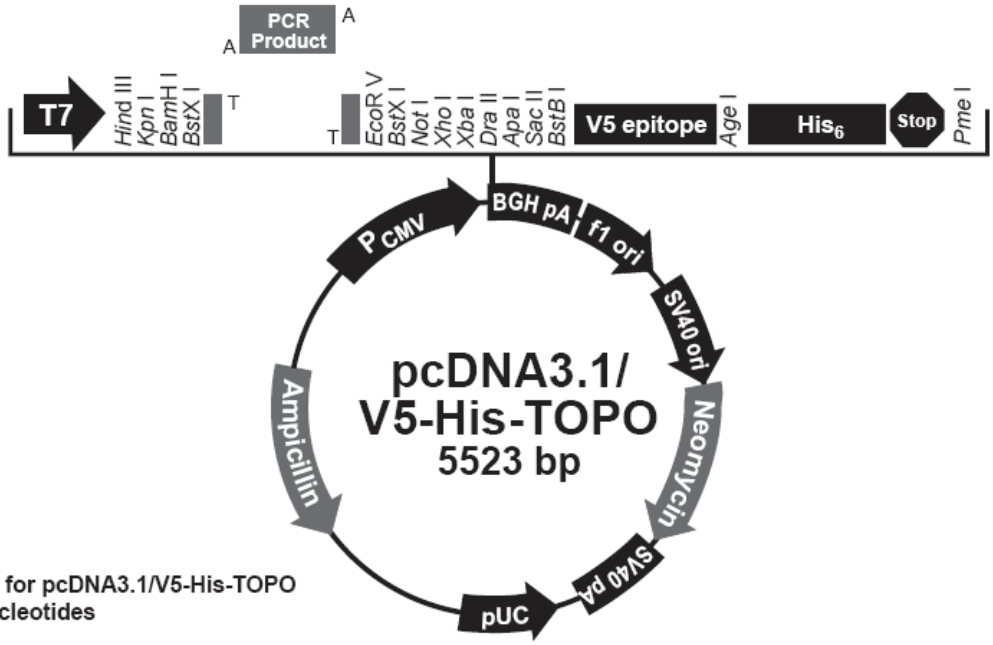
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

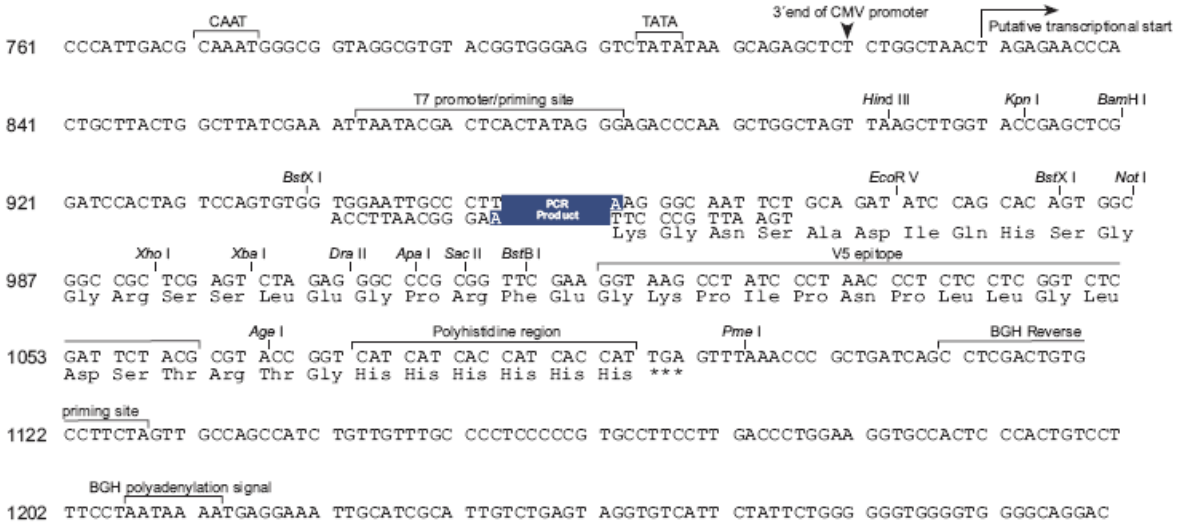


Appnedix 1. Map of pGEM-T Easy vector (Promega).

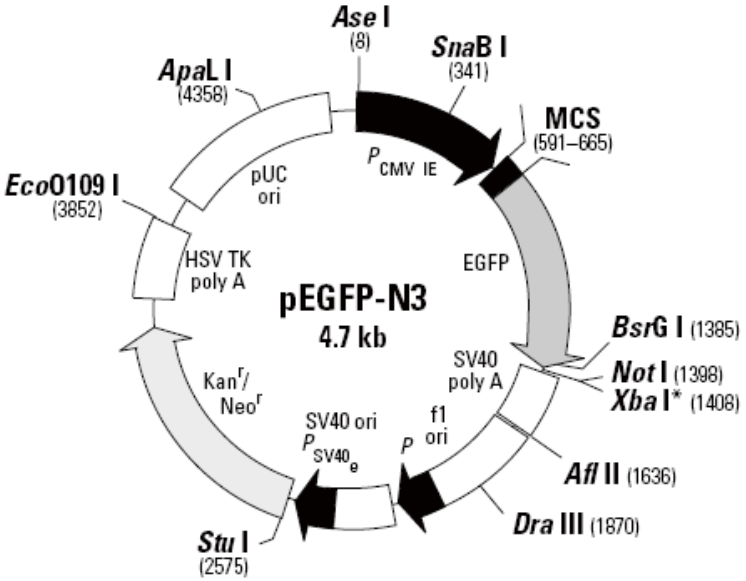


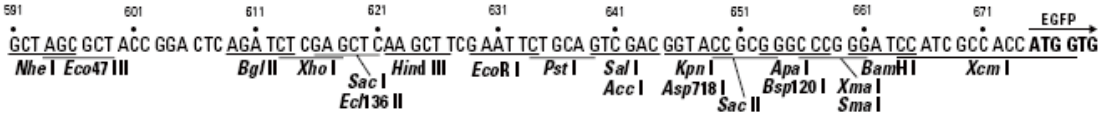
Comments for pcDNA3.1/V5-His-TOPO
5523 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 902-1019
TOPO® Cloning site: 953-954
V5 epitope: bases 1020-1061
Polyhistidine tag: bases 1071-1088
BGH reverse priming site: bases 1111-1128
BGH polyadenylation signal: bases 1110-1324
f1 origin of replication: bases 1387-1800
SV40 promoter and origin: bases 1865-2190
Neomycin resistance gene: bases 2226-3020
SV40 polyadenylation signal: bases 3039-3277
pUC origin: bases 3709-4382
Ampicillin resistance gene: bases 4527-5387



Appendix 2. Map of pcDNA3.1/V5-His-TOPO (Invitrogen).





Appendix 3. Map of pEGFP-N3 vector (Clontech).

Appendix Table 1. Plasmids used in this study.

Plasmid name	Gene	Vector
PCDH10.v1	PCDH10 variant 1	pcDNA3.1/V5-His-TOPO
PCDH10.V1-GFP		pEGFP-N3
PCDH10.v2	PCDH10 variant 2	pcDNA3.1/V5-His-TOPO
PCDH10.V2-GFP		pEGFP-N3

Appendix Table 2. Buffers used in this study.

Buffer	Formula
RIPA	50mM Tris, pH=7.5 150mM NaCl 1% NP40 0.5% Sodium deoxycholate 0.1% SDS 1mM EDTA 1mM PMSF 1x protease inhibitor was added before used
5x SDS-PAGE running buffer	125 mM Tris-HCl 960 mM Glycine 0.05% SDS pH=8.3
1x western transfer buffer	25 mM Tris-HCl 192 mM Glycine 20% Methanol pH=8.3
5x TBST	100 mM Tris-HCl 2.5 M NaCl 0.25% Tween 20 pH=7.4
1x PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4 pH=7.4