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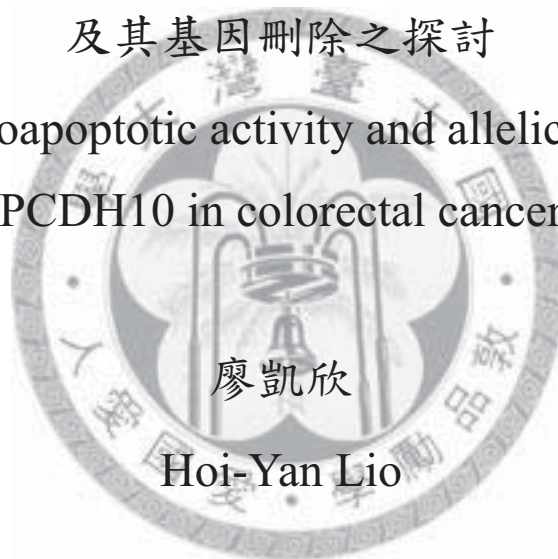
Master Thesis

PCDH10 於大腸直腸癌促進細胞凋亡

及其基因刪除之探討

Study of proapoptotic activity and allelic deletion of

PCDH10 in colorectal cancer



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## 摘要

根據台灣行政院衛生署 2008 年死因統計資料，大腸直腸癌在男性或女性中都是排名第三位的癌症死因。之前本研究室針對大腸直腸癌腫瘤檢體進行第四號染色體之微衛星標記發生失異合性(loss of heterozygosity, LOH)的分析，發現 4q25-4q28.3 中有 3 個發生頻率較高(23.1%-28.0%)的刪除區域。而在其他癌症亦被報導第四號染色體之長臂(q arm)有發生刪除的現象，進而推論第四號染色體可能存在抑癌基因。在本研究室發現的一個頻率較高的刪除區域(4q28.3)，存在一細胞吸附分子 *Protocadherin 10* (*PCDH10*) 基因。目前知道 *PCDH10* 有兩種 RNA 產物，分別為 *PCDH10* variant 1 (*PCDH10 V1*) 和 *PCDH10* variant 2 (*PCDH10 V2*)。近來的研究發現在其他癌症當中，*PCDH10* 可能扮演著抑癌基因的角色。除了染色體的刪除，本研究室之前針對 *PCDH10* 於大腸直腸癌中基因後生遺傳修飾的研究，發現 *PCDH10* 基因啟動子(promoter)有過度甲基化的現象。再者，其 mRNA 在大腸直腸癌的腫瘤組織之表現，明顯地比其成對之正常黏膜組織較低。之後亦針對 *PCDH10* 進行了功能性的探討，發現當 *PCDH10* 在大腸直腸癌細胞株中再度表現後，可以抑制癌細胞侵犯以及群落形成，進一步顯示 *PCDH10* 可能為抑癌基因。本論文利用重新挑選的 *PCDH10 V1* 和 *PCDH10 V2* 之穩定表現細胞株，先進行細胞增生的實驗，發現當 *PCDH10 V1* 於大腸直腸癌細胞再表現時，會抑制細胞的生長，但再表現 *PCDH10 V2* 則觀察不到同樣的現象。之後再進行促進細胞凋亡的實驗，無論是 *PCDH10 V1* 或是 *PCDH10 V2* 皆可促進細胞凋亡。除此之外，利用藥物 camptothecin 處理 *PCDH10 V1* 或 *PCDH10 V2* 再表現之細胞株，結果顯示當 *PCDH10* 存在時會增加癌細胞對藥物的感受性。另外，本論文亦設計四個分佈在 *PCDH10* 基因 5 端和 3 端的微衛星標記，針對大腸直腸癌腫瘤探討 *PCDH10* 發生對偶基因缺失(allelic deletion)的情形。於 191 個大腸直腸癌腫瘤檢體中，有 57 個(29.8%)發生 *PCDH10* 對偶基因的缺失，同時，此基因變異與大腸直腸癌後

期的轉移相關，且病人之存活率亦較差。由於研究室之前曾針對 *PCDH10* 進行基因後生遺傳修飾的探討，故利用其結果，配合對偶基因缺失與否，進行病人存活分析，而當同時探討此兩種基因變化時，其與病人的存活率並沒有顯著的相關性。最後，本論文亦利用 DNA 套數檢測(copy number assay)以研究大腸直腸癌腫瘤中 *PCDH10* 基因套數的變異，並與 LOH 研究的結果相比較，結果顯示此二方法的結果並不完全符合。綜合以上結果，*PCDH10* 的再表現可以促進大腸直腸癌細胞之凋亡，同時此基因在大腸直腸癌腫瘤中有高比例之腫瘤發生對偶基因缺失的現象，皆進一步支持 *PCDH10* 在大腸直腸癌化過程中具有抑癌的功能。

關鍵詞：大腸直腸癌、失異合性、*PCDH10*、細胞凋亡、對偶基因缺失

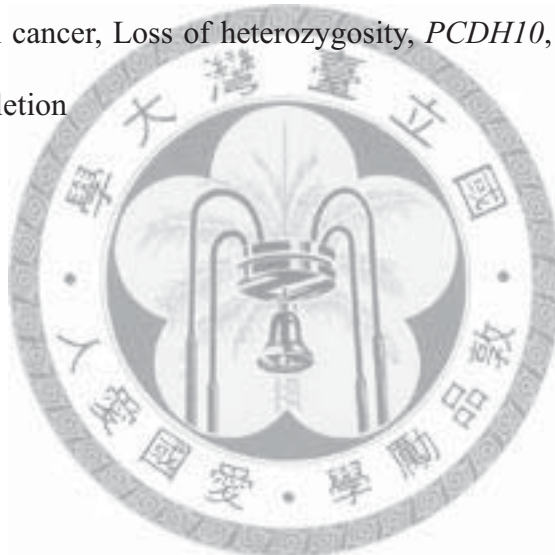


## Abstract

Colorectal cancer (CRC) is the third leading cause of cancer death for both genders in Taiwan according to the report by the Department of Health, Executive Yuan, R.O.C., in 2008. Our previous investigation of allelic losses of microsatellites at chromosome 4q25-4q28.3 in CRC revealed three regions with higher frequency (23.1%-28.0%) of incidences. Deletions of chromosomal 4q were also reported in various carcinomas, suggesting putative tumor suppressor genes may exist within the chromosome region. *Protocadherin 10 (PCDH10)* is located at 4q28.3, which is one of the three common deletion regions defined. There are two transcript variants of *PCDH10*, namely *PCDH10* variant 1 (*PCDH10* V1) and *PCDH10* variant 2 (*PCDH10* V2). Recent studies of *PCDH10* in multiple carcinomas suggested that it may be a candidate tumor suppressor gene. Besides genomic deletion, studies of *PCDH10* at our lab also indicated that *PCDH10* may be regulated by epigenetic modification in CRC cells. Consistently, *PCDH10* mRNA expression was significantly lower in CRC tumor tissues compared with the paired normal mucosae. Functional studies, such as invasion assay and colony formation assay, also indicated that *PCDH10* as a tumor suppressor. In the present study, new single stable clones of *PCDH10* V1 and *PCDH10* V2 were selected. A reduction in cell proliferation was observed when *PCDH10* V1, but not *PCDH10* V2, was re-expressed in CRC cells. Investigation of proapoptotic activities of *PCDH10* V1 and *PCDH10* V2 revealed increased apoptosis and enhanced camptothecin susceptibility when either one was re-expressed in CRC cells. On the other hand, *PCDH10* allelic deletion was studied by using four newly designed microsatellite markers flanking the 5' and 3' regions of the gene. Out of the 191 CRC studied, 57 (29.8%) tumors exhibited allelic deletion of *PCDH10*. Importantly, the genetic alteration is correlated with distal metastasis of CRC and poorer overall

survival. However, simultaneous analysis of patient survival related to both the incidence of LOH and promoter hypermethylation of *PCDH10* revealed no significant correlation. Copy number assay was also used to detect copy number variation of *PCDH10* in CRC. Nevertheless, no positive correlation could be found between copy number variation and the results of the LOH study. In conclusion, induction of apoptosis by *PCDH10* re-expression in CRC cells and high incidence of *PCDH10* allelic deletion in CRC tumors support its role as a tumor suppressor in colorectal tumorigenesis.

Keywords: Colorectal cancer, Loss of heterozygosity, *PCDH10*, Proapoptotic activity, Allelic deletion



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## Abbreviation

Abbreviation	Full name
APC	Adenomatous polyposis coli
BSP	Bisulfite-sequencing polymerase chain reaction
CGH	Comparative genomic hybridization
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
DFS	Disease-free survival
DMSO	Dimethyl sulfoxide
EMT	Epithelial-to-mesenchymal transition
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
GI tract	Gastrointestinal tract
HNPCC	Hereditary non-polyposis colorectal cancer
LOH	Loss of heterozygosity
MMR	Mismatch repair
MSI	Microsatellite instability
MSP	Methylation specific polymerase chain reaction
MSS	Microsatellite stable
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCDH10	Protocadherin 10
PCDH10 V1	Protocadherin 10 variant 1
PCDH10 V2	Protocadherin 10 variant 2
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
RT-PCR	Reverse transcriptase polymerase chain reaction
TBST	Tris-HCl buffered saline with Tween-20
WHO	World Health Organization

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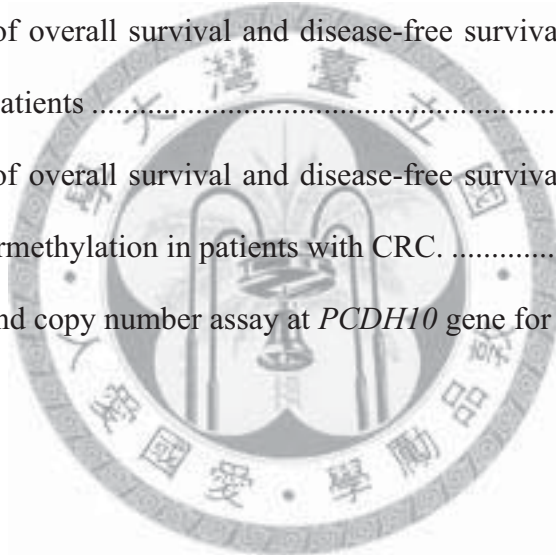
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# 1. Introduction

## 1.1 Colon and rectum

Colon and rectum belong to the large intestine which is the terminal part of the gastrointestinal tract (GI tract).

Colon is divided into ascending, transverse, descending and sigmoid colon.

Ascending colon ascends from the lower right side of the abdomen, turns at the right colic (hepatic) flexure, then continues to the left and makes up the transverse colon. At the left colic (splenic) flexure, the colon turns and descends to the lower left side of the abdomen which forms the descending colon, followed by the sigmoid colon and ends at the rectum. The rectum is the last part of the large intestine, with anal canal at its terminal. The opening of the anal canal is the anus.

The main function of the colon is water, ions and vitamins absorption through the absorptive cells. Bacteria inside the colon also help to break down proteins and ferment any remaining carbohydrates. Feces formed will then be eliminated from the body. Colon is different from the small intestine in a way that there are no villi found in the mucosa, while the submucosa is similar to the rest of the GI tract. The next two layers come the muscularis and serosa, with serosa being part of the visceral peritoneum.<sup>1</sup>

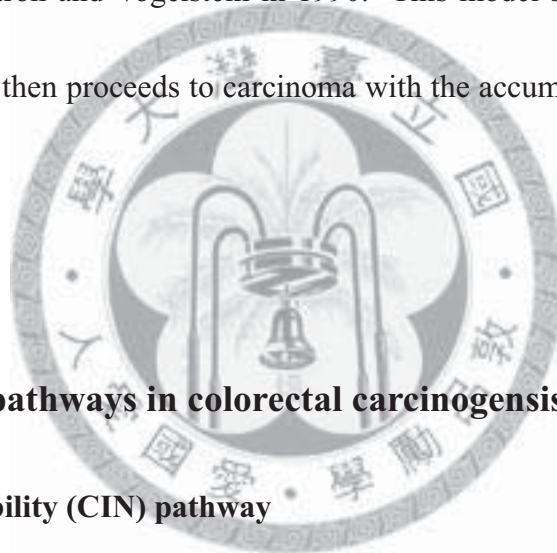
## 1.2 Colorectal cancer

According to the World Health Organization (WHO), cancer is the leading cause of death worldwide which accounted for 7.9 million deaths in 2007. The WHO estimates that cancer will cause 12 million deaths in 2030.<sup>2</sup> In Taiwan, according to the Department of Health, Executive Yuan, R.O.C. (Taiwan), cancer is the leading cause of death in Taiwan in 2008, with colorectal cancer (CRC) being the third leading cause of cancer death for both genders (Appendix Table 1 & 2).<sup>3</sup>

CRC refers to the cancer formed in the tissue of colon and rectum. Approximately 75% of CRCs are sporadic, while the remaining 25% are familial disease.<sup>4</sup> The two most common familial CRCs are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP patients inherit mutated germline Adenomatous polyposis coli (APC), a tumor suppressor gene at chromosome 5q in CRC which leads to the development of hundreds to thousands colorectal adenomas and therefore, the early onset of carcinomas.<sup>5</sup> Contrary to FAP, patients of HNPCC do not exhibit unusual number of polyps. The cause of HNPCC is due to inherited mutations in the DNA mismatch repair genes which cannot repair DNA errors whenever they occur. As a result, errors may contribute to the mutations in some tumor suppressor genes, and finally lead to the occurrence of carcinomas. HNPCC is also

known as Lynch syndrome.<sup>6</sup>

CRC is a multistep process, which may be resulted from genetic alterations in the oncogene such as *ras* gene mutations or the tumor suppressor genes inactivation, chromosomal losses or allelic losses of tumor suppressor genes, as in the case of the loss of chromosome 17p which contains the p53 gene, etc. The above multistep process was proposed as the “adenoma-carcinoma sequence” model for the tumorigenesis of sporadic CRC by Fearon and Vogelstein in 1990.<sup>7</sup> This model states that CRC arises from adenoma which then proceeds to carcinoma with the accumulations of a series of genetic alterations.



### **1.2.1 Two major pathways in colorectal carcinogenesis**

#### **Chromosomal instability (CIN) pathway**

Chromosomal instability refers to the loss, gain or translocation of chromosomal segments. Around 85% of CRCs belong to this group. When chromosomal instability takes place where tumor suppressor gene is located, tumorigenesis may occur due to the loss of functions of the tumor suppressor gene. Deletion at 1p and 8p, as well as loss of heterozygosity (LOH) of 17p and 18q, are frequently found in CRC.<sup>5 8 9</sup>

#### **Microsatellite instability (MSI) pathway**

Microsatellites are short nucleotide repeat sequences dispersed throughout the genome. When DNA replicates, inappropriate base insertion or DNA replication slippage may occur at these repeat sequences, resulting in insertion or deletion loops formation. In normal condition, loops are usually repaired. However, if there are defects in the DNA mismatch repair (MMR) enzymes, these loops cannot be repaired and mutations tend to accumulate in these microsatellite regions, resulting in alleles of different sizes. Microsatellite instability takes place in tumor suppressor genes may lead to the malfunction of its tumor suppressing ability, thus leading to tumorigenesis. Tumor carrying alleles of different sizes due to defect in MMR genes is called microsatellite instability (MSI) tumor. Around 15% of sporadic CRCs belong to this group and more than 95% HNPCCs have MSI identified in them.<sup>589</sup>

### **1.2.2 Epigenetic pathway in colorectal carcinogenesis**

Hypermethylation of CpG islands in the 5' promoter region of gene usually leads to the silencing of gene. This is known as epigenetic regulation. Recently, epigenetic modification was proposed to be involved in CRC tumorigenesis. Silencing of the tumor suppressor genes or DNA mismatch repair genes such as *hMLH1* was reported. Around 30% to 50% of CRCs show evidences of CpG island hypermethylation. Tumors demonstrating hypermethylation and thus silencing of genes are known as CpG island



methylator phenotype-positive (CIMP) tumors.<sup>10</sup>

### 1.3 Protocadherin 10 (PCDH10)

Protocadherins are members of a non-classic subfamily of calcium-dependent cell–cell adhesion molecules in the cadherin superfamily. Subfamily members of protocadherins contain similar extracellular domain structure, a transmembrane domain but a different cytoplasmic tail.<sup>11</sup> *Protocadherin 10 (PCDH10/OL-PCDH /KIAA1400)* is the human orthologue of mouse *Pcdh10*. It is located at chromosome 4q28.3. There are two transcript variants of *PCDH10*, namely *PCDH10* variant 1 (*PCDH10* V1) and *PCDH10* variant 2 (*PCDH10* V2). The first exon of *PCDH10* V1 encodes the extracellular domain which contains six ectodomain repeats (extracellular repeat domains, EC domains), as well as the transmembrane domain and the cytoplasmic domain, while the remaining exons encode an extension for the cytoplasmic tail. On the other hand, transcript of *PCDH10* V2 ends in the first intron, resulting a single exon gene that encodes the three domains stated above.<sup>11</sup>

*PCDH10* is mainly expressed in the olfactory bulb, most parts of the limbic system (hence the name *OL-PCDH*) and in the cerebellum, suggesting its function in cerebellum development.<sup>11</sup> Recent analysis of *PCDH10* knockout mice demonstrated that the striatal neurons could not extend their axons normally in the brain. Since

*PCDH10* was highly concentrated in the axons' growth zones, it suggests that *PCDH10* may play a role in sustaining the normal migration of striatal axons.<sup>12 13</sup> *PCDH10* is also found to be expressed in other organs such as heart, kidney, lung, trachea and colon as revealed by reverse transcriptase polymerase chain reaction (RT-PCR).<sup>14 15</sup>

*PCDH10* was also reported to induce homophilic cell-cell adhesion ability.<sup>16</sup> However, the cell-cell adhesion ability was weaker than that of the classical cadherins, suggesting that protocadherins may be involved in other functions such as signal transduction besides physical linking of cells.

Recently, regulatory elements in *PCDH10* gene promoter was further identified and characterized by Li *et al.* using human prostate cancer cells. It was shown that the region between nucleotides -144 and -99 was essential to the promoter activity, and within this region, a CAAT box and a GT box were found to be important to the promoter regulation.<sup>17</sup>

### **1.3.1 Recent studies of PCDH10**

In a study by Miyamoto *et al.* in 2005, CpG islands in the 5' promoter region of *PCDH10* was found to be aberrantly methylated in breast cancer cell lines and in 42.8% primary breast cancer tested. On the contrary, there is no CpG islands hypermethylation found in normal human mammary epithelial cells.<sup>18</sup> In 2006, Ying *et*

*al.* identified *PCDH10* as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. Moreover, transcriptional silencing of *PCDH10* could be reversed by pharmacologic demethylation drug, suggesting *PCDH10* may be regulated by epigenetic modification. In the same study, ectopic expression of *PCDH10* in several carcinoma cell lines (nasopharyngeal carcinoma cell line CNE-1 and the esophageal carcinoma cell line EC109) strongly suppressed tumor cell growth, migration, invasion and colony formation ability.<sup>15</sup> In 2007, the same research group identified the frequent methylation and silencing of *PCDH10* in lymphoma cell lines as well as in primary tumors but not in normal peripheral blood mononuclear cells (PBMCs) or lymph nodes, suggesting methylation of *PCDH10* may also involve in lymphomagenesis.<sup>19</sup> Methylation of *PCDH10* in gastric cancer cell lines and in 81.7% primary gastric tumors tested was reported by Yu *et al.* in 2009,<sup>20</sup> supporting its role as a tumor suppressor in various carcinomas. Monosomy 4 and hypermethylation of promoter region of *PCDH10* were also reported by Narayan *et al.*<sup>21</sup> and Wang *et al.*<sup>22</sup> in invasive cervical cancer. However, loss of heterozygosity using microsatellite markers (D4S2423) adjacent to *PCDH10* was not observed.<sup>22</sup> Recently, Cheung *et al.* reported the first application of methylated DNA immunoprecipitation-chip to detect epigenetically regulated regions in human testicular cancer. In this study, one of the genes identified was *PCDH10* and its promoter region

was found to be hypermethylated in testicular embryonal carcinoma cell line NT2.<sup>23</sup>

On the other hand, Yu *et al.* utilized high-resolution melting analysis to detect the methylation status of *PCDH10* in gastric, colorectal and pancreatic cancers and reported that the methylation level of *PCDH10* was significantly higher in tumor tissues than in normal tissues in all three type of cancers, supporting its role as a tumor suppressor.<sup>24</sup>

### 1.3.2 PCDH10 and apoptosis

In Yu *et al.* study in 2009, *PCDH10* re-expression in gastric cancer cell line MKN45 was found to induce apoptosis and reduce cell proliferation. cDNA array analysis of MKN45 stably transfected with *PCDH10* revealed that several genes involved in apoptosis, proliferation, and invasion/metastasis pathways were modulated by *PCDH10*.<sup>20</sup>

Another study by Ding *et al.* in 2009 reported that downregulation of human brain expressed X-linked 1 (hBex1) inhibited apoptosis induced by imatinib in *Bcr/Abl*<sup>+</sup> leukemic cells. Further investigations pointed out that hBex1 is associated with *PCDH10*, and silencing of *PCDH10* also attenuated apoptosis induced by imatinib in hBex1 transfected cells, suggesting that *PCDH10* may participate in the apoptosis of cancer cells.<sup>25</sup>

### 1.3.3 Deletion of chromosome 4q28 and cancer

High loss of heterozygosity (LOH) frequency in 4q28 region was first reported in primary hepatocellular carcinoma cases by Hammond *et al.* in 1999, indicating important tumor suppressors within or in vicinity of this region.<sup>26</sup> In 2004, Knösel *et al.* used comparative genomic hybridization (CGH) to screen CRCs for chromosomal aberrations that were associated with metastatic phenotype. Deletions were prominent at 4q27-4q28.<sup>27</sup> In addition, loss in copy number at 4q28.3 in pancreatic adenocarcinoma,<sup>28</sup> a 4.5-megabase deletion at 4q28 in prostate cancer cells<sup>29</sup> and chromosomal loss at 4q28-32 in breast cancer were also reported.<sup>30</sup>

### 1.3.4 Studies of PCDH10 at our lab

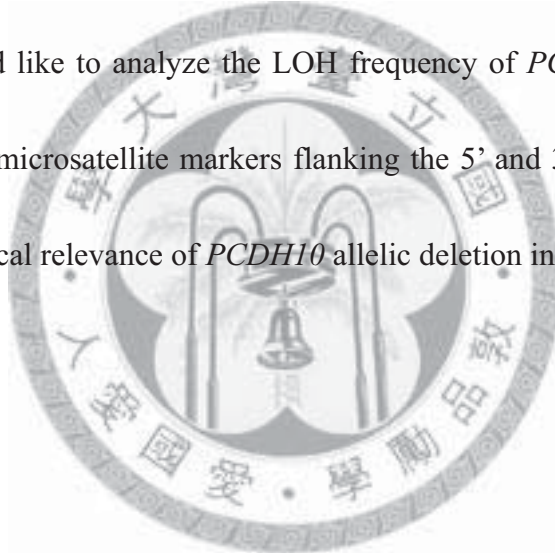
Previous studies of LOH at chromosome 4q25-4q28.3 identified 3 regions with high frequency of LOH; *PCDH10* is located within one of these regions. Epigenetic studies of *PCDH10* in CRC cell lines and patient tumor samples revealed that 5' promoter region of *PCDH10* was highly methylated when compared to the paired normal tissue. Treatment of five CRC cell lines with demethylation drug 5-aza-2'-deoxycytidine also restored RNA expression of *PCDH10*, implying that *PCDH10* was regulated by epigenetic modification. Investigation of *PCDH10* V1 RNA expression in CRC

primary samples also indicated a decreased expression level in tumor than in normal tissue. However, expression pattern of *PCDH10* V2 was inconsistent; though predisposed to co-express with *PCDH10* V1 when the expression level of it was high. Functional assays using *PCDH10* V1 single stable expression clones in CRC cell line HCT116 showed that both proliferation and colony formation ability of cells decreased, but no similar observations were found in *PCDH10* V2 single stable expression clones. Moreover, *PCDH10* V1 and *PCDH10* V2 were found to co-localize in cell membrane. In conclusion, *PCDH10* may be a putative tumor suppressor gene associated with colorectal carcinogenesis.



## 2. Specific aims

Since *PCDH10* was reported as a putative tumor suppressor gene in multiple carcinomas, and several functional assays by our lab and others also revealed supportive results, we therefore want to investigate the proapoptotic activity of *PCDH10* in CRC cells. On the other hand, since chromosomal deletion is frequently found at chromosome 4q28 in other various carcinomas and *PCDH10* is located within this region, we would like to analyze the LOH frequency of *PCDH10* in CRC using four newly designed microsatellite markers flanking the 5' and 3' regions of the gene and to assess the clinical relevance of *PCDH10* allelic deletion in CRC.

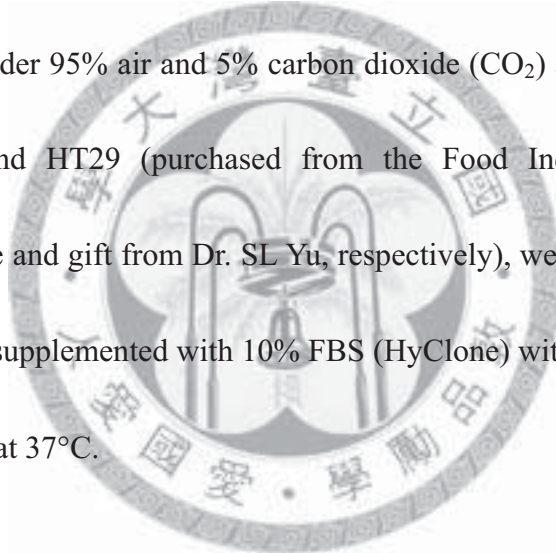


### 3. Materials and methods

#### 3.1 Study of cell biological functions of PCDH10

##### 3.1.1 Cell culture

Human CRC cell line HCT116 (gift from Dr. SL Yu), was cultured in RPMI-1640 (GIBCO-Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone) without antibiotics under 95% air and 5% carbon dioxide (CO<sub>2</sub>) at 37°C. Human CRC cell lines SW480 and HT29 (purchased from the Food Industry Research and Development Institute and gift from Dr. SL Yu, respectively), were cultured in DMEM (GIBCO-Invitrogen) supplemented with 10% FBS (HyClone) without antibiotics under 95% air and 5% CO<sub>2</sub> at 37°C.



##### 3.1.2 Vector construction

Vector construction was done by previous senior coworkers Yen-Chun Lai and Chao-Ha Fu. Briefly, *PCDH10* V1 and *PCDH10* V2 cDNA was obtained from MCF7 and were cloned into the mammalian expression vector pcDNA3.1/V5-His-TOPO (Invitrogen, Appendix Figure 1). The two constructs were named as pcDNA3.1/PCDH10.v1 and pcDNA3.1/PCDH10v.2 for *PCDH10* V1 and *PCDH10* V2, respectively.



### 3.1.3 Cell transfection and stable clone selection

Transfection was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 18h before transfection,  $5 \times 10^5$  cells per well of a 6-well plate were seeded (~90% confluence) in 2mL RPMI-1640 containing 10% FBS. During transfection, Lipofectamine™ 2000 and plasmid DNA were mixed in a 2.5:1 ratio (10 $\mu$ L Lipofectamine™ 2000 and 4 $\mu$ g plasmid DNA purified by QIAGEN Plasmid Midi Kit). Both were diluted with 250 $\mu$ L of Opti-MEM I Reduced Serum Medium (Invitrogen) before complexing and allowed to stand at room temperature for 5 min. The diluted Lipofectamine was then mixed with the diluted plasmid DNA and incubated at room temperature for 20 min. The complexes were then added to the cells and incubated at 37°C in a 5% CO<sub>2</sub> incubator for 6 h. Medium will be changed back to RPMI-1640 with 10% FBS only and incubated at the same condition for a further 18h (Total 24h since the time of introduction of the complexes). Part of the cells will then be collected for western blotting to confirm protein expression. For stable clone selection, 1200 $\mu$ g/mL G418 (Geneticin, GIBCO) was added 24h after transfection and kept selecting for at least 2-3 weeks. Single stable clones obtained were then kept at a final concentration of 600 $\mu$ g/mL G418.

### 3.1.4 Western blotting

Total cellular extracts were obtained by RIPA lysis buffer with protease inhibitor. Protein concentrations were determined by the Bradford method (Bio-Rad). 4 $\mu$ L 4x sodium dodecyl sulfate loading dye was added to 12 $\mu$ L sample, and the sample was boiled for 10 min. Protein samples (50 $\mu$ g per lane) were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was then blocked with 5% skim milk in Tris-HCl buffered saline containing 0.025% Tween-20 (TBST) at room temperature for 1h followed by incubation with the primary antibody for 1.5h at room temperature or overnight at 4°C. The membrane was then washed with TBST thrice (10 min per wash) and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. The membrane was then washed as previously described. The bands were visualized using the Western Lightning Chemiluminescence Reagent *Plus* (PerKinElemer) and detected by Fujifilm *LAS 4000* Imager.

### 3.1.5 Cell proliferation assay

MTT assay for cell proliferation was performed for single stable clones of *PCDH10*

V1 and *PCDH10* V2. Briefly, 3000 cells per well were seeded in a 96-well plate in four replicates. The colorimetric MTT (Amresco) assay was used to measure cell numbers at 24, 48, 72, 96 and 120h. 50 $\mu$ g MTT was added to each well and incubated for 90 min at 37°C, followed by dissolving the formazan by 100 $\mu$ L Dimethyl sulfoxide (DMSO). Absorbance at 570nm was then measured.

### 3.1.6 Camptothecin treatment

Camptothecin was isolated from the bark and stem of *Camptotheca acuminata* (Camptotheca, Happy tree). It binds irreversibly to the DNA-topoisomerase I complex, and traps the enzyme in a covalent linkage with DNA, thus depleting cellular topoisomerase I. However, due to its low solubility and adverse drug effect, it is no longer adapted in chemotherapy, though derivatives of it are developed and approved for use in chemotherapy such as irinotecan.<sup>31</sup> Camptothecin (Sigma-Aldrich) is employed in our study to induce apoptosis in CRC cells.

### 3.1.7 Flow cytometry analysis for apoptosis

1 x 10<sup>6</sup> cells per well were seeded in a 6-well plate or 1.5 x 10<sup>5</sup> cells per well were seeded in a 12-well plate. For medium only condition, cells were incubated in RPMI-1640 with 10% FBS with or without 600 $\mu$ g/mL G418 for 48h. For camptothecin treatment, cells were first plated for 24h, then 10ng/mL camptothecin (Sigma-Aldrich)

will then be added in the medium and incubated for a further 18h. Cells treated with 0.01% DMSO at the same time interval act as the control group since camptothecin has to be dissolved in DMSO. Cells will then be collected and detected for apoptosis using ANNEXIN V-FITC Kit (Beckman Coulter) as instructed by the manufacturers. Briefly, cells were washed by cold PBS (pH7.4) twice, followed by addition of 100 $\mu$ L cold 1x binding buffer, and stained with 1 $\mu$ L of Annexin V-FITC and 5 $\mu$ L propidium iodide (PI). After incubation for 15 min in the dark on ice, the cells are ready to be analyzed by flow cytometry.

### 3.1.8 Study of epithelial-to-mesenchymal transition

TGF- $\beta$ 1 (PeproTech) was used to induce microsatellite stable (MSS) CRC cell lines SW480 and HT29 to undergo epithelial-to-mesenchymal transition (EMT).<sup>32</sup> Briefly, 5, 10 or 20ng/mL TGF- $\beta$ 1 was used to treat the above CRC cell lines for 24 or 48 hours with 1% or 10% FBS in order to test the optimal condition for TGF- $\beta$ 1 treatment. Afterwards, cells were collected for investigation of the EMT markers, E-cadherin and vimentin by western blotting (Mouse anti-E-cadherin antibody, BD Transduction Laboratories<sup>TM</sup> and mouse anti-vimentin monoclonal antibody, Chemicon). For the investigation of involvement of *PCDH10* in EMT pathway, cells were seeded in a 6-well plate to achieve 90% confluence for each cell line used for 18h, then either 4 $\mu$ g

pcDNA3.1/V5-His-TOPO or *PCDH10 V1* plasmid was transfected to the cells. 24h after transfection (procedure as described above), 10ng/mL TGF- $\beta$ 1 was added to each well and incubated for a further 24h or 48h. Cells were then collected for investigation of the above EMT markers by western blotting.

## 3.2 Study of allelic deletion of PCDH10

### 3.2.1 Patients

A total of 211 patients with sporadic CRC, who underwent surgery between August 1997 and June 2009 at the Cardinal Tien Hospital, Taiwan, were recruited. Patient clinicopathological characteristics were summarized in Table 1. Median age of patients at disease diagnosis was 73 (range 37-98) years. Male to female ratio was 110:101. Out of the 211 patients, 49 patients with their tumors located at the proximal colon (cecum, ascending colon, and transverse colon), while the remaining 162 patients with their tumors located at the distal colon (descending colon, sigmoid colon, rectosigmoid junction, and rectum). Tumors were staged using the modified Dukes' classification<sup>33</sup>. There were 22 patients in Dukes' A, 83 in Dukes' B, 66 in Dukes' C and 40 in Dukes' D. Tumor stage was further subdivided into early stage (Dukes' A and B) and late stage (Dukes' C and D), with 105 and 106 patients, respectively. Distal metastasis (Dukes' D) and no distal metastasis (Dukes' A, B and C), with 40 and 171 patients, respectively.

For pathological differentiation, 155 tumors were graded as moderately differentiated, 54 as poorly differentiated and two without pathological reports. No patients received preoperative chemotherapy and/or radiotherapy. For disease recurrence assessment, only 140 patients with either Duke's B or C, who underwent surgery before December 31<sup>st</sup>, 2008 were recruited. Follow-up was continued until April 2010. Out of 140 patients, there were five patients without follow-up after surgery, five patients who died of post-operative complications, and one patient without curable surgery, they were excluded from disease recurrence analysis. For the remaining 129 patients, 34 exhibited disease recurrence and 95 patients did not.

### **3.2.2 DNA extraction**

DNA extraction was performed using the QIAamp DNA mini kit (QIAGEN) according to the manufacturers' protocol. DNA concentration was tested by Nanodrop 2000 (Thermo Scientific). Final DNA concentration was adjusted to 10ng/ $\mu$ L for the polymerase chain reaction (PCR).

### **3.2.3 Loss of heterozygosity study**

Four markers for the LOH study were newly designed flanking the 5' and 3' regions of *PCDH10* with the help of a web software for MicroSatellite Marker Development.<sup>34</sup>

Polymorphic loci contain dinucleotide and trinucleotide were selected. Microsatellite

repeats were amplified by PCR using the primers labeled with fluorescence at the 5' end of each forward primer (Applied Biosystems) as in Table 2. Briefly, 20 ng DNA template was mixed with 5  $\mu$ M of each sense and antisense primer, 2.5 mM dNTP, 10x reaction buffer and 0.3 units of HOT FIREPol<sup>®</sup> DNA polymerase (Solis BioDyne). Final reaction volume is 6 $\mu$ L. DNA template was amplified using the TGradient Thermocycler (Biometra). Thermal cycling consisted of initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 30 sec, with final elongation for an additional 10 min at 72°C. If necessary, PCR products were visualized in 2% agarose gel with ethidium bromide. (Appendix Table 3)

### **3.2.4 Automated fluorescence-based PCR assay**

Fluorescent PCR products of the four markers were mixed in a fixed ratio (D4P1: D4P2: D4P3: D4P4= 0.5 $\mu$ L: 0.5 $\mu$ L: 1 $\mu$ L: 3 $\mu$ L). Formamide and size standard Rox-400 (Applied Biosystems) were then added to the mixed PCR product, boiled at 94°C for 5 min and then kept on ice. Then, the mixture was subjected to ABI Prism 377 DNA Sequencer for electrophoresis-laser detection and computer analysis. Results were analyzed using the software GeneScan (Applied Biosystems). For LOH analysis, presence of heterozygous alleles was prerequisite for the evaluation of allelic losses.

LOH index was calculated using the formula: ratio of allele peak heights of normal tissue divided by the ratio of allele peak heights of the paired tumor tissue. When LOH index is  $\leq 0.67$  or  $\geq 1.5$ , which corresponds to a minimal 33% decrease of the tumor allele, we defined the presence of allelic losses.<sup>35</sup>

### 3.2.5 Copy number assay

Copy number assay (Taqman<sup>®</sup> Copy number assay, Applied Biosystems) was performed according to the manufacturers' protocol. Briefly, 20ng DNA was mixed with 2x TaqMan<sup>®</sup> Genotyping Master Mix, fluorescence labeled probe targeting exon 4 of *PCDH10* and fluorescence labeled probe targeting endogenous reference gene RNaseP, which is known to be present in two copies in a diploid genome. The reaction is a duplex real-time PCR. The instrument used is the 7500 Fast System. Data is analyzed by the CopyCaller<sup>™</sup> software from ABI.

### 3.3 Statistical analysis

For continuous variables, Students' *t*-test was used. For categorical variables, Fisher's exact test or chi-square test was used. Overall and disease-free survival was estimated by the Kaplan-Meier method and the survival curve was compared by the Log-Rank test. Statistical significance was considered when *p* value  $< 0.05$ . All statistical analyses



were performed using SPSS 17.0 software package (SPSS, IL, USA) for Windows and GraphPad Prism (GraphPad Software, Inc.).



## 4. Results

### 4.1 Cell biological functions of PCDH10

#### 4.1.1 Selection of *PCDH10* variant 1 and *PCDH10* variant 2 single stable clones

Protein level of both PCDH10 V1 and PCDH10 V2 single stable clones selected earlier dropped rapidly which were no longer suitable for functional assays. This may be a result of re-expression of PCDH10 which was disadvantageous to tumor cell growth. Therefore, new single stable clones for both variants were re-selected. For PCDH10 V1, there were four new single stable clones, namely PCDH10 V1S6, PCDH10 V1S9, PCDH10 V1S12 and PCDH10 V1S14. For PCDH10 V2, there were also four new single stable clones, namely PCDH10 V2S5, PCDH10 V2S6, PCDH10 V2S9 and PCDH10 V2S11. The stable cell line transfected with pcDNA3.1/V5-His-TOPO vector only will be abbreviated as “Mock” in the followings. Protein expression was confirmed by western blotting (Figure 1). Expression level of PCDH10 was the strongest in PCDH10 V1S6 for PCDH10 V1 and PCDH10 V2S9 for PCDH10 V2.

Since the previous results of proliferation assay from senior coworkers were controversial about the effect of PCDH10 expression. Therefore, we would like to perform the MTT proliferation assay with the newly selected single stable clones to

confirm the effect of PCDH10 re-expression (Figures 2 and 3). Since protein expression might decrease during cell subculturing, protein level was detected in parallel for each assay. The MTT assay of PCDH10 V1 single stable clones showed that the proliferation rate of all single stable clones with PCDH10 V1 re-expression decreased significantly when compared to Mock. However, for PCDH10 V2 single stable clones, the proliferation rates were all similar to Mock.

#### 4.1.2 Apoptosis induced by PCDH10

Since *PCDH10* was hypothesized as a putative tumor suppressor gene, we would like to investigate whether overexpression of PCDH10 can induce apoptosis in CRC cells or not. PCDH10 V1 and PCDH10 V2 single stable clones were recruited for the apoptosis study. The experiment was repeated three times independently.

Firstly, the ability of PCDH10 to induce apoptosis was tested using PCDH10 V1 and PCDH10 V2 single stable clones without any drug treatment. Outcomes of PCDH10 single stable clones were then compared with Mock, which is HCT116 with pcDNA3.1/V5-His-TOPO vector only. Results were shown in Figure 4. Apoptosis increased with the re-expression of PCDH10, regardless of PCDH10 V1 or PCDH10 V2, when compared to Mock. Meanwhile, Mock showed similar apoptosis percentage to the parental cell line, HCT116.

Secondly, we used camptothecin (10ng/mL) to induce apoptosis in PCDH10 V1 and PCDH10 V2 single stable clones to see whether re-expression of PCDH10 would increase the susceptibility of CRC cells to drug treatment. Since camptothecin has to be dissolved in DMSO, therefore, a control group using the same single stable clones treated with 0.01% DMSO was prepared to offset the effect of DMSO. Outcomes were compared with Mock treated with DMSO. Results were shown in Figure 5. The results indicated an increase to drug susceptibility of CRC cells when PCDH10 was re-expressed.

#### 4.1.3 Regulation of epithelial-to-mesenchymal transition by PCDH10

Since *PCDH10* is a transmembrane protein related to cytoskeleton, therefore, we would like to investigate whether *PCDH10* played a role in EMT or not. One of the hallmark EMT marker, E-cadherin,<sup>36</sup> was investigated in all *PCDH10* single stable clones including both PCDH10 V1 and PCDH10 V2. Results were shown in Figure 6. However, no significant differences were found between Mock and *PCDH10* expressing single stable clones. As a result, other strategies for studying EMT were employed.

A study of Pino *et al.* revealed that SW480 and HT29 were responsive to TGF- $\beta$  –induced EMT<sup>32</sup>. Therefore, we recruited these two cell lines for the following

EMT study. We tested the optimal condition for the experiments by using different concentration of TGF- $\beta$ 1 (5, 10 and 20ng/mL) to treat SW480 and HT29 for different time points (24h or 48h) to induce EMT (Figure 7). Protein expression of the mesenchymal marker, vimentin, cannot be detected in TGF- $\beta$ -untreated or -treated HT29 cells in our lab, which was different from Pino's report. Therefore, we excluded this cell line for our study. As for TGF- $\beta$  treatment in SW480, the results showed more prominent changes in E-cadherin and vimentin protein expression with 5 or 10 ng/mL of TGF- $\beta$  treatment for 24h. Finally, condition using 10ng/mL TGF- $\beta$  treatment for 24h was employed for the study of *PCDH10* involvement in EMT, which was the same concentration used as in previous studies. The parental CRC cell line used for *PCDH10* transfection, HCT116, is a microsatellite unstable cell line, and harbors biallelic mutational inactivation of TGF- $\beta$ R II.<sup>37</sup> As a result, HCT116 is insensitive to the treatment of TGF- $\beta$ . Therefore, the *PCDH10* expressing cell clones are not suitable for the TGF- $\beta$ -induced EMT study.

## **4.2 Allelic deletion of *PCDH10***

### **4.2.1 Loss of heterozygosity of *PCDH10***

Seven microsatellite markers were recruited for the establishment of gene deletion study of *PCDH10* (Figure 8A). Primers were designed to amplify the seven

microsatellite regions. PCR was then performed to check whether the primers can be used to amplify the desired products (Figure 8B). Since all the primers can be used for PCR, therefore, optimization of the PCR condition was performed (Figure 9). A panel of eight individual DNA from normal PBMCs was used to detect the polymorphism in each marker. Five out of eight markers (Marker No.1, 3, 4, 5 and 6) showed polymorphisms in primary screening (Figure 10). Two markers at each side flanking *PCDH10* upstream and downstream regions were chosen for the study (Marker No.1 and 3, and Markers No.4 and 6, respectively). The markers were renamed as D4P1 for Marker No.1, D4P2 for Marker No.3, D4P3 for Marker No.4 and D4P4 for Marker No.6 (Figure 11). LOH study was carried out by fluorescence-based PCR using ABI Prism 377 DNA sequencer. Figure 12 showed the results analyzed by the software GeneScan. For the calculation of the LOH index, only heterozygous markers were considered as informative. Table 3 showed the summary of the number of informative samples and the LOH frequency for each marker. There were 211 cases in total, except for D4P4 marker which contained only 209 cases due to failure in the PCR amplification for two samples. The four markers displayed a heterozygosity rate ranging from 56.9% to 72.2%, while the LOH frequency ranged from 15.8% to 32.8%. Among the four markers, marker D4P3, which is located nearest to the *PCDH10* gene, displayed the highest frequency of LOH (32.8%).

Next we try to correlate the relationship between clinicopathological characteristics of patients and the incidence of LOH for each marker. Table 4 showed the statistical results. Markers D4P2 and D4P3 showed statistically significant correlation between incidence of LOH and distal metastasis of tumor ( $p=0.0383$  and  $p=0.0440$ , respectively). For the other variables, there is no statistical significance ( $p>0.05$ ) between the groups that are compared with LOH (+) and LOH (-) for each marker.

The four markers surrounding *PCDH10* allow us to determine whether allelic deletion of *PCDH10* occurred. Therefore, analysis of relationship between patient clinicopathological characteristics and the incidence of allelic deletion at *PCDH10* was performed (Table 5). Only patients who obtained curable surgery before December 31<sup>st</sup>, 2008, were recruited for the study. Out of 211 pairs of CRC samples, ten were collected after the above date, so only 201 samples remained. Among these 201 samples, ten were identified as non-informative due to homozygosity in all the four markers studied, therefore, these ten cases were also excluded from the analyses. Out of the 191 cases, 57 cases were defined as having allelic deletion at the *PCDH10* gene. The correlation between distal metastasis of tumor and the incidence of LOH was statistically significant ( $p=0.0250$ ). For the other variables, there is no statistical significance between the groups that are compared with *PCDH10* allelic deletion (+) and allelic deletion (-).

Overall survival (OS) analysis was performed on 174 patients out of 191 because of five patients without follow-up after surgery and 12 patients died of postoperative complications. Disease-free survival (DFS) was performed on 118 patients because only patients with Dukes' B or C were recruited (Table 6 and Figure 13). Allelic loss of *PCDH10* gene predicted poor OS ( $p=0.0082$ ). Among 54 patients with allelic deletion, the OS was 55.6% and had a mean survival of 35 months, while the other 120 patients without allelic loss had OS of 72.5% and mean survival of 49 months. However, there was no statistically significant correlation between allelic loss and DFS among Dukes' B and C patients ( $p=0.6135$ ).

Previously our lab had identified the methylation status of the 5' promoter region of *PCDH10* of 74 pairs of CRC samples. Here, relationship between incidence of LOH and promoter hypermethylation on patients' survival was analyzed. Survival analysis was first independently performed depending on the incidence of LOH or methylation status of these 74 pairs. Among the 74 patients, two died of postoperative complications, three were without follow-up after surgery, and four had non-informative results in the LOH study; therefore, these nine cases were excluded from the survival analysis relating LOH. OS analysis was performed for the remaining 65 pairs of CRC samples depending on the incidence of LOH (Table 7 and Figure 14A). For DFS analysis, only 47 patients in Dukes' B and C were recruited (Table 7



and Figure 14B). No significant correlations were found between incidence of LOH and OS or DFS survival ( $p=0.1273$  and  $p=0.4680$ , respectively). For correlation between promoter methylation and survival, 69 patients were included for the study because two died of postoperative complications and three were without follow-up after surgery. For DFS analysis, only 51 patients with Dukes' B and C were recruited. Results as shown in Table 7 and Figure 15. No statistically significant correlations were found between promoter hypermethylation and OS or DFS survival ( $p=0.3212$  and  $p=0.1354$ , respectively).

Combined influence of incidence of LOH and promoter methylation on OS was investigated. Out of the 74 patients, as in the above OS analysis related to the LOH status, only 65 patients were recruited for the analysis. Patients were divided further into four groups: neither with the presentation of LOH nor promoter hypermethylation of *PCDH10*, with presentation of LOH but no promoter hypermethylation, with no presentation of LOH but with promoter hypermethylation, and with presentation of both of LOH and promoter hypermethylation. Results as shown in Table 7 and Figure 16A. There was no statistically significant correlation between the incidence of LOH and promoter hypermethylation with OS ( $p=0.4655$ ). Then, patients were divided into two groups: neither with the presentation of LOH nor promoter hypermethylation of *PCDH10*, and with presentation of LOH and/or promoter hypermethylation of

*PCDH10*. Results as shown in Table 7 and Figure 16B. There was also no statistically significant correlation between the two groups and OS ( $p=0.7704$ ).

#### 4.2.2 Copy number variation of *PCDH10*

Ten normal PBMC samples were recruited to investigate the copy number variation of *PCDH10* in normal individuals. Results were shown in Figure 17A. Normal PBMCs revealed a copy number of two for *PCDH10*. In addition, five pairs of CRC samples defined as having allelic deletion at *PCDH10* by LOH study, and five pairs of CRC samples without, were also selected to validate the copy number assay (Figure 17A). Results showed that only one tumor sample (CRC19T, copy number=1) exhibited corresponding results as that in LOH study. The rest showed a copy number of two regardless of the incidence of LOH. Copy number assay was also performed in a panel of CRC cell lines, which included HCT116, HCT15, HT29, COLO205, SW480, SW620, KM12, Caco2, SW48 and LoVo (Figure 17B). Only Caco2 revealed a change in copy number (copy number=3), the others had a copy number of two. Previous study of *PCDH10* V1 expression using RT-PCR revealed no expression of *PCDH10* V1 in Caco2. In addition, RT-PCR showed that *PCDH10* V1 expressed in HCT116. Methylation study of *PCDH10* in Caco2 and HCT116 by Methylation-specific PCR (MSP) showed that the CpG sites investigated were methylated. In addition,

bisulfite-sequencing PCR (BSP) study on these two cell lines also revealed high percentage of methylated CpG sites.

Since it was unable to observe a positive correlation between LOH study and copy number assay, samples with apparent LOH indexes were recruited for a final validation of the copy number assay. An apparent LOH index often reflects low contamination of tumors with normal cells. A total of 13 CRC cases were studied, only two samples (2CRC3T and 2CRC81T) showed a copy number of one, while the copy numbers of the remaining samples ranged from two to seven (Figure 17C and Table 8).



## 5. Discussion

In the present study, when PCDH10 V1 and PCDH10 V2 single stable clones were treated with camptothecin, increased drug susceptibility of HCT116 cells was observed. Although camptothecin is no longer used in the chemotherapy for the treatment of CRC due to its low solubility and adverse drug effect, however, analogues or derivatives of camptothecin, such as Irinotecan, are used in routine treatment. These kinds of analogues act in the same mechanism as that of camptothecin.<sup>38</sup> Based on the above results, re-expression of PCDH10 V1 or PCDH10 V2 exerted impacts on apoptosis after HCT116 treated with camptothecin. This is an interesting result because PCDH10 V2 possesses a much shorter cytoplasmic tail than PCDH10 V1. Cytoplasmic domain of PCDH10 is assumed to transduce signals to the cells. Previous results from our lab on the investigation of *PCDH10* V2 RNA expression reported that the expression level of *PCDH10* V2 was very low and it was often associated with strong *PCDH10* V1 expression. Therefore, the results observed by over-expression of *PCDH10* V2 may not be compatible with *in vivo* situation. Future studies on apoptosis will recruit chemotherapeutic drugs such as 5-Fluorouracil, that are used in present clinical regimens.<sup>39 40</sup>

Loss or gain of chromosomal regions is one of the most important mechanisms

underlying tumorigenesis, in which tumor cells may gain abnormal growth control. Besides, loss or gain of chromosomal regions also leads to alteration in gene expression. It is postulated that tumor suppressor genes often exist in regions where losses occurred, and is often reflected as allelic imbalance or LOH at molecular level.<sup>41</sup>

According to the allelic deletion study of *PCDH10* gene, heterozygosity rates for all the four markers, except for D4P4, were relatively low. D4P1 showed the lowest heterozygosity rate (56.9%) and its location is the farthest from *PCDH10*. Because of its low heterozygosity rate and low LOH frequency, we conclude that this marker may be dispensable and can be excluded when determining allelic deletion of *PCDH10* gene. On the other hand, marker D4P3, which is located 388bp downstream of *PCDH10*, showed the highest LOH frequency (32.8%). This marker alone allows us to determine the status of allelic deletion of *PCDH10* for most cases. Statistical analysis of LOH status in each marker and in allelic deletion of *PCDH10* with clinicopathological characteristics of patients showed that incidence of LOH in markers D4P2 and D4P3, and allelic deletion of *PCDH10*, were significantly associated with distal metastasis of CRC. This result corresponded to the results from the previous studies in our lab, in which invasion ability decreased when *PCDH10* V1 was re-expressed in CRC cells. Since markers D4P2 and D4P3 were critical when determining the status of allelic deletion, so when LOH of these two markers was

observed, allelic deletion of *PCDH10* was defined. The results showed that allelic deletion of *PCDH10* were correlated to a poor OS of patients as estimated by the Kaplan-Meier method and compared by log-rank test, however, it was not found to be correlated to DFS in Duke's B and C patients. Patients in Dukes' D, who have the worst overall survival, may contribute bias when considering overall survival. However, in our study of allelic deletion of *PCDH10* and overall survival, the number of patients in Dukes' D was similar to the remaining groups, which can eliminate the bias from this group of patients in survival analysis. In addition, when investigating the impacts of allelic deletion of *PCDH10* on survival, change in survival rate and duration of patients with CRC should be considered because due to the advances in the treatment of CRC, five-year survival rate of patients will change over time. This change may also cause a bias in the current survival analysis. At present, approximately one-fourth of patients have metastatic CRC at diagnosis, with a five-year survival rate of less than 10%. Nevertheless, with the development of target therapy and combination of chemotherapy, patients with metastatic CRC have their median overall survival raised from six to eight months, up to 20 months over the past ten years.<sup>40 42</sup> Though patients in our study were collected throughout ten years of time, however, even with the advances in the treatment of CRC, significant difference in overall survival can still be observed in patients with *PCDH10* allelic deletion. In the

future, multi-variable analysis on survival will be carried out.

With the availability of *PCDH10* methylation status of 74 paired CRC samples, OS analysis on combined influence of incidence of LOH and promoter hypermethylation was performed. The outcome was not statistically significant, however, this may be due to the small sample size. Moreover, patients with allelic deletion only displayed poorer OS than any other conditions, while patients without both of allelic deletion and promoter hypermethylation of *PCDH10* gene showed relatively better OS. More samples should be included in future study.

*PCDH10* is reported as a transmembrane protein, this prompts us to investigate whether *PCDH10* is involved in the EMT pathway or not. Preliminary results using TGF- $\beta$  to treat CRC cell line SW480 showed induction of cells to undergo EMT. In future study, *PCDH10* V1 will be transiently transfected to SW480 and treated with TGF- $\beta$  in order to study *PCDH10* effect on EMT. Besides, EMT is reported to be one of the mechanism underlying tumor metastasis,<sup>43</sup> and study from the above allelic deletion of *PCDH10* also revealed association of *PCDH10* with distal metastasis. Therefore, study of involvement of *PCDH10* in EMT was of great interest.

Taqman<sup>®</sup> Copy number assay was used to compare with the results of LOH study, in hope of finding a method to substitute the laborious process when carrying out the LOH study. Normal PBMCs showed a copy number of two of *PCDH10* gene, and up

to date, no copy number variations for *PCDH10* have been reported in the database of genomic variants.<sup>44</sup> Nevertheless, we cannot observe a positive correlation between copy number assay and LOH study. Most cases with incidence of allelic deletion of *PCDH10* showed no evidence of a reduced copy number, and by contrast, a few cases showed an increased copy number. This may be a consequence of normal cell contamination using patients' primary tumors<sup>41</sup> because our samples were not obtained by laser capture, microdissection, or because of intratumor heterogeneity. In addition, only one specific region in exon four was investigated by this copy number assay, it may not be enough to detect the loss of other regions of *PCDH10*. Many other target sites have to be included in further comparison of LOH study to copy number loss. Recently, frequent occurrence of uniparental disomy revealed by SNP arrays was reported in CRC and other cancers.<sup>45 46</sup> Uniparental disomy is a genetic change in which one allele is lost while the remaining allele gains through duplication, resulting in LOH but no copy number change. Genomic alterations of this kind were also reported to impose significant changes on gene expression, suggesting that when gene dosage was unchanged due to uniparental disomy, the gene expression level may mainly remained unchanged. This has to be taken into consideration when performing LOH study and copy number assay.

Controversial roles of *PCDH10* V2 were found in the proliferation assay and



apoptosis assay. Investigation of the effect of PCDH10 V2 on PCDH10 V1 will be carried out using transient overexpression in PCDH10 V1 single stable clones. If the results are conclusive, we will move on to stable clones selection using pEF6/V5-His-TOPO which contains different selection marker from PCDH10 V1. The *PCDH10* V2 sequence was obtained from the original *PCDH10* V2/pcDNA3.1/V5-His-TOPO through enzyme digestion and ligation to the pEF6 vector. Nevertheless, as mentioned previously, *in vitro* culture systems may not be able to reflect *in vivo* condition and PCDH10 was reported to exist cell-cell adhesion, therefore, interaction of the cellular matrix or extracellular environments may have trigger different influence of PCDH10 V2 on PCDH10 V1.

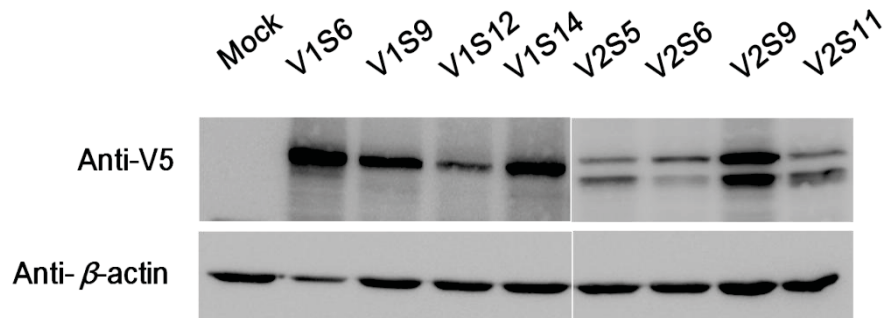
Other protocadherins were also reported to be candidate tumor suppressors in various carcinomas besides Protocadherin 10.<sup>47</sup> Decreased expression of Protocadherin 1 (*PCDH1*) was shown to be a predictor for poorer overall survival in medulloblastoma patients.<sup>48</sup> Loss of Protocadherin 8 (*PCDH8*), which is the human ortholog of Paraxial Protocadherin in frog, fish and mouse, through mutation and epigenetic silencing was also found in breast carcinomas.<sup>49</sup> Protocadherin 20 (*PCDH20*) exhibited hypermethylation at the promoter region in non-small-cell lung cancer cell lines. Hypermethylation of *PCDH20* was also shown to be associated with a poorer overall survival in patients.<sup>50</sup> These further support that the non-classic

cadherins may play a role in tumor suppression aside from cell-cell adhesion.

In conclusion, apoptosis and gene deletion studies on *PCDH10* support its role as a putative tumor suppressor gene in CRC. The sequence of which LOH or hypermethylation takes place, occurs at which tumor stage, or which mechanism play a more important role in CRC tumorigenesis remain to be answered.



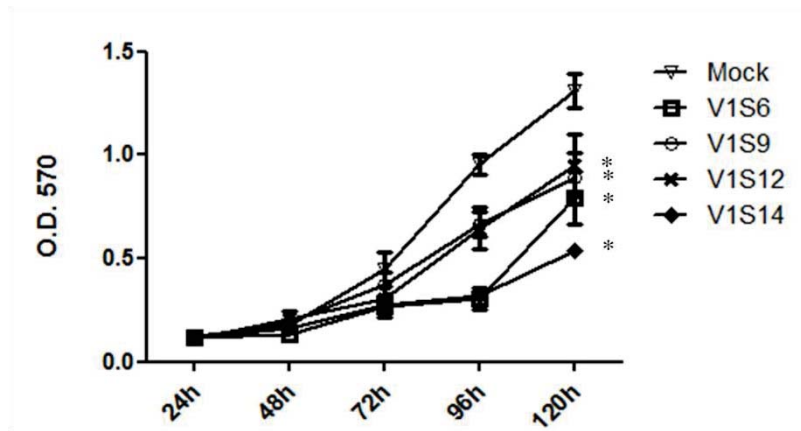
## Figures



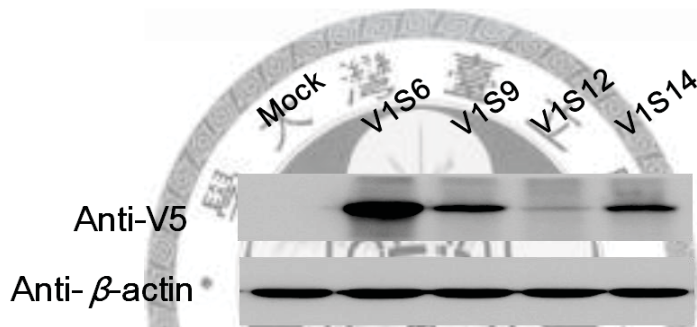
**Figure 1. Protein expression of newly selected PCDH10 variant 1 and PCDH10 variant 2 single stable clones.**

Protein levels of PCDH10 V1 and PCDH10 V2 single stable clones were determined by Western blotting with anti-V5 antibody.  $\beta$ -actin was used as a protein loading control. Molecular weight of PCDH10 V1 and PCDH10 V2 is 116 kDa and 100 kDa, respectively according to NCBI. Mock represents pcDNA3.1/V5-His-TOPO. V1 and V2 stand for PCDH10 V1 and PCDH10 V2, respectively.

A.



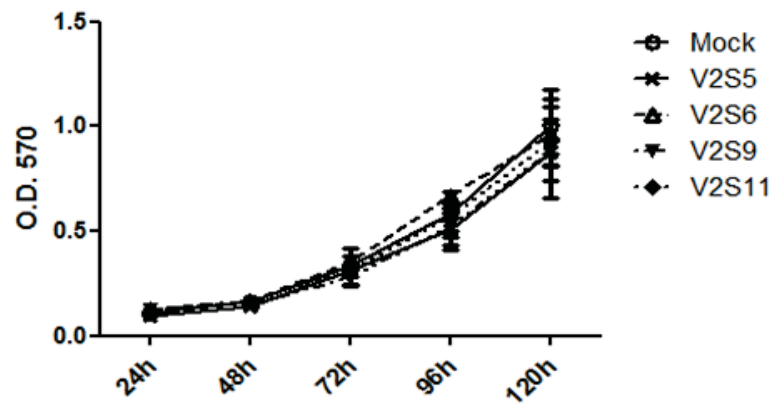
B.



**Figure 2. Cell proliferation of four new single stable clones of PCDH10 V1 using MTT assay (A) and the corresponding protein expression (B).**

The proliferation rates of four PCDH10 V1 single stable clones were compared with a pcDNA3.1 only stable line (Mock) by MTT assay (A), and the corresponding protein expression was determined by Western blotting (B). Cell proliferation decreased significantly with PCDH10 V1 re-expression when compared to Mock ( $*p < 0.01$ ). V1 stands for PCDH10 V1.

A.

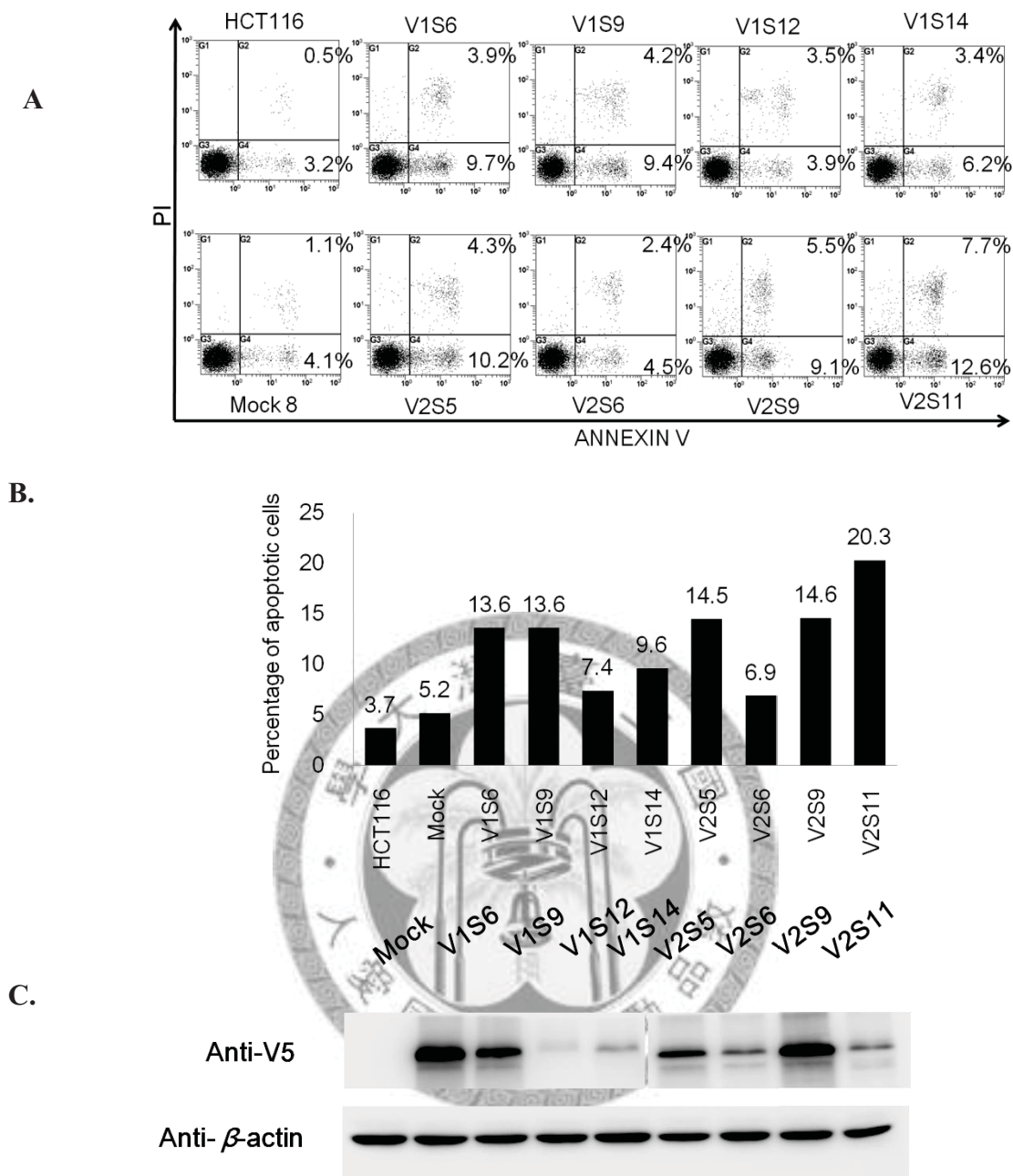


B.



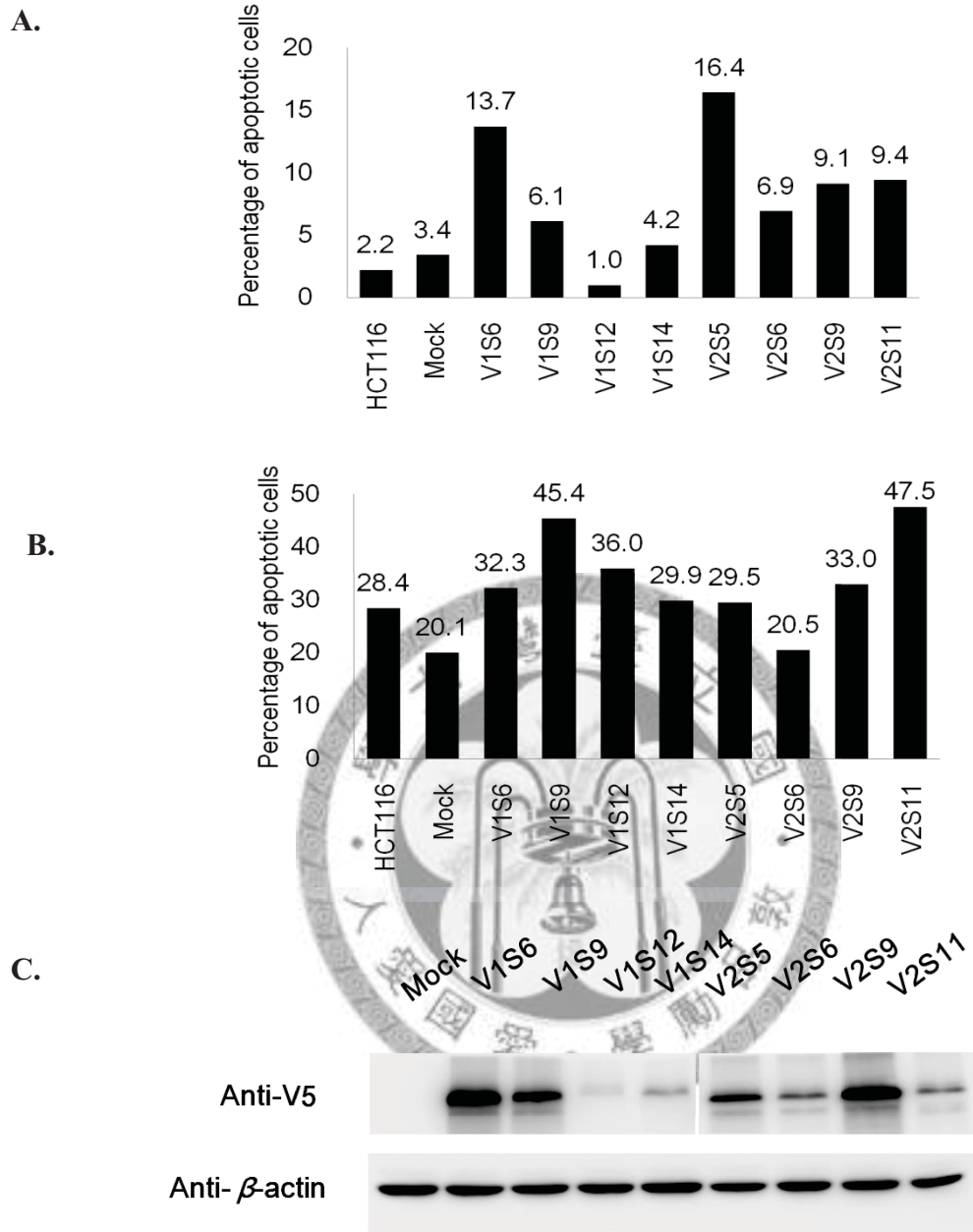
**Figure 3. Cell proliferation of four new single stable clones of PCDH10 V2 using MTT assay (A) and the corresponding protein expression (B).**

The proliferation rates of four PCDH10 V2 single stable clones were compared with a pcDNA3.1 only stable line (Mock) by MTT assay (A), and the corresponding protein expression was determined by Western blotting (B). Cell proliferation was similar to Mock with PCDH10 V2 re-expression. V2 stands for PCDH10 V2.



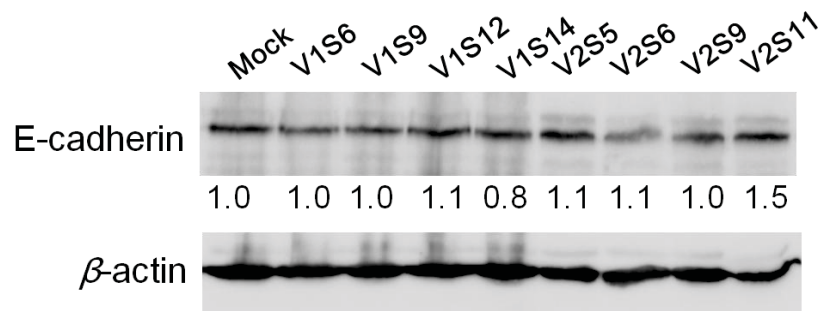
**Figure 4. Re-expression of PCDH10 V1 and PCDH10 V2 induced spontaneous apoptosis of CRC cell line, HCT116.**

The apoptosis percentages of PCDH10 V1 and PCDH10 V2 single stable clones were compared with a pcDNA3.1 only stable line (Mock) by Annexin V-PI staining (A), and quantitative results of flow cytometry were shown in (B). Corresponding protein expression was detected by Western blotting (C). V1 and V2 stands for PCDH10 V1 and PCDH10 V2, respectively. Apoptosis percentages increased with the re-expression of PCDH10 compared to Mock. Mock showed similar apoptosis percentage to the parental cell line, HCT116.



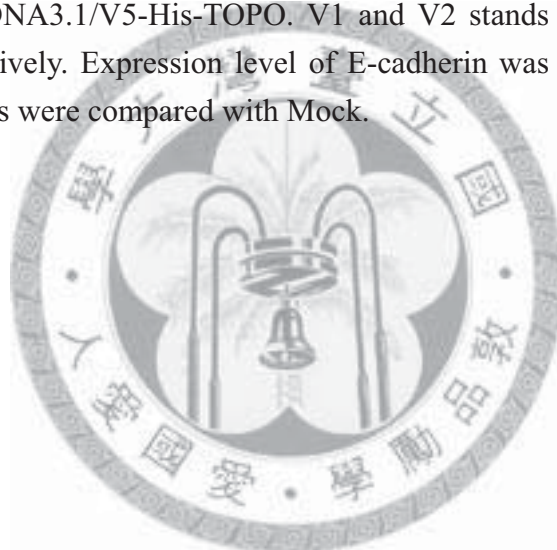
**Figure 5. Camptothecin-induced apoptosis of PCDH10 V1 and PCDH10 V2 single stable clones.**

DMSO was used to treat PCDH10 V1 and PCDH10 V2 single stable clones as control since camptothecin was dissolved in DMSO. Percentage of apoptotic cells of DMSO-treated group was shown in (A). Percentages of apoptotic cells in PCDH10 V1 and PCDH10 V2 single stable clones were shown after subtracting that of the DMSO-treated Mock, except for HCT116, which shows the percentage after subtracting its own DMSO-treated control (B). Corresponding protein expression was detected by Western blotting (C). Mock represents pcDNA3.1/V5-His-TOPO. V1 and V2 stands for PCDH10 V1 and PCDH10 V2, respectively.



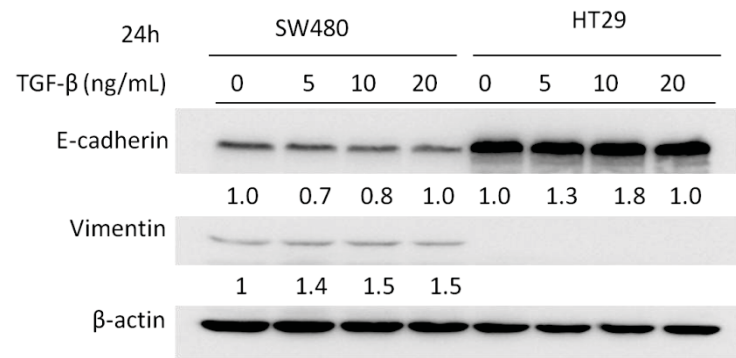
**Figure 6. Relative expression of E-cadherin in all PCDH10 single stable clones.**

Western blot analysis shows protein expression of E-cadherin in various cell clones. Mock represents pcDNA3.1/V5-His-TOPO. V1 and V2 stands for PCDH10 V1 and PCDH10 V2, respectively. Expression level of E-cadherin was normalized to that of  $\beta$ -actin, and the results were compared with Mock.

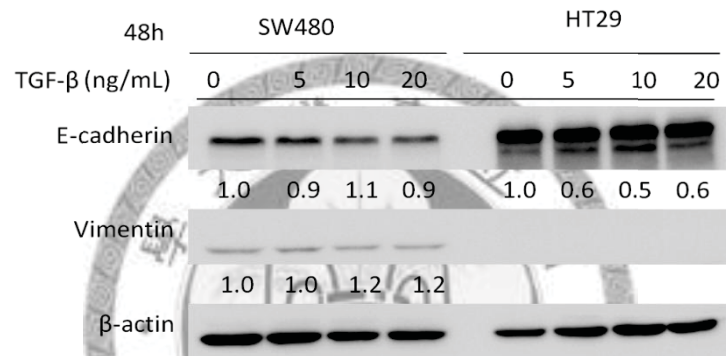




A.



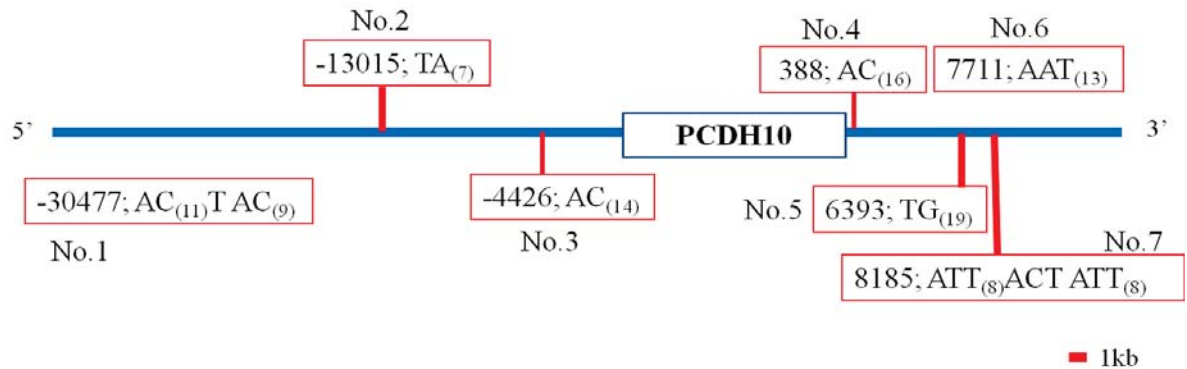
B.



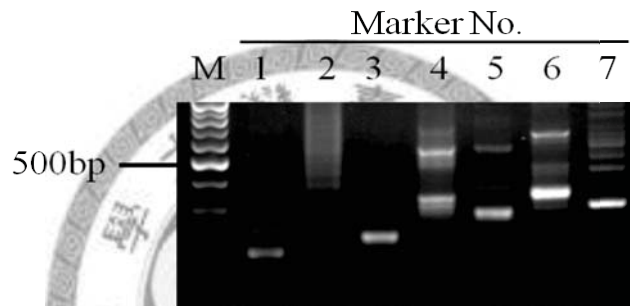
**Figure 7. Different concentration of TGF- $\beta$  treatment on CRC cell lines, SW480 and HT29, for 24 or 48h.**

5, 10 or 20ng/mL TGF- $\beta$  was used to treat CRC cell lines SW480 and HT29 for 24h (A) or 48h (B) to undergo EMT. Protein expression of vimentin in HT29 cannot be detected. All protein expression level was normalized to  $\beta$ -actin. Values are expressed as fold change compared with the untreated group.

A.

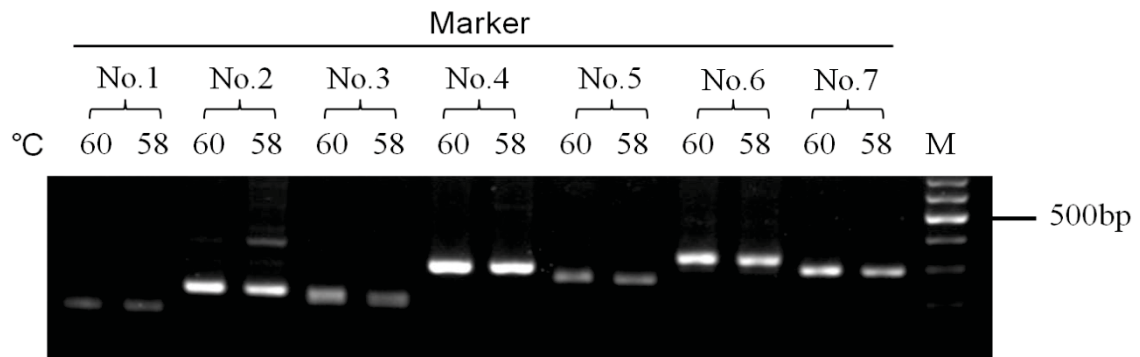


B.



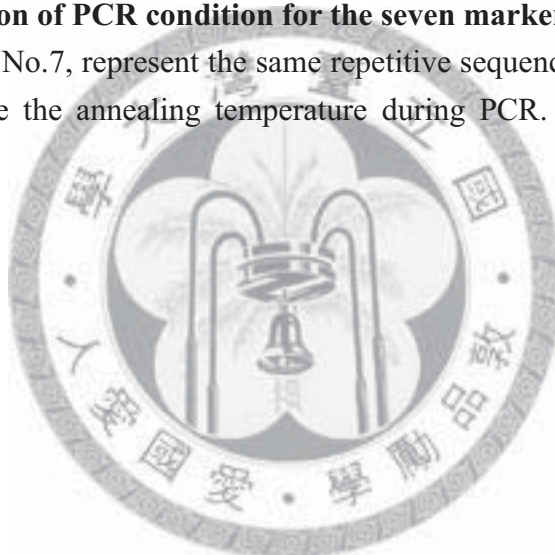
**Figure 8. Seven microsatellite markers for the detection of allelic deletion of *PCDH10* by LOH study.**

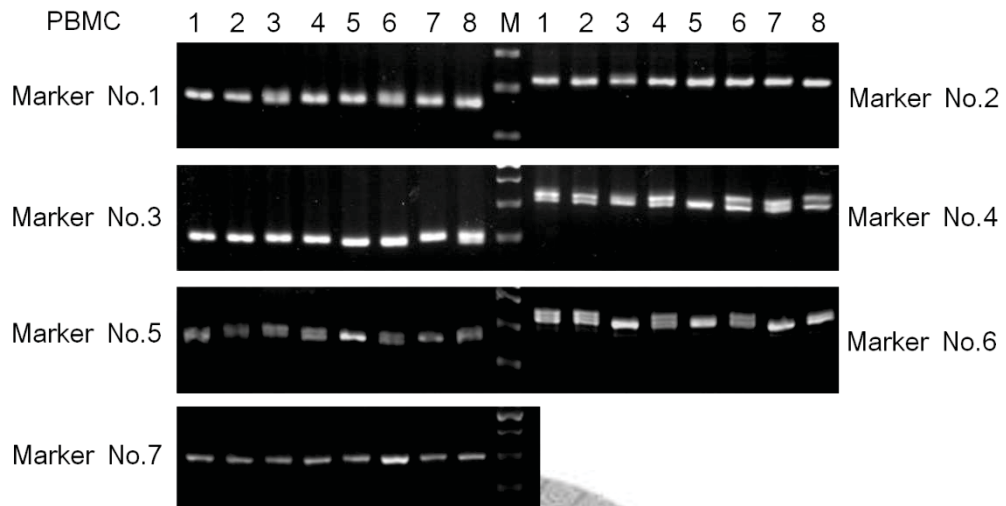
Preliminary test of the seven primer sets for amplifying the repetitive sequence No.1 to No.7 surrounding the *PCDH10* gene. Figure 8A shows the location of the markers, in which negative values represent the numbers of base pairs (bp) upstream from the first nucleotide of the *PCDH10* gene, and positive values represent the numbers of bp downstream from the last nucleotide of the *PCDH10* gene. Figure 8B shows the result of PCR visualized by gel electrophoresis. DNA was obtained from normal PBMCs. M: 100bp DNA size marker.



**Figure 9. Optimization of PCR condition for the seven markers designed.**

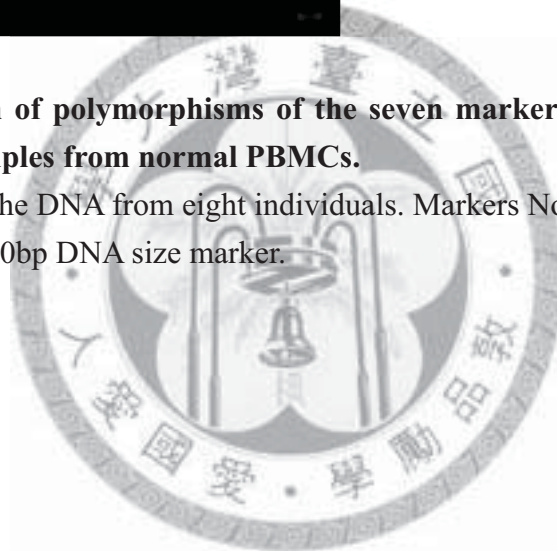
The markers, No.1 to No.7, represent the same repetitive sequences designed in Figure 8. 60 and 58 indicate the annealing temperature during PCR. M: 100bp DNA size marker.

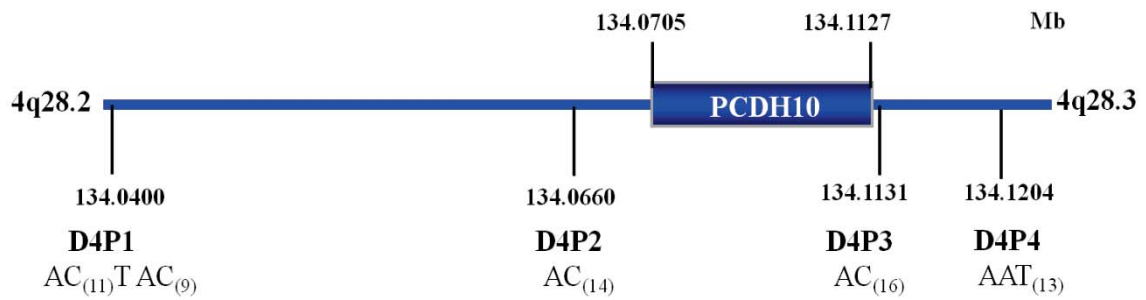




**Figure 10. Detection of polymorphisms of the seven markers in a panel of eight DNA samples from normal PBMCs.**

PBMCs 1-8 indicate the DNA from eight individuals. Markers No.1, 3, 4, 5 and 6 show polymorphism. M: 100bp DNA size marker.

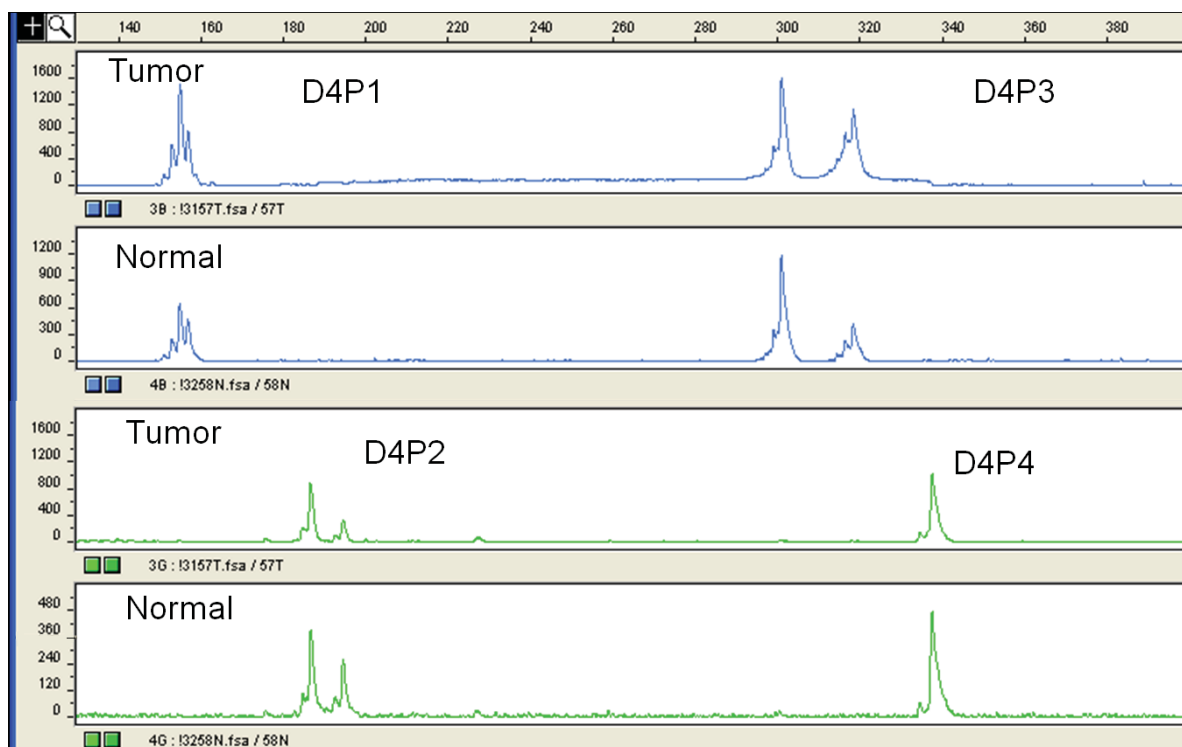




Marker	Location	Repeat unit	Product size (bp)
D4P1	-30477	AC <sub>(11)</sub> T AC <sub>(9)</sub>	149
D4P2	-4426	AC <sub>(14)</sub>	189
D4P3	388	AC <sub>(16)</sub>	304
D4P4	7711	AAT <sub>(13)</sub>	324

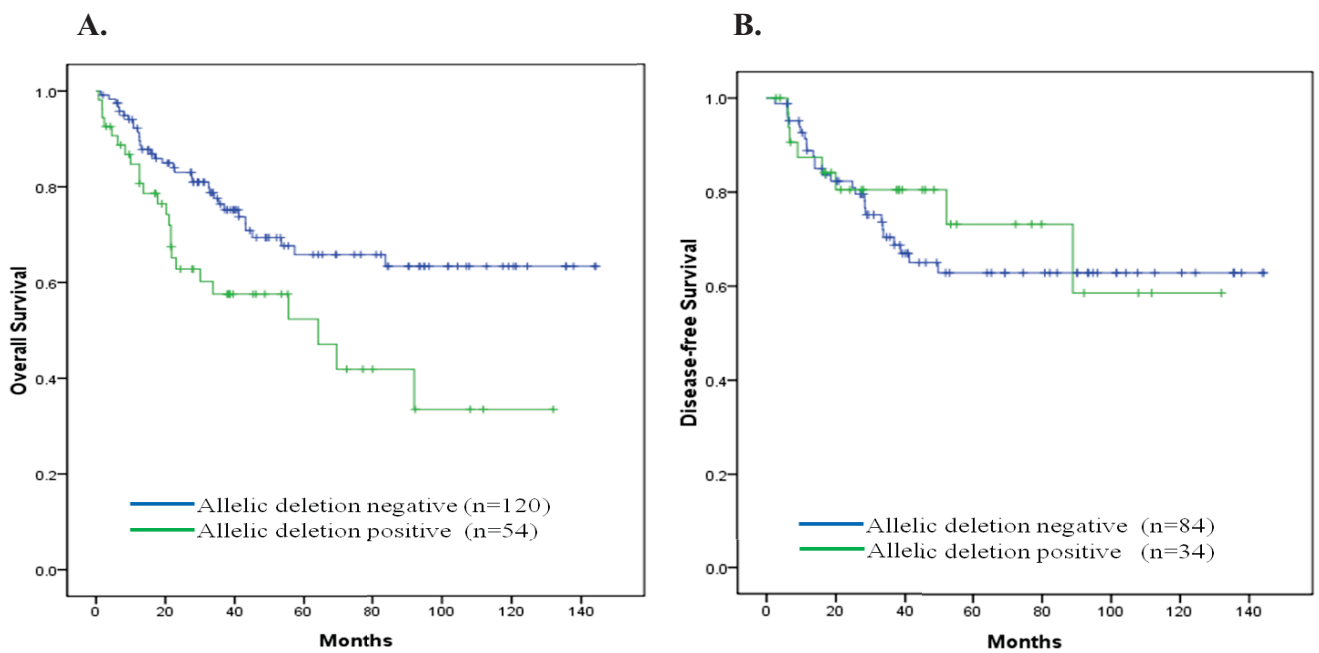
**Figure 11. Four markers designed for the study of allelic deletion of *PCDH10*.**

Negative values in location represent the numbers of base pairs (bp) upstream from the first nucleotide of the *PCDH10* gene, and positive values represent the numbers of bp downstream from the last nucleotide of the *PCDH10* gene. Repeat numbers in repeat unit and product sizes are according to the sequence from Ensembl (ENSG00000138650).



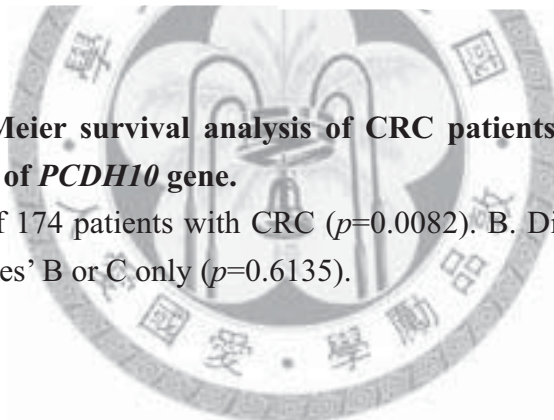
**Figure 12. Results of LOH study of one paired CRC sample analyzed by GeneScan.**

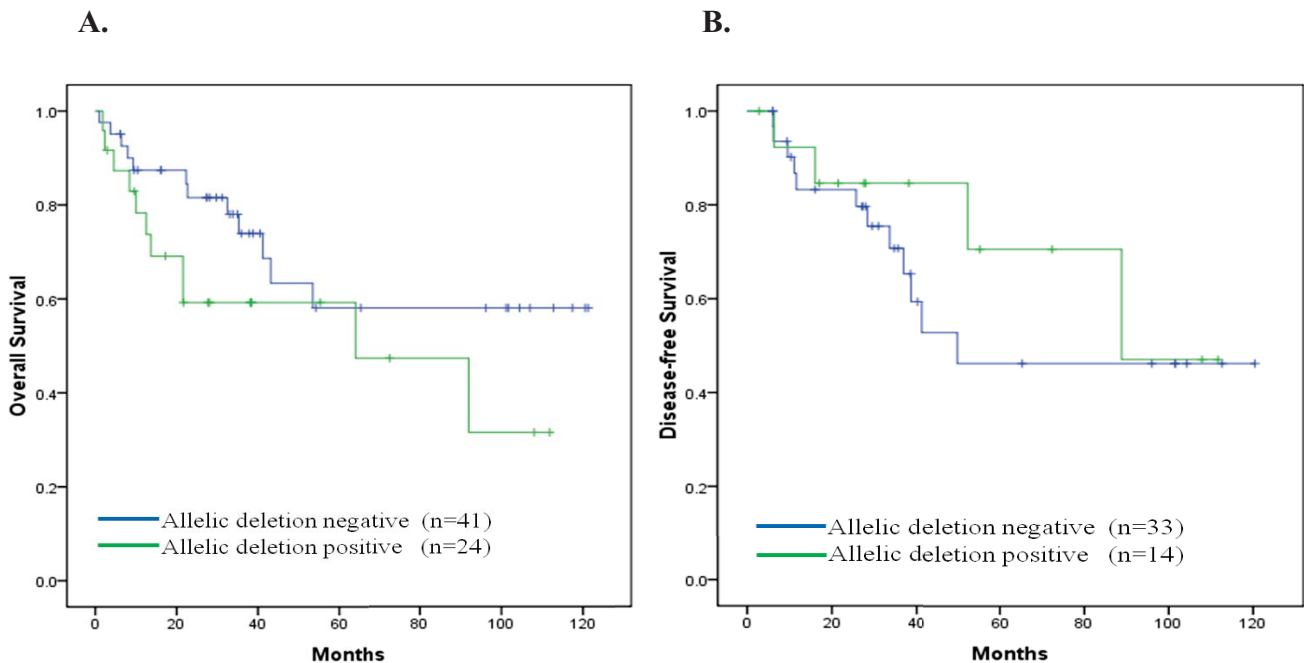
X-axis represents the size of the PCR product, and y-axis represents the intensity of the fluorescence. Marker D4P4 shows a result of homozygous alleles while the other markers show a result of heterozygous alleles. Markers D4P2 and D4P3 exhibit LOH.



**Figure 13. Kaplan-Meier survival analysis of CRC patients according to allelic deletion of *PCDH10* gene.**

A. Overall survival of 174 patients with CRC ( $p=0.0082$ ). B. Disease-free survival of 118 patients with Dukes' B or C only ( $p=0.6135$ ).

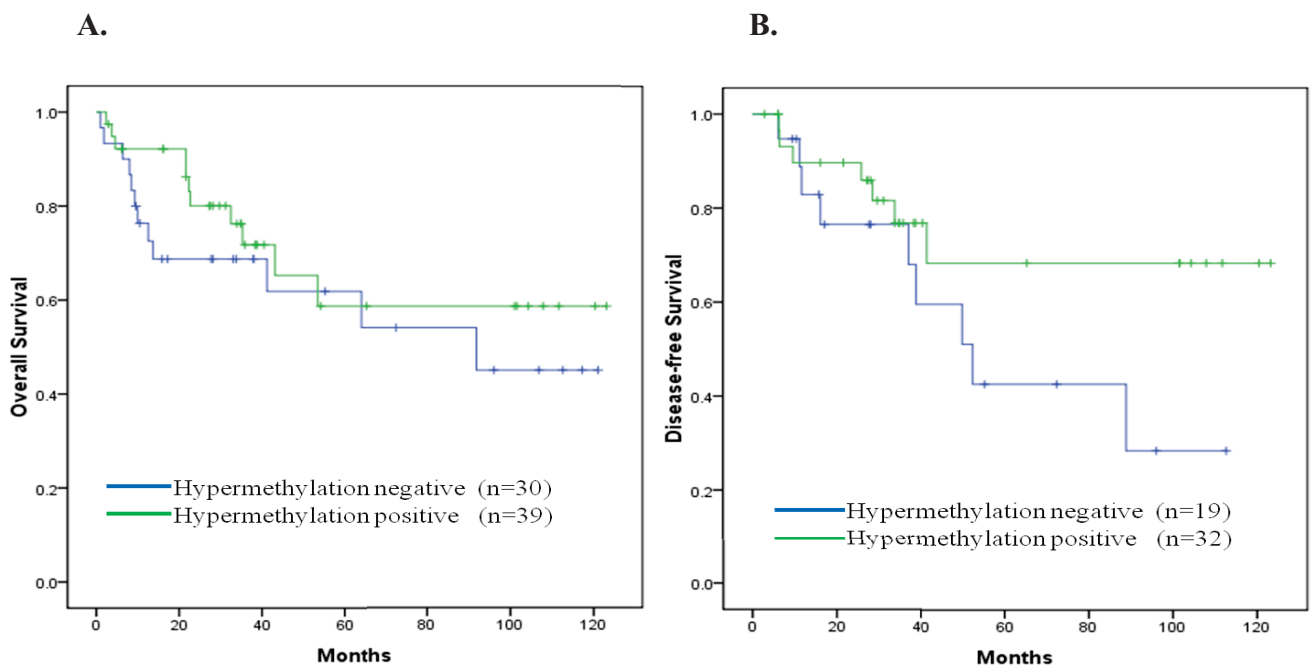




**Figure 14. Kaplan-Meier survival analysis of CRC patients according to allelic deletion of *PCDH10* gene.**

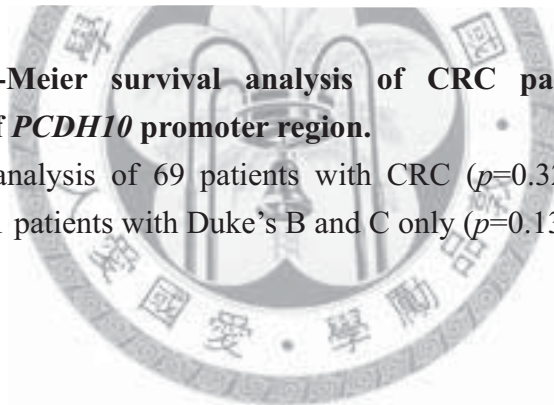
Since promoter hypermethylation status was available for 74 patients in CRC, therefore, investigation of incidence of LOH of *PCDH10* and promoter hypermethylation with OS and DFS was carried out. Independent analysis for incidence of LOH and promoter hypermethylation with OS and DFS was first performed. Here shows the result of OS for 65 patients according to allelic deletion of *PCDH10* gene. (A) There are total 65 patients for OS analysis because of two patients died of postoperative complications, three without follow-up after surgery and four without informative results in LOH study. (B). For DFS analysis, only 47 patients in Dukes' B and C were recruited. No correlation was found between incidence of LOH and OS or DFS ( $p=0.1273$  and  $p=0.4680$ , respectively).

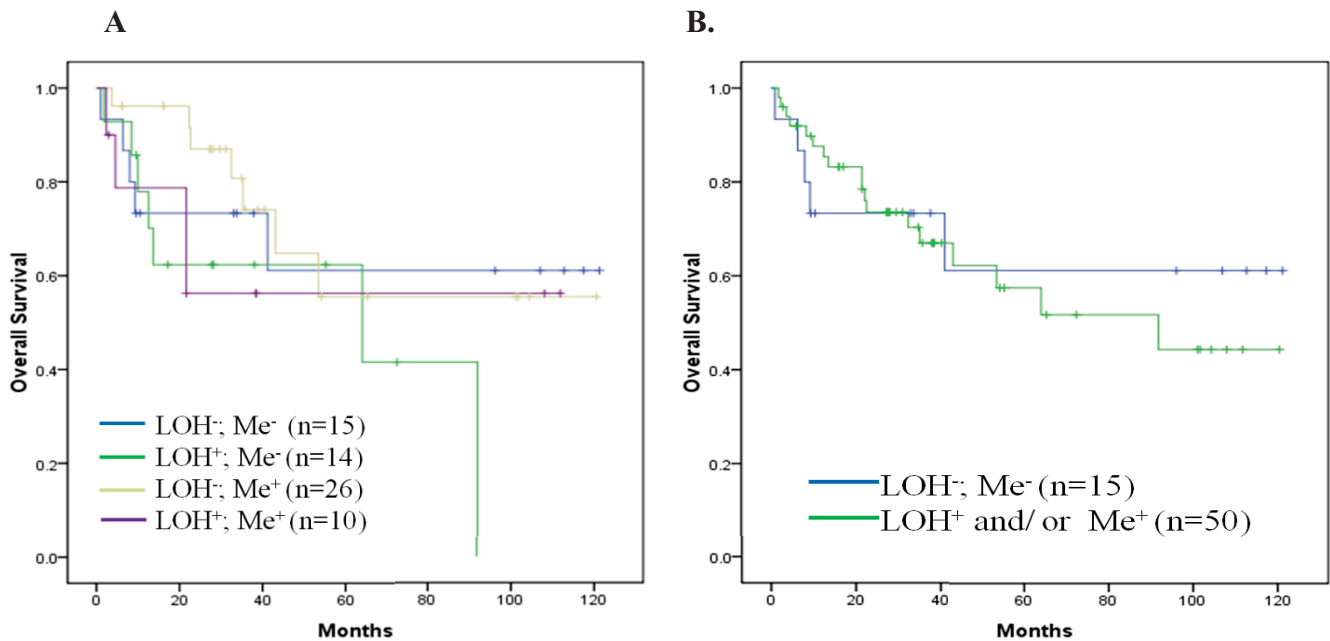




**Figure 15. Kaplan-Meier survival analysis of CRC patients according to methylation status of *PCDH10* promoter region.**

A. Overall survival analysis of 69 patients with CRC ( $p=0.3212$ ). B. Disease-free survival analysis of 51 patients with Duke's B and C only ( $p=0.1354$ ).

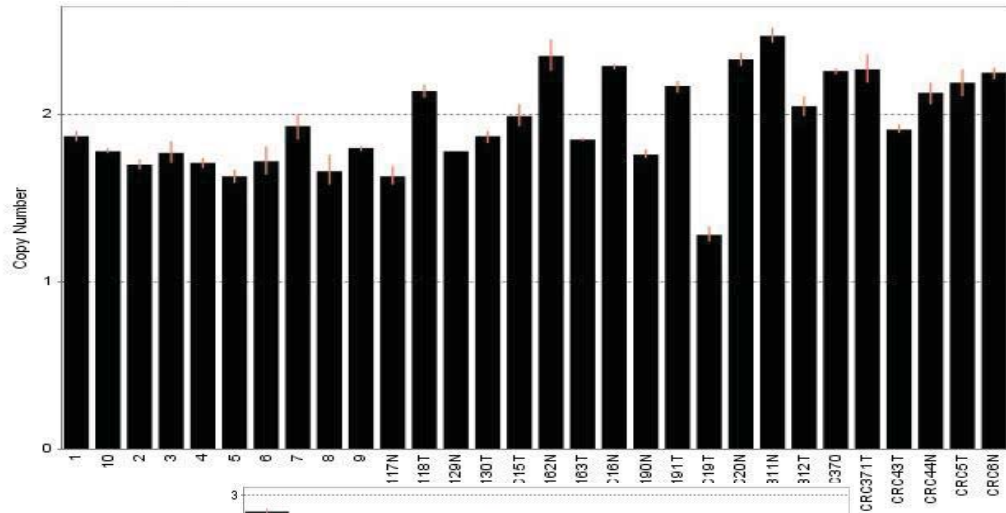




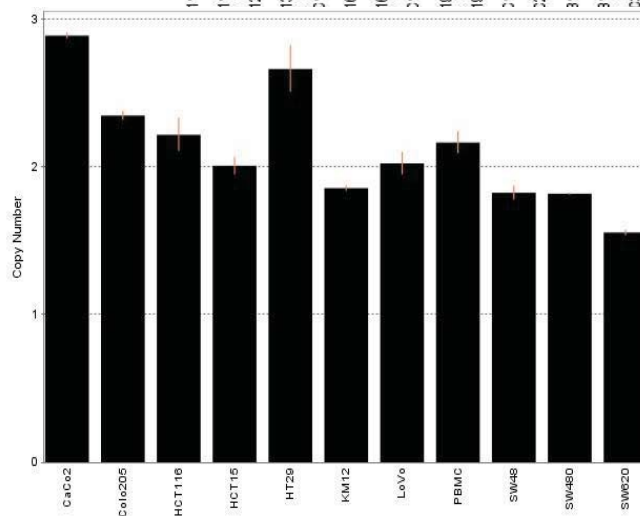
**Figure 16. Kaplan-Meier survival analysis of 65 CRC patients according to LOH and promoter hypermethylation (Me) of *PCDH10* gene**

A. There was no statistically significant correlation between the incidence of LOH and promoter hypermethylation of *PCDH10* with OS in the four groups ( $p=0.4655$ ). B. Patients in A. are divided into two groups: neither with the presentation of LOH nor promoter hypermethylation, and with presentation of LOH and/or promoter hypermethylation. There was no statistically significant correlation between the incidence of LOH and/or promoter hypermethylation of *PCDH10* with OS in the two groups ( $p=0.7704$ ).

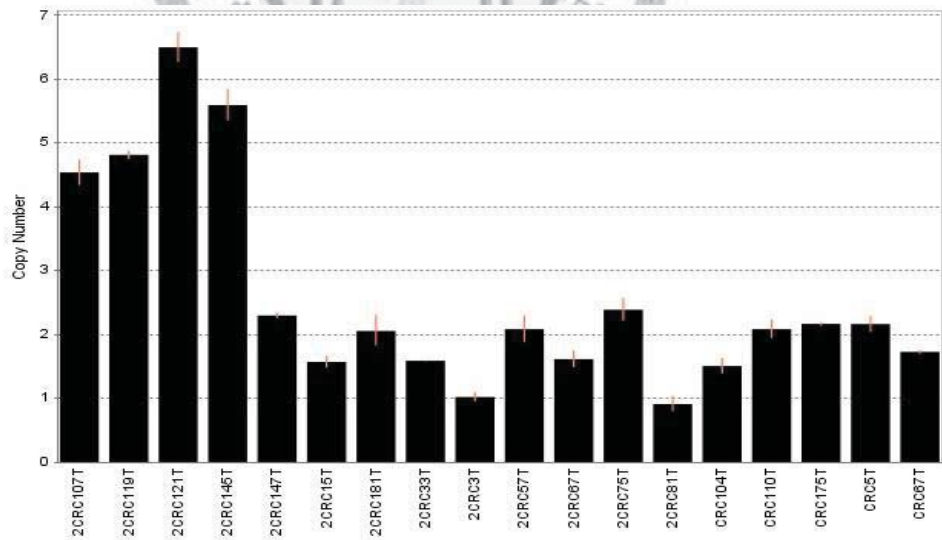
A.



B.



C.



**Figure 17. Copy number assay of *PCDH10* gene.**

A. Normal PBMCs are obtained from ten individuals (1-10), and the others are paired CRC samples. B. *PCDH10* copy numbers were determined with a panel of CRC cell lines. C. *PCDH10* copy numbers were determined with 18 colorectal tumors with apparent LOH.

## Tables

**Table 1. Patient clinicopathological characteristics (n=211)**

Characteristic	No. of patients (%)
Age (yr)	
Median	73
Range	37-98
Gender	
Male	110 (52.1)
Female	101 (47.9)
Tumor location <sup>a</sup>	
Proximal colon	49 (23.2)
Distal colon	162 (76.8)
Dukes' stage	
A	22 (10.4)
B	83 (39.3)
C	66 (31.3)
D	40 (19.0)
Tumor stage <sup>b</sup>	
Early stage	105 (49.8)
Late stage	106 (50.2)
Distal metastasis <sup>c</sup>	
Yes	40 (19.0)
No	171 (81.0)
Pathological differentiation <sup>d</sup>	
Moderate	155 (74.2)
Poor	54 (25.8)
Disease recurrence <sup>e</sup>	
Yes	34 (26.4)
No	95 (73.6)

<sup>a</sup> Proximal colon includes the cecum, ascending colon and transverse colon. Distal colon includes the descending colon, sigmoid colon, rectosigmoid junction and the rectum.

<sup>b</sup> Early stage refers to Dukes' A and B. Late stage refers to Dukes' C and D.

<sup>c</sup> Distal metastasis: Dukes' D; no distal metastasis: Dukes' A, B and C.

<sup>d</sup> 209 patients in total because of two patients without pathological reports.

° 129 patients with Dukes' B and C, who obtained curable surgery before December 31<sup>st</sup>, 2008, are assessed for disease recurrence.



**Table 2. Primers for LOH study**

Marker	Location <sup>a</sup>	Repeat unit <sup>b</sup>	Primer sequence <sup>c</sup>	Fluorescence dye labeled <sup>d</sup>	Product size (bp) <sup>b</sup>
D4P1	-30477	AC <sub>(11)</sub> TAC <sub>(9)</sub>	5' GGG ATT GTT TCT AAG GCC AA 3' 5' <u>GTT TCT TCA TAC CCA TAC ACA AAC ATC ACC</u> 3'	6-FAM	149
D4P2	-4426	AC <sub>(14)</sub>	5' CTA CCA AAA GAC ACA ATA CGT GA A G 3' 5' <u>GTT TCT TTT AAA TTG GGC ACT GGT TTG</u> 3'	VIC	189
D4P3	+388	AC <sub>(16)</sub>	5' CAA TGA CAG TTT GAG CTG CAC 3' 5' <u>GTT TCT TGA CTT CAA AAG GCT GGA ACA TT</u> 3'	6-FAM	304
D4P4	+7711	AAT <sub>(13)</sub>	5' TCC CAG ATA CTT GAA AGG CTG AA 3' 5' <u>GTT TCT TCC GAA ATG CCT CTC AAT TC</u> 3'	VIC	324

<sup>a</sup> Negative values in location represent the numbers of base pairs (bp) upstream from the first nucleotide of *PCDH10* gene, and positive values represent the numbers of bp downstream from the last nucleotide of *PCDH10* gene.

<sup>b</sup> Repeat numbers in the parentheses of repeat unit and product sizes are according to sequence from Ensembl (ENSG00000138650).

<sup>c</sup> Seven nucleotides GTT TCT T is added to the 5' end of each reverse primer in order to reduce the minus-A phenomenon in PCR.

<sup>d</sup> Fluorescence dye is labeled at the 5' end of each forward primer.

**Table 3. LOH frequencies of the four microsatellite markers in 211 patients with CRC**

Marker	Informative no. <sup>a</sup>	Heterozygosity (%) <sup>b</sup>	LOH no.	LOH frequency (%) <sup>c</sup>
D4P1	120	56.9	19	15.8
D4P2	130	61.6	26	20.0
D4P3	137	64.9	45	32.8
D4P4 <sup>d</sup>	151	72.2	38	25.2

<sup>a</sup> Total no. of samples with heterozygous alleles in normal mucosa.

<sup>b</sup> Percentage of the incidence of heterozygosity among 211 samples.

<sup>c</sup> Percentage of the incidence of LOH among informative cases.

<sup>d</sup> 209 cases in total due to failure in the PCR amplification of D4P4 in 2 samples.



**Table 4. Relationship between patient clinicopathological characteristics and the incidence of LOH for each marker (n=201)<sup>a</sup>**

Characteristic	No. of patients (%)											
	D4P1			D4P2			D4P3			D4P4		
	LOH	Retained	<i>p</i>	LOH	Retained	<i>p</i>	LOH	Retained	<i>p</i>	LOH	Retained	<i>p</i>
Number <sup>b</sup>	19 (16.2)	98 (83.8)		26 (20.8)	99 (79.2)		43 (32.6)	89 (67.4)		36 (25.0)	108 (75.0)	
Age (yr)			0.6623			1.0000			0.7420			0.9112
Median	73	73		73	76		74	71		76	73	
Range	45-93	37-98		46-97	37-98		46-93	37-98		46-97	37-98	
Gender			0.7708			0.9634			0.9484			0.6304
Male	10 (17.2)	48 (82.8)		13 (21.0)	49 (79.0)		22 (32.8)	45 (67.2)		19 (26.8)	52 (73.2)	
Female	9 (15.3)	50 (84.7)		13 (20.6)	50 (79.4)		21 (32.3)	44 (67.7)		17 (23.3)	56 (76.7)	
Tumor location			0.5833			0.7405			0.9198			0.3217
Proximal colon	6 (19.4)	25 (80.6)		6 (18.8)	26 (81.2)		10 (33.3)	20 (66.7)		7 (18.9)	30 (81.1)	
Distal colon	13 (15.1)	73 (84.9)		20 (21.5)	73 (78.5)		33 (32.4)	69 (67.6)		29 (27.1)	78 (72.9)	
Dukes' stage			0.5574			0.2690			0.1992			0.3417
A	1 (8.3)	11 (91.7)		2 (14.3)	12 (85.7)		2 (18.2)	9 (81.8)		1 (8.3)	11 (91.7)	
B	7 (15.9)	37 (84.1)		8 (18.2)	36 (81.8)		18 (32.1)	38 (67.9)		13 (24.1)	41 (75.9)	
C	4 (12.1)	29 (87.9)		6 (15.8)	32 (84.2)		11 (26.8)	30 (73.2)		12 (24.0)	38 (76.0)	
D	7 (25.0)	21 (75.0)		10 (34.5)	19 (65.5)		12 (50.0)	12 (50.0)		10 (35.7)	18 (64.3)	



**Table 4. Relationship between patient clinicopathological characteristics and the incidence of LOH for each marker (n=201)<sup>a</sup> (continued)**

Characteristic	No. of patients (%)								p	
	D4P1		D4P2		D4P3		D4P4			
	LOH	Retained	LOH	Retained	LOH	Retained	LOH	Retained		
Tumor stage <sup>c</sup>										
Early stage	8 (14.3)	48 (85.7)	10 (17.2)	48 (82.8)	20 (29.9)	47 (70.1)	14 (21.2)	52 (78.8)	0.3342	
Late stage	11 (18.0)	50 (82.0)	16 (23.9)	51 (76.1)	23 (35.4)	42 (64.6)	22 (28.2)	56 (71.8)		
Distal metastasis <sup>d</sup>		0.1495							0.1446	
Yes	7 (25.0)	21 (75.0)	10 (34.5)	19 (65.5)	12 (50.0)	12 (50.0)	10 (35.7)	18 (64.3)		
No	12 (13.5)	77 (86.5)	16 (16.7)	80 (83.3)	31 (28.7)	77 (71.3)	26 (22.4)	90 (77.6)	0.1739	
Pathological differentiation <sup>e</sup>		0.2540								
Moderate	11 (13.8)	69 (86.2)	17 (19.5)	70 (80.5)	31 (31.6)	67 (68.4)	30 (28.0)	77 (72.0)		
Poor	8 (22.2)	28 (77.8)	9 (24.3)	28 (75.7)	11 (33.3)	22 (66.7)	6 (16.7)	30 (83.3)	0.3081	
Disease recurrence <sup>f</sup>		0.7152								
Yes	2 (10.5)	17 (89.5)	3 (15.0)	17 (85.0)	5 (20.8)	19 (79.2)	9 (32.1)	19 (67.9)		
No	9 (17.3)	43 (82.7)	10 (18.2)	45 (81.8)	21 (32.8)	43 (67.2)	13 (19.7)	53 (80.3)		

<sup>a</sup> Only samples obtained before December 31<sup>st</sup>, 2008 were recruited.

<sup>b</sup> Samples with homozygous alleles were excluded.

<sup>c</sup> Early stage refers to Dukes' A and B. Late stage refers to Dukes' C and D.

<sup>d</sup> Distal metastasis refers to Dukes' D. No distal metastasis refers to Dukes' A, B and C.

<sup>e</sup> Pathological report of 1 case is not available.

<sup>f</sup> Patients with Dukes' B and C, who obtained curable surgery before December 31<sup>st</sup>, 2008, are assessed for disease recurrence.

Student's *t* test for continuous variables; Fisher's exact test or chi-square test for categorical variables.

**Table 5. Relationship between patient clinicopathological characteristics and allelic deletion of *PCDH10* gene (n=191)<sup>a</sup>**

Characteristic	No. of patients (%)		<i>p</i> value
	Allelic deletion (+)	Allelic deletion (-)	
Number	57 (29.8)	134 (70.2)	
Age (yr)			0.3831
Median	74	73	
Range	46-97	37-98	
Gender			0.9868
Male	29 (29.9)	68 (70.1)	
Female	28 (29.8)	66 (70.2)	
Tumor location			0.7878
Proximal colon	13 (28.3)	33 (71.7)	
Distal colon	44 (30.3)	101(69.7)	
Dukes' stage			0.1189
A	3 (15.8)	16 (84.2)	
B	20 (27.4)	53 (72.6)	
C	17 (27.9)	44 (72.1)	
D	17 (44.7)	21 (55.3)	
Tumor stage <sup>b</sup>			0.1585
Early stage	23 (25.0)	69 (75.0)	
Late stage	34 (34.3)	65 (65.7)	
Distal metastasis <sup>c</sup>			<b>0.0250</b>
Yes	17 (44.7)	21 (55.3)	
No	40 (26.1)	113 (73.9)	
Pathological differentiation <sup>d</sup>			0.8611
Moderate	41 (29.3)	99 (70.7)	
Poor	15 (30.6)	34 (69.4)	
Disease recurrence <sup>e</sup>			0.5284
Yes	8 (23.5)	26 (76.5)	
No	26 (29.2)	63 (70.8)	

<sup>a</sup> Only 201 patients who had their surgery before December 31<sup>st</sup>, 2008 were recruited for the study. Among them, 10 patients got non-informative results in all four markers studied, and were excluded from this analysis.

<sup>b</sup> Early stage refers to Dukes' A and B. Late stage refers to Dukes' C and D.

<sup>c</sup> Distal metastasis refers to Dukes' D. No distal metastasis refers to Dukes' A, B and C.

<sup>d</sup> Pathological reports of 2 cases are not available.

<sup>e</sup> 123 patients with Dukes' B and C, who obtained curable surgery before December

31<sup>st</sup>, 2008, are assessed for disease recurrence.



**Table 6. Correlation of overall survival and disease-free survival with allelic deletion of *PCDH10* in CRC patients**

<i>PCDH10</i>	Overall survival			Disease-free survival <sup>a</sup>			<i>p</i> value <sup>b</sup>
	Case no.	OS (%)	Mean/Median (months)	Case no.	DFS (%)	Mean/Median (months)	
n	174			118			
Allelic deletion							0.6135
Yes	54	30 (55.6)	35/35	34	26 (76.5)	48/35	
No	120	87 (72.5)	49/33	84	58 (69.0)	63/33	

<sup>a</sup> Only patients with Dukes' B and C are assessed.

<sup>b</sup> By Log-Rank test.



**Table 7. Correlation of overall survival and disease-free survival with allelic deletion and/or promoter hypermethylation in patients with CRC.**

Variable	Overall survival				Disease-free survival <sup>a</sup>			
	Case no.	OS (%)	Mean (months)	<i>p</i> value <sup>b</sup>	Case no.	DFS (%)	Mean (months)	<i>p</i> value <sup>b</sup>
Allelic deletion (n=65)				0.1273				0.4680
Yes	24	13 (54.2)	34		14	10 (71.4)	48	
No	41	29 (70.7)	45		33	21 (63.6)	50	
Promoter hypermethylation (n=69)				0.3212				0.1354
Yes	39	28 (71.8)	50		32	25 (78.1)	52	
No	30	18 (60.0)	52		19	10 (52.6)	45	
Allelic deletion /promoter hypermethylation (n=65)				0.4655				
No/No	15	10 (66.7)	68					
Yes/No	14	7 (50.0)	35					
No/Yes	26	19 (73.1)	47					
Yes/Yes	10	6 (60.0)	54					
Allelic deletion /promoter hypermethylation (n=65)				0.7704				
No/No	15	10 (66.7)	68					
Except No/No	50	32 (64.0)	45					

<sup>a</sup> Only patients with Dukes' B and C are assessed. DFS was not analyzed for allelic deletion/promoter hypermethylation group due to small sample size.

<sup>b</sup> By Log-Rank test



**Table 8. LOH study and copy number assay at *PCDH10* gene for three CRC cases<sup>a</sup>**

Sample	D4P1	D4P2	D4P3	D4P4	Gene copy number
CRC5T	LOH (0.39)	LOH(2.64)	LOH (2.69)	Homo	2
CRC110T	Homo	LOH (0.33)	LOH (3.09)	Homo	2
CRC175T	LOH (0.34)	LOH (0.41)	LOH (2.70)	Homo	2

<sup>a</sup> CRC tumors with apparent LOH indexes (in parentheses). A LOH index  $\leq 0.67$  or  $\geq 1.5$  is used to indicate allelic loss.

CRC : colorectal cancer; LOH : loss of heterozygosity; Homo : homozygous allele; T : tumor



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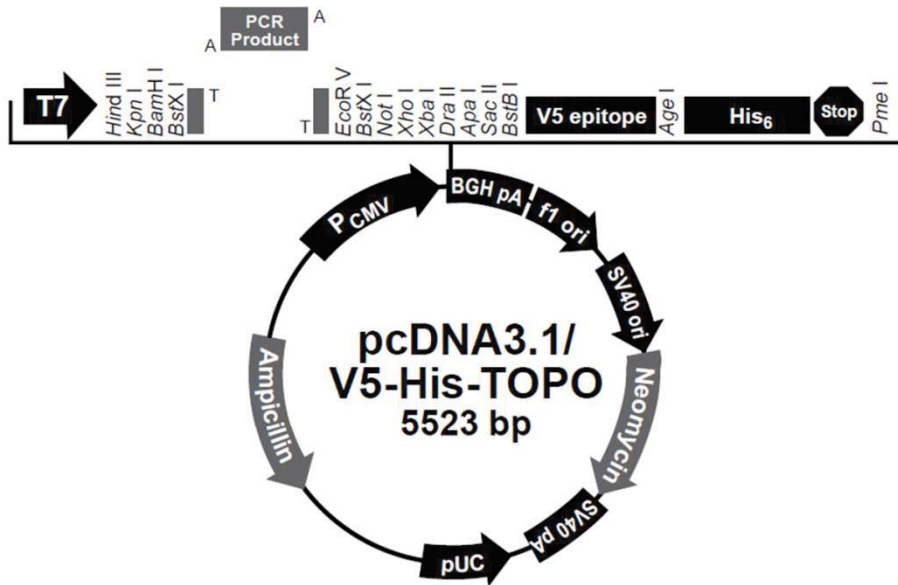


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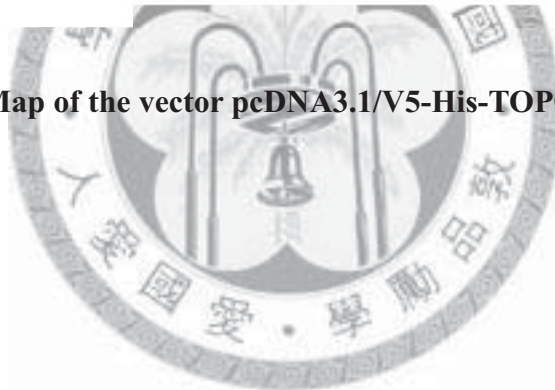
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## Appendix



Appendix Figure1. Map of the vector pcDNA3.1/V5-His-TOPO



**Appendix Table 1: 10 leading causes of death in Taiwan in 2008**

Rank	Causes of death	Percentage
1	Malignant neoplasms	27.35
2	Diseases of heart	11.05
3	Cerebrovascular diseases	7.49
4	Pneumonia	6.09
5	Diabetes mellitus	5.65
6	Unintentional injuries	4.97
7	Chronic lower respiratory diseases	3.78
8	Chronic liver disease and cirrhosis	3.46
9	Suicide	2.90
10	Nephritis, nephrotic syndrome, and nephrosis	2.82

Source : Department of Health, Executive Yuan, R.O.C. (Taiwan)



**Appendix Table 2: 10 leading causes of cancer death in Male and Female in Taiwan 2008**

Rank	Male & Female	Male	Female
1	Lung cancer	Liver cancer	Lung cancer
2	Liver cancer	Lung cancer	Liver cancer
3	Colorectal cancer	Colorectal cancer	Colorectal cancer
4	Breast cancer	Oral cancer	Breast cancer
5	Stomach cancer	Stomach cancer	Stomach cancer
6	Oral cancer	Esophageal cancer	Cervical Cancer
7	Prostate cancer	Prostate cancer	Pancreatic cancer
8	Cervical Cancer	Pancreatic cancer	Ovarian cancer
9	Esophageal cancer	nasopharyngeal carcinoma	Leukemia
10	Pancreatic cancer	Non-Hodgkin's lymphoma	Non-Hodgkin's lymphoma

Source : Department of Health, Executive Yuan, R.O.C. (Taiwan)

### Appendix Table 3. PCR for microsatellite markers

#### 1. Preparation of PCR premix

ddH <sub>2</sub> O	1.54μL	*
dNTP (2.5mM)	0.6μL	*
MgCl <sub>2</sub> (25mM)	0.6μL	*
HOTFIREPol (5U/μL)	0.06μL	*
10X buffer	0.6μL	*
Total	3.4μL	*

$$*X = (S + 2) \times M + 2 = \underline{\hspace{2cm}}$$

X=final volume of PCR premix; S = Numbers of Samples; M = Numbers of Markers)

#### 2. Preparation of PCR cocktail mix

PCR premix	3.4μL	#
Primer Mix (5μM for each primer)	0.6μL	#
#Y=S+2		

#### 3. Addition of 2μL of 10ng/μL DNA to 6μL of PCR cocktail mix

#### 4. PCR reaction:

Denaturation	95°C for 10 min
	95°C for 30 s
35 cycles	60°C for 30 s
	72°C for 30 s
Elongation	72°C for 10 min
Hold	4°C ∞