行政院國家科學委員會專題研究計畫 成果報告

先天性巨腸症及其相關假性巨腸之相關因子與分子診斷研究
研究成果報告(精簡版)

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Introduction:

Hirschsprung disease (HSCR) is characterized by the absence of intramural ganglion cells in the nerve plexuses of the distal gut [1, 2].Typically, HSCR are present in neonates or early childhood with symptoms ranging from chronic constipation to acute ileus, but late manifestation in adults have occasionally been described [3]. It is the most common cause of neonatal intestinal obstruction, affecting one in 5000 live newborns with a male predominance (3:1 to 5:1) [1, 2, 4, 5]. The disease has a complex genetic etiology and is likely to be of multifactorial inheritance with a couple of susceptibility genes including members of the RET [4-6]. As described in the above section, in the past seven years we collected genomic DNA samples from 64 HSCR patients in central Taiwan and analyzed the coding regions of the RET and EDNRB gene by PCR amplification and DNA sequencings. Even though only one point mutation at critical site for the signaling pathways was identical in a pair of twin brothers, differences between patients and controls in allele distribution of the five RET polymorphic sites are statistically significant.

Anorectal malformations (ARM) are diagnosed due to the absence or ectopic location of anus. The incidence of associated ARM with Hirschsprung disease is variously reported from 0.4%-3.4% [7, 8]. ARM with syndromic Hirschsprung disease often associated with rectal ectasia, a state of massive dilation of the rectum and distal sigmoid [9]. It may be primary presenting at birth with characteristic features as a dilated recto sigmoid with a thin bowel wall without hypertrophy of smooth muscles, or as rectal ectasia with balloon-like rectum [10], colonic intertia [11], megarectum [12], and pseudo-Hirschsprung disease [13]. Some ARM patients developed secondary rectal ectasia after birth as a result of bowel reaction to distal obstruction or inadequate evacuation [14] postoperatively. We also collected anorectal malformations patients with co-existing syndromic Hirschsprung disease (syndromic HSCR). The allele distributions of all five RET SNPs of the syndromic HSCR patients do not statistically deviate from those of the normal population. The results strengthen the association of specific RET SNP alleles with typical HSCR in Taiwan.

Even though we have conducted the genetic analyses of the Hirschsprung disease and the syndromic HSCR patients in Taiwan, we would like to further develop more sensitive and definite molecular diagnosis and to identify more markers.

Methods:

Collecting samples

Tissue blocks

We retrieved tissue blocks from patients diagnosed as HSCR and related colonic neuropathies diseases (HSCR with Down syndrome or anorectal malformation co-existing HSCR) at Chung-Shan Medical University Hospital from 1999 to 2006.

Tissue collection

By now 21 different fresh tissue samples from pull-through operation separated as aganglionosis, oligoganglionosis and normal ganglionosis segments. Besides, we also collected the tissues from anorectal malformation co-existing HSCR patients. They are frozen in liquid nitrogen (small quantity) and at –80C (larger quantity) for further protein analysis.

Preparation of colon tissue protein extract and two-dimensional (2-D) gel electrophoresis

Total protein in the colon tissue will be extracted by homogenizing the tissues with a Polytron homoginizer in the IPG (immubilized pH gradient) sample buffer for isoelectrophocusing (IEF). After determination of the protein concentration in the extract, protein samples will be loaded to IPG strips through rehydration. In our previous pilot studies, most of the proteins in the colon samples will be present in the pH4-7 range. We have set the condition for using the pH 4-7, 18 cm strips for the 1st dimension IEF (Tsai, Wu and Li, unpublished results). After the first dimension IEF electrophoresis, the IPG strips will be equilibrated and analyzed for the second dimension by gradient SDS polyacrylamide electrophoresis. The protein patterns will be detected by staining with colloidal blue or silver stain. Basically, the protein samples form the same patient (aganglionosis vs normal) will run in parallel for comparison. The protein patterns will be analyzed by Melanie 3. After comparing the gels for differentially expressed spots, the spots will be excised, digested with protease (such as trypsin) and then the peptide fragment will be analyzed by matrix-assisted laser desorption/ionization time of fight (MALDI-TOF) mass spectrometry or mass/mass spectrometry.

Immunnohistochemisty

Sections will be deparaffinized, rehydrated, and endogenous peroxidase-blocked with 1% H_2O_2 in d_2H_2O. Antigen retrieval will be performed by autoclave using citrate buffer. After blocking with antibody dilution (DakoCytomation), at room temperature with the 1st antibody are performed, follow by a PBS(phosphate-buffer saline) rinse. A secondary antibody with peroxidase-conjugated (horseradish peroxidase) is added. After, PBS rinsing, immunoreactivity is visualized by using 3,3'diaminobenzidine.

結果與討論:

1.2D 分析:

將巨腸症病人正常與異常組織以 2D分離後，以質譜儀鑑定其表現量差異的蛋白，發現為 Heat-shock protein 27 (HSP27) 蛋白 (Fig 1)。Heat-shock protein 27 (HSP27) 普遍存在很多細胞中 [15]，於心肌與平滑肌細胞中會存在actin的相同位置 [16, 17]。人類HSP27 蛋白於 Ser^{15}, Ser^{78}, and Ser^{82}. Ser^{82} 會有磷酸化的修飾[18, 19]。HSP27 在等電點電泳實驗所測得的pI值約為 5.8，與分析上HSP27 理論pI值不同，可能因isoform不同所造成。我們在分子量為 27 KDa處較鹼端取另一點鑑定，發現其亦為HSP27 (pI 6.8) 其分布似有異常多於正常情況。若以此兩點相對量來看，異常組織中HSP27 (pI 6.8) 明顯較HSP27 (pI 5.8) 為高，但在正常組織中此差異則較不明顯。

以 2D 分析無肛症併有直腸擴張病人 (pt 148、pt154) 其正常與異常組織蛋白 (Fig 2)，比對後在 pt148 病人檢體上發現，HSP27 (pI 5.8)正常組織較異常組織多但並不明顯，但是 HSP27 (pI 6.8)在正常組織中的量較異常組織少。在正常組織中此兩點的分布較平均，但在異常組織 HSP27 (pI 6.8)表現量大於 HSP27 (pI 5.8)。pt154 病人的 HSP27(pI 5.8) 表現量正常組織大於異常組織，但 HSP27 (pI 6.8)的表現無明顯差異，而且在正常與異常組織中 HSP (pI 6.8)表現量皆大於 HSP27 (5.8)，此差異在正常組織中更為顯著。總和以上正常與異常組織 HSP27 表現量的差異，可能是因為 HSP27 有不同 isoform 所致。

2.PRMT1 分析

細胞中 85%的精胺酸甲基化反應由 PRMT1 催化 [20]，PRMT1 廣泛地存在細胞核及細胞質，其主要受質為核苷酸結合蛋白及核仁蛋白，包含了組織蛋白 H3、H4 和 hnRNP (heterogeneous nuclear ribonucleoprotein)、fibrillarin、nucleolin 等，涉及了 mRNA 代謝運輸及核糖體生合成。在小鼠中剔除 PRMT1 基因會造成胚胎植入後快速死亡 [21]，證實 PRMT1 為胚胎發育所必要的酵素；在神經細胞發育方面，先前的研究指出在神經生長因子 (nerve growth factor, NGF) 所誘發的神經細胞 PC12 細胞分化中，細胞內 PRMT1 的活性會上升 [22]；而在非洲爪蟾胚胎中抑制 Xprmt1 基因的表現，則會造成胚胎中神經細胞標示蛋白的表現量降低 [23]。推測 PRMT1 與神經細胞的生成有其相關性，取巨腸症病人正常組織、擴大組織 (寡神經節) 與不正常組織 (無神經節) 做免疫組織染色 (Fig. 3)，發現 PRMT1 會表現在腸道組織中的黏膜層、血管內皮與神經節細胞。而不正常組織中，因缺乏神經節細胞的關係，並無 PRMT1 表現。取無肛症病人正常組織 (近端腸道)、擴大組織與不正常組織 (遠端腸道) 做免疫組織染色 (Fig. 4)，同樣發現 PRMT1 會表現在腸道組織中的黏膜層、血管內皮與神經節細胞，而不正常組織的神經節部份也有 PRMT1 的表現。總和以上結果，神經細胞相對於週邊肌肉組織，會有較高的 PRMT1 表現量，未來可用於鑑別 HSCR 與其他相關腸道疾病。

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Figure1: 2-DE analysis of HSP27 isoforms in normal and abnormal tissues of HSCR patients.Proteins were separated by 2-DE followed by colloidal Coomassie brilliant blue staining. The circles indicate the spots identified as HSP27 in normal and abnormal tissues from different patients. Arrows highlight HSP27 isoforms.

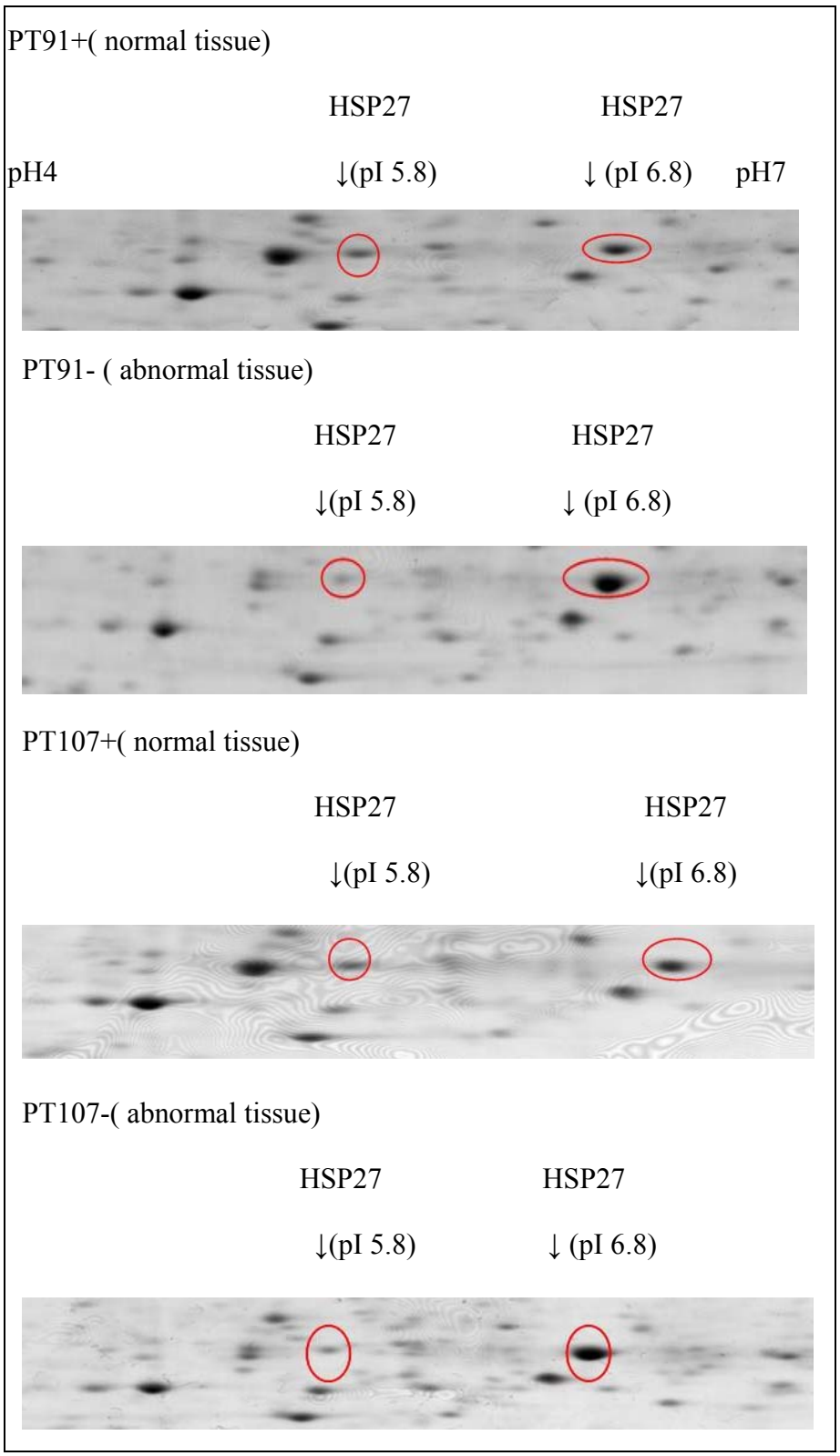


Figure2: 2-DE analysis of HSP27 isoforms in normal and abnormal tissues of anorectal malformations co-existing syndromic HSCR patients: Proteins were separated by2-DE followed by colloidal Coomassie brilliant blue staining. The circles indicate spots identified as HSP27 in normal and abnormal tissues from different patients. Arrows highlight HSP27 isoforms

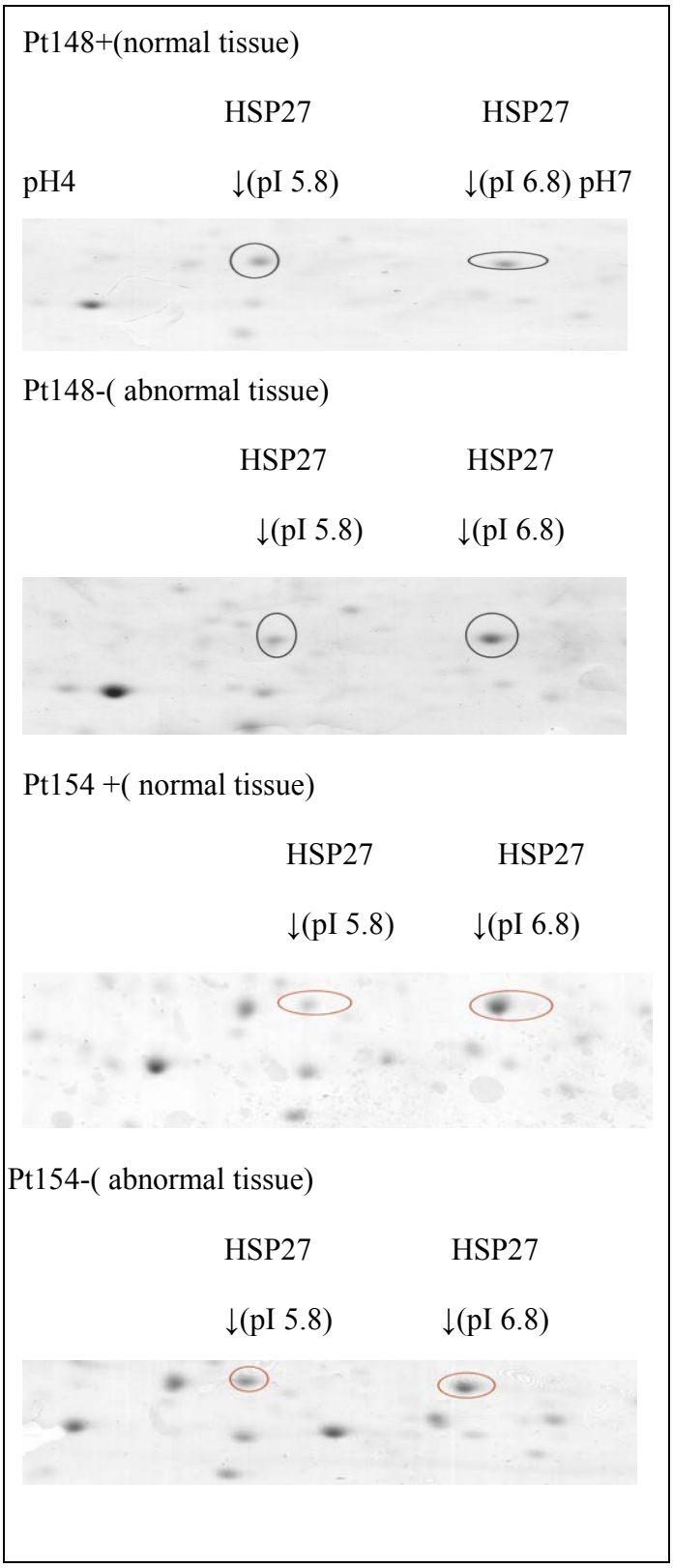


Figure 3 Immunodetection of PRMT1 in HSCR.

Overall view of the intestinal wall in (A) normal ganglionosis, (B) oligoanglionosis and (C) aganglonosis colon specimens. PRMT1 mucosal, submucosal and muscularis staining in normal ganglionosis (D,G,J), oligoanglionosis (E,H,K) and aganglonosis (F,I,L) colon specimens: positive staining in all crypts, capillary endothelial cells (arrowhead) , submucosal and muscularis plexuses (arrows). Absence of staining of hypertrophic nerve bundles (asterix).

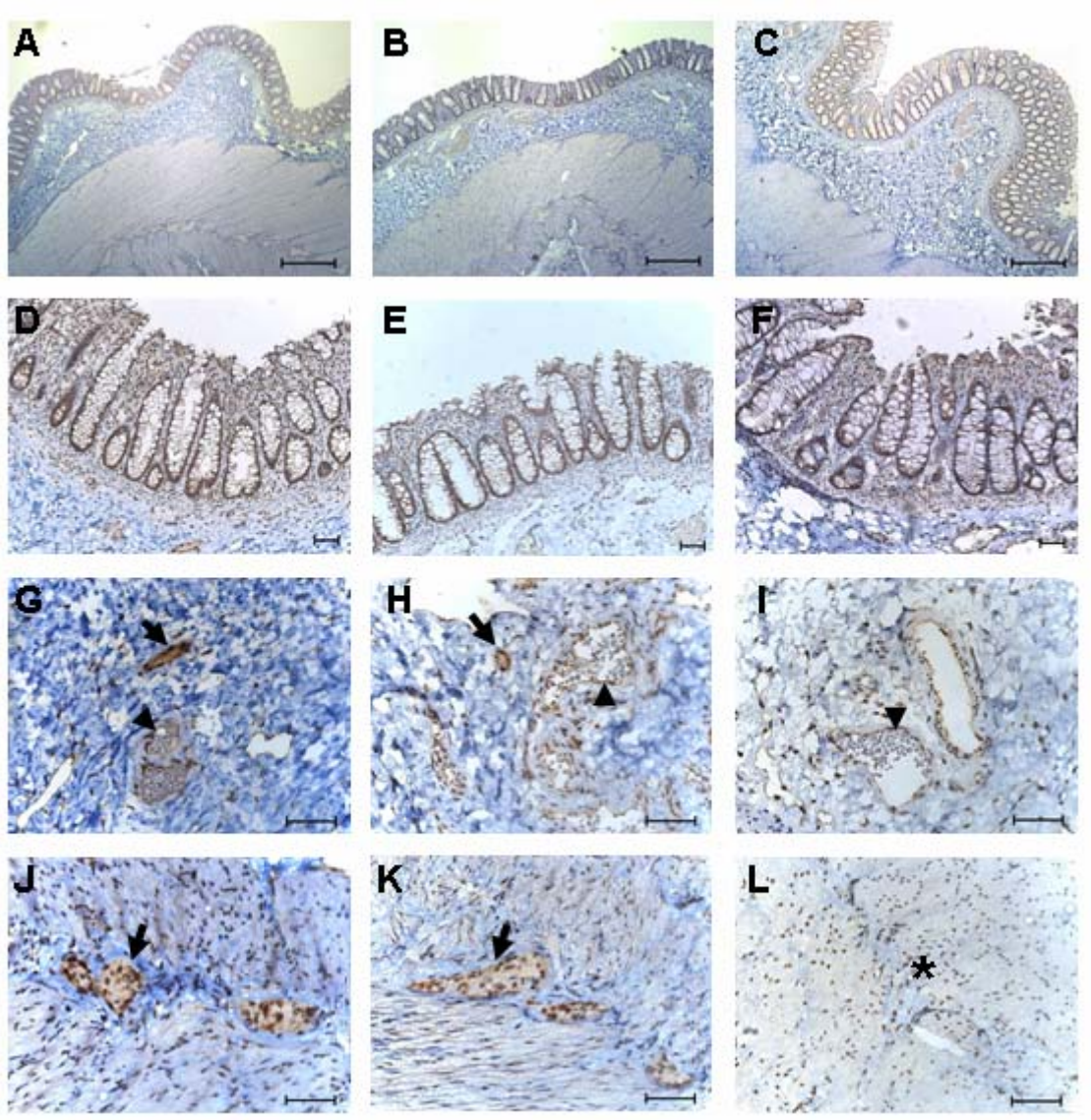
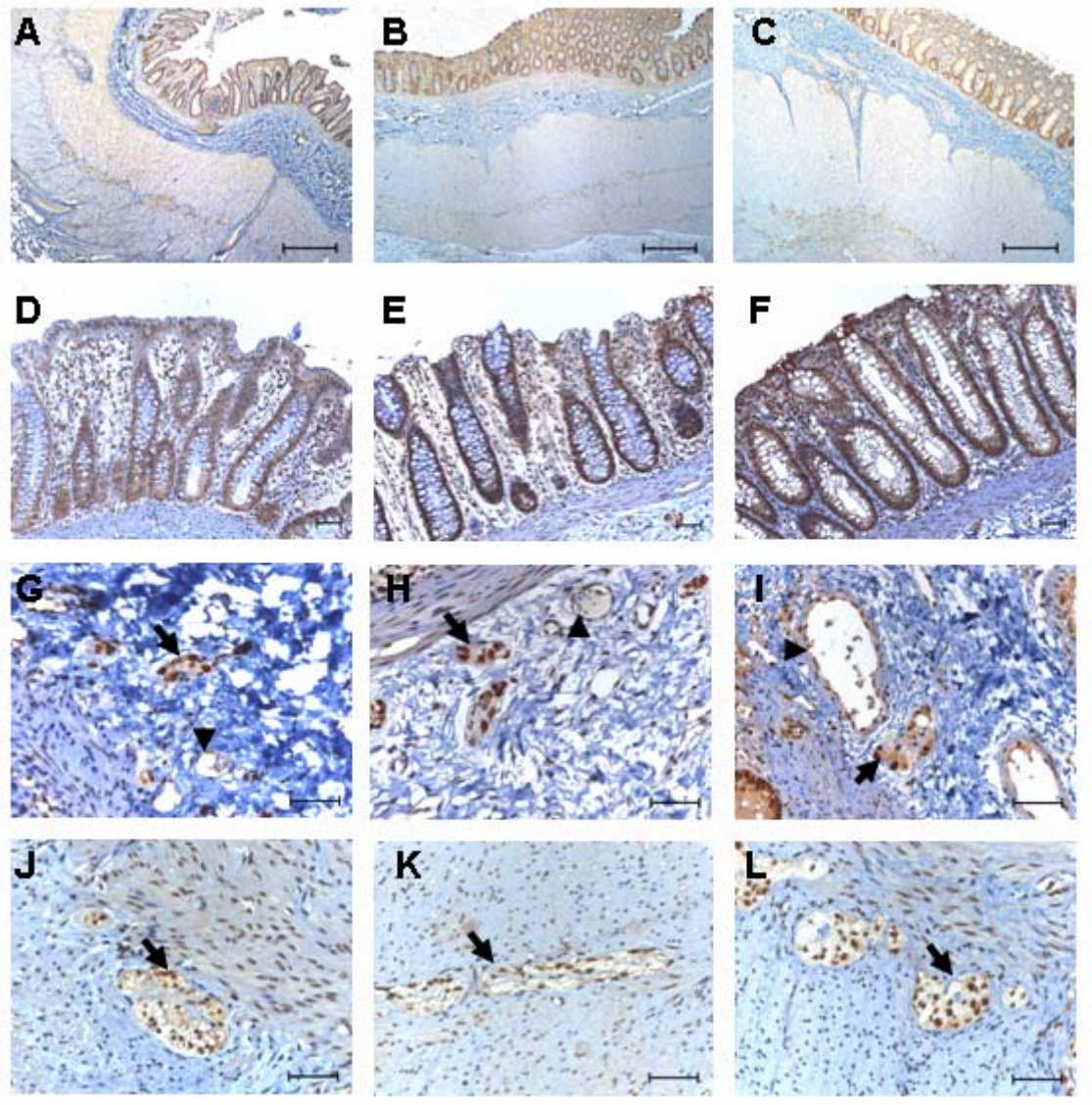


Figure 4 Immunodetection of PRMT1 in ARM.

Overall view of the intestinal wall in (A) normal, (B) dilated and (C) abnormal colon specimens. PRMT1 mucosal, submucosal and muscularis staining in normal (D,G,J), dilated (E,H,K) and abnormal (F,I,L) colon specimens: positive staining in all crypts, capillary endothelial cells (arrowhead) , submucosal and muscularis plexuses (arrows). Scale bars: A-C:500 mm; D-L: 50 mm.



計畫成果自評：

原計畫預定利用蛋白質體學方法，進行 HSCR 病人正常組織與異常組織的分析，了解蛋白質體的變化，並對於其致病原因有進一步了解；且尋找用於臨床檢定上更方便的標示蛋白。由我們的研究成果看來，在蛋白質體分析方面，HSP27 (pI 6.8)的表現量皆高於 HSP27 (pI 5.8)，在 HSCR 與 ARM 病人中正常組織與異常組織皆有些許的差異，但不同個體間的差異性會使結果不同，所以對於 HSP27 表現量的差異與 HSCR 致病原因相關性並不顯著，且在疾病鑑別方面所能提供的能力仍不足。但是在 PRMT1 結果方面來說，對於未來 HSCR 與其他腸道相關疾病，提供了另一個臨床檢定的選擇。達到原計畫預定尋找的臨床鑑定標示蛋白。