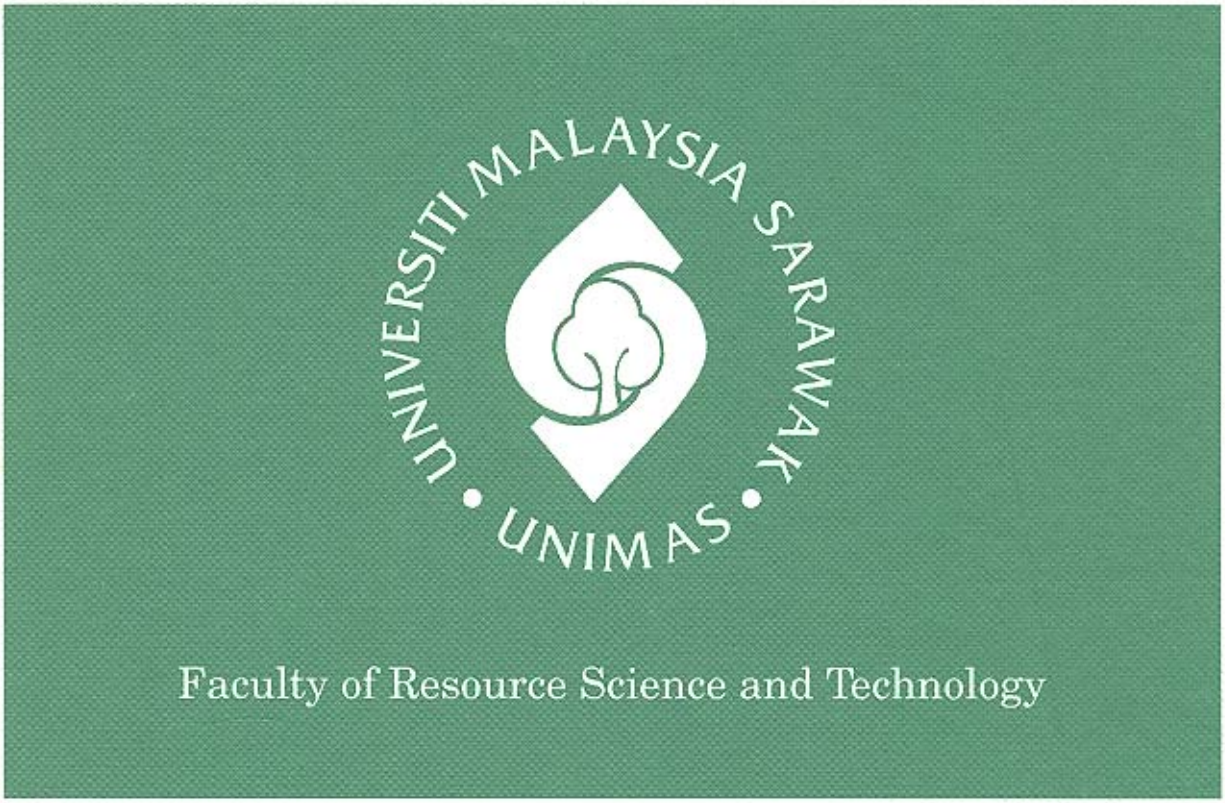

ISOLATION AND RESTRICTION SITE CHARACTERIZATION OF HUMAN TUMOR SUPPRESSOR GENE, *APC*, PARTIAL cDNA FRAGMENT FROM HUMAN COLON TISSUE RNA

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Pusat Khidmat Maklumat Akademik
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Isolation and Restriction Site Characterization of the Human Tumor Suppressor Gene, *APC*, Partial cDNA Fragment from Human Colon Tissue RNA

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ABSTRACT

Mutation in *Adenomatous Poliposis Coli* (*APC*) tumor suppressor gene plays a significant role in the initiation of colorectal tumorigenesis, especially in familial adenomatous poliposis (FAP). Although *APC* mutation has been intensively studied, there is still a lack of effort in finding a rapid and economical technique to survey nucleotide aberrancy in *APC*. In this report, cDNA fragments of *APC* from normal and tumor colorectal tissue are isolated for the purpose of mutation site identification via restriction endonuclease site characterization. Information generated on the restriction site of *APC* can be used for effective and cost-saving strategy of *APC* mutation analysis by means of restriction enzyme-based method. Using commercially available total RNA for normal colon tissue as the starting material, reverse transcription PCR (RT-PCR) was performed to obtain the *APC* gene fragment. A partial fragment of *APC* gene with a predicted size of 1172 bp was successfully amplified and subcloned. A preliminary restriction site characterization was performed with *Eco*RI. An attempt to procure nucleotide sequence was carried out.

Key words: Adenomatous Poliposis Coli (*APC*) gene, Reverse Transcription Polymerase Chain Reaction (RT-PCR), Normal colon tissue, Total RNA

ABSTRAK

Gen Adenomatous Poliposis Coli (APC) memainkan peranan penting dalam menghalang kejadian barah usus dan rektum. Walaupun banyak kajian telah dijalankan ke atas mutasi APC, usaha untuk mencari satu teknik yang cepat dan ekonomis untuk mengkaji aberansi nucleotida dalam APC masih kurang. Dalam laporan ini, fragmen cDNA bagi APC daripada sel usus normal dan tumor telah diasingkan untuk mengenalpasti tapak mutasi melalui pengenalpastian tapak enzim penghad. Kaedah ini akan memberikan maklumat tentang tapak enzim penghad pada APC yang dapat digunakan untuk mengkaji mutasi APC dengan lebih berkesan dan murah berasaskan kaedah yang melibatkan enzim penghad. Dengan menggunakan keseluruhan RNA daripada tisu usus yang normal sebagai bahan permulaan, Reverse Transcription Ploymerase Chain Reaction (RT-PCR) telah dijalankan bagi mendapatkan fragmen gen APC. Fragmen gen APC dengan saiz 1172 bp telah berjaya diamplifikasi dan diklon. Usaha awal pengenalpastian tapak enzim penghad telah dijalankan menggunakan EcoRI. Percubaan untuk mendapatkan jujukan nukleotida juga telah dijalankan.

Kata kunci: Gen Adenomatous Poliposis Coli (APC), Reverse Transcription Polymerase Chain Reaction (RT-PCR), Tisu usus normal, Keseluruhan RNA

INTRODUCTION

Previous studies on the *APC* tumor suppressor gene have identified it as a major regulatory gene that is linked to cancer, particularly colorectal cancer. As reported by Kinzler and Vogelstein (1996), the initiation of both familial and sporadic colorectal tumorigenesis is strongly attributed to the functional loss of Adenomatous Poliposis Coli (*APC*) tumor suppressor gene. This is evident in the finding of germline mutation of *APC* gene in 90% of patients suffering from the hereditary colorectal cancer, familial adenomatous poliposis or FAP (Esteller *et al.*, 2000). *APC* mutations also accounted for 20% of Hereditary Nonpolyposis Colorectal Cancer cases (Miyaki *et al.*, 1999).

In terms of incidence, mortality and prevalence, a total of 924,717 colorectal cancer cases were reported worldwide in the year 2000 of which 492,411 cases have resulted in death (Ferlay *et al.*, 2000). In Malaysia, 193 or 8.9 % out of 2512 cancer mortality cases were attributed to colorectal cancer in the year 1997 (Ministry of Health of Malaysia, 1998). Locally in Sarawak, colorectal cancer cases made up 11% of 1156 cancer cases diagnosed in 1996 (Sarawak Health Department, 2002). Despite a significant number of cases reported, there is little evidence of mutation survey performed on *APC* using tumor specimens from local patients.

Montera *et al.*, 2001 stated that the Adenomatous Polyposis Coli or *APC* is a type of tumor suppressor gene localised on chromosome 5q21-22. The *APC* gene (8.9kb) is made up of 15 exons with a particularly large exon 15 that spans over 6.5kb (Hamelin 1998, Dobbie *et al.*, 1996).

Fodde (2001) described *APC* as the coding gene for functional protein products required by cells in WNT signaling pathway, cell adhesion, migration and apoptosis as well as chromosomal segregation during mitosis. Despite having a range of functions, the most important cancer-linking function of this gene lies in its capacity to regulate the levels of intracellular ß-catenin (Albuquerque *et al.*, 2002). Under normal circumstances, the *APC* gene codes for the APC protein that form complex with glycogen synthase kinase 3 ß (GSK 3 ß), ß-catenin and the proteins axin and conductin. Formation of such complex results in the degradation of intracellular ß-catenin and thus preventing it from activating downstream growth-promoting gene such as *c-myc*, *cyclin D1*, *matrilysin* and *c-jun*, thereby signaling the stop of cell proliferation (Fujimori *et al.*, 2001).

APC mutations often result in truncated protein product lacking all the axin/conductin binding motifs as well as a variable number of amino acid repeats that are essential in regulating the level of intracellular ß-catenin (Albuquerque *et al.* 2002). Increase of ß-catenin level results in hyperproliferation of the colorectal cell. Taking FAP as an example, the mutation will give rise to the development of hundred to thousand adenomatous polyps in the colorectum; the earliest manifestation of the disease which can be detected in patients in their teenager phase (Laken *et al.*, 1999).

In western nations with high incidence of colorectal cancer cases, intensive studies are continuously conducted by various cancer research groups to generate interesting facts that explained the effects of *APC* mutations to both hereditary and sporadic colorectal cancer. As far as Malaysia is concerned, extensive study performed in regards to *APC* gene mutation and its link to colorectal cancer is still lacking. Local *APC* mutation studies reported so far focus

mainly on exon 8 of the gene obtained using genomic blood samples from local colorectal cancer patients (Mohamed *et al.*, 2003).

The objective of this project is to obtain cDNA fragments of *APC* from normal colon tissue. Primers used for amplification of *APC* cDNA are designed in the lab and therefore, the feasibility of these primers in producing consistent results is yet to be known. The project serves as a platform to qualitatively and quantitatively determine the efficiency of these primers. Costly expenses of DNA sequencing often limit the use of this technique for mutation detection. Cheaper method with high reproducibility such as Restriction Fragment Length Polymorphism (RFLP) is commonly used as an alternative (Stratchan and Read, 1999). Comparison between the *APC* gene sequence of normal and tumor colon tissue will facilitate the finding of potential *APC* mutation markers. Restriction site characterization of the gene will reveal the possible flanking restriction sites of the mutation markers. Subsequently, restriction enzyme-marker combinations for detecting *APC* gene mutation via restriction enzyme-based method can be established. Findings from this project will assist in future colorectal cancer research in UNIMAS as in facilitating rapid and cost-saving strategy of *APC* mutation screening in local colorectal cancer cases.

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MATERIAL AND METHODS

Materials

The starting material utilized in this project is the total human RNA derived from normal colon tissue that was purchased from BD Biosciences, Clontech Laboratories, Inc.

Methods

Reverse Transcription PCR:

Two step reverse transcription or RT-PCR consisting of a first strand cDNA synthesis and a subsequent PCR was performed.

First strand cDNA synthesis (Reverse transcription)

The commercially available total human RNA derived from normal colon tissue need to be converted into its complementary cDNA in order for amplification via polymerase chain reaction (PCR) to be carried out. The first strand cDNA synthesis was done according to the protocol as described in Life Technologies Technical Product Information. The protocol that results in the synthesis 20µl of cDNA per reaction is as follows: 1µl of 100ng oligodT, 2µl of 1-5ng total RNA and 10.5µl of nuclease free water were added into a nuclease free microcentrifuge tube. The mixture was heated to 70°C for 10 minutes followed by quick chill on ice before subjected to brief centrifugation. Subsequently, 4µl of 5X MMLV Reverse Transcriptase buffer, 1 µl of 10mM dNTP mix and 0.5µl RNase inhibitor were added to the initial mixture. The content of the microcentrifuge tube was gently mixed and then incubated at 42°C for 2 minutes. Following incubation, 1µl of MMLV reverse transcriptase was added

into the reaction mixture and the whole reaction was mixed by pipetting gently. The reaction mixture is subjected to further incubation at 42°C for 50 minutes, after which, the reaction was inactivated by heating at 70°C for 15 minutes. Removal of RNA complementary to the cDNA was then performed by addition of 0.2 µl (2 unit) of *E.coli* RNase H followed by incubation at 37°C for 20 minutes. The resulting cDNA which serves as the template for PCR amplification was then stored at -20°C.

PCR Amplification

Polymerase chain reaction (PCR) was performed to amplify the *APC* gene fragment using the previously synthesized first strand cDNA as template. 8 pairs of specific PCR primers were used to amplify the whole *APC* gene (see Table 1).

Table 1: Sets of primer used in PCR amplification of *APC* gene, designation and predicted size of each *APC* fragments

Sets of primer	Type	Primer name	Primer Sequence	Designation of APC fragment primed	Expected Size of product
1	Forward	APC-F1	5'-caagggtagccaaggatg-3'	*APC* 1	1172 bp
	Reverse	APC-R1	5'-gtgaatgatgttgtggagtg-3'		
2	Forward	APC-F2	5'-catccagcttttacatggca-3'	*APC* 2	1132 bp
	Reverse	APC-R2	5'-catccttgtacttcgcaggc-3'		
3	Forward	APC-F3	5'-gaagcattatgggacatggg-3'	*APC* 3	1103 bp
	Reverse	APC-R3	5'-ttccttgattgtctttgctcac-3'		
4	Forward	APC-F4	5'-cagatgagcagttgaactctgg-3'	*APC* 4	1126 bp
	Reverse	APC-R4	5'-cattccactgcatggttcac-3'		
5	Forward	APC-F5	5'-cacccaaaagtccacctgaa-3'	*APC* 5	1169 bp
	Reverse	APC-R5	5'-tgatttttgttgggtgcaga-3'		
6	Forward	APC-F6	5'-cccaaagggaaaagtcacaa-3'	*APC* 6	1148 bp
	Reverse	APC-R6	5'-agcagcagcagcttgatgta-3'		
7	Forward	APC-F7	5'-ttcagaacatggtctatcccc-3'	*APC* 7	1171 bp
	Reverse	APC-R7	5'-aaactggagtttgtgcctgg-3'		
8	Forward	APC-F8	5'-tggaggaatctgcttcatttg-3'	*APC* 8	1290 bp
	Reverse	APC-R8	5'-tggcttccagaacaaaaacc-3'		

Each PCR amplification using respective sets of primers was performed using a total of 25ul PCR reaction mixture. The exact amount of each ingredient in each PCR reaction mixture is as follows:

Table 2: Ingredients and their appropriate amount used in each PCR reaction

Ingredient	Amount (µl)
Template DNA	2
10X PCR buffer + 1.5 mM $MgCl_2$ (Promega , USA)	5
10mM dNTP mix	1
25 pmol/µl Forward Primer	1
25 pmol/µl Reverse Primer	1
5 U/µl GoTaq polymerase (Promega, USA)	0.16
Sterile ultrapure water	14.84

In order to monitor the validity of PCR, a negative control in which the template is substituted with sterile ultrapure water was included in every PCR run. Each PCR process consisting of 35 cycles was carried out using thermal cycler (Perkin Elmer Model No. 2400) using the following parameters:

Table 3: PCR parameters

Stages	Temperature	Duration
Initial denaturation	95°C	1 minute
Denaturation	94°C	30 second
Annealing	Varies according to the set of primers used	1 minute
Extension	72°C	2 minute
Final extension	72°C	10 minutes

Analysis of RT-PCR products: Gel electrophoresis

Screening for RT-PCR products obtained was performed by means of agarose gel electrophoresis using 2% agarose. For each RT-PCR product, 5 µl of DNA sample was mixed with 1 µl of 6X gel loading dye and subsequently loaded into the wells. 2 µl of GeneRuler® 1 kb DNA ladder (Fermentas, USA) was loaded into an adjacent well as a standard size marker for DNA fragments. The submerged gel was electrophorised in 1X TAE buffer at 100V for

approximately 50 minutes. Following gel electrophoresis, the gel was stained in ethidium bromide at 0.5 µg/ml for 15 minutes. The gel was then viewed under a UV transiluminator and images were captured onto 667 black and white Polaroid films using Ultralum's Direct Screen Instant Polaroid Camera DS34.

Gel Extraction

Prior to downstream applications of RT-PCR products as in subcloning and sequencing, the products were purified using the Viogene® Gel Extraction System according to manufacturer's instruction. A brief summary of the protocol is as follows: A clean, sharp scalpel was used to excise the DNA fragment of interest from the gel that was viewed under UV light. The gel slice was weighed and transferred into a clean 1.5 microcentrifuge tube. Half a ml of buffer GEX was added into the tube after which, incubation was carried out at 60 °C for 10 minutes to solubilize the gel slice. Solubilized gel mixture was transferred into a gel extraction column in a collection tube and centrifuged at 13000 rpm for 60 second. The filtrate was discarded and the column was further washed with 500ml of Wash buffer I and subsequently with 700 ml Wash buffer II. Centrifugation for 60 second at 13000 rpm was performed following each addition of wash buffers. Following washing, remaining ethanol residue was removed by further centrifugation at 13000 rpm for 3 minutes. DNA elution was then performed. The column was transferred to a new 1.5 ml tube and added with 30 µl of ddH_2O. The column assembly was left to stand for 1 minute after which centrifugation is carried out at 13000 rpm for 2 minutes. Three µl of the filtrate was screened for purified DNA whilst the remainder was stored at -20 °C.

Subcloning of DNA fragments

RT-PCR products were subcloned using pGEM®-T Easy Vector System (Promega, USA). The subcloning involves ligation and transformation processes that were carried out according to the manufacturer's instruction.

Ligation was performed to insert the desired RT-PCR products into the pGEM®-T Easy Vector. Positive experimental control using a Control Insert DNA provided by the manufacturer and background control were performed to access the performance of the pGEM®-T Easy Vector System used. In brief, the ligation protocol is as follows: Prior to setting up a ligation reaction, the pGEM®-T Easy Vector and Control Insert DNA tubes were briefly centrifuged whilst the 2X Rapid Ligation Buffer were subjected to vigorous vortexing. The ligation reactions were set up as follows:

Table 4: Set up of Ligation reactions

Reagent	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T_4 DNA ligase	5µl	5µl	5µl
pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	3µl	-	-
Control Insert DNA	-	2µl	-
T_4 DNA Ligase (3 Weiss ubits/µl)	1µl	1µl	1µl
Deionized water to a final volume of	10	10µl	10µl

The reactions are prepared into 0.5 ml tube with low DNA binding capacity, mixed by gentle pipetting and then incubated for 1 hour at room temperature.

Following ligation, the ligated vectors were transformed into E. coli JM109 competent cells. These cells were prepared following the protocol by Sambrook *et al.* (1989).

Basically, the tubes containing ligation reactions were centrifuged to collect the contents at the bottom of the tube after which 2 µl of each ligation reactions were transferred into different 1.5 ml microcentrifuge tubes on ice. The frozen JM109 competent cells were removed from -80°C fridge, allowed to thaw in an ice bath, mixed gently by flicking and subsequently, 50-100 µl of cells are carefully transferred into each 1.5ml tube containing respective ligation product. The tubes were mixed by gentle flicking and incubated on ice for 20 minutes. Consequently, the tubes were subjected to heat shock for 45-50 second in a water bath at exactly 42°C and later returned immediately to ice for 2 minutes. Nine hundred and fifty µl of room temperature SOC medium was added into each tube containing transformed cells followed by incubation for one and a half hour at 37°C with shaking at approximately 150 rpm. After incubation, 100 µl of each transformation culture was plated onto LB/ampicilin/IPTG and X-Gal plates prepared earlier. The plates were then incubated overnight (16-24 hours) at 37 °C to obtain desired colonies of transformants.

Screening for colonies containing PCR product

Insertion of DNA fragment into pGEM®-T Easy Vectors will disrupt the nucleotide sequence that codes for ß-galactosidase and thus, recombinants can be identified by blue-white screening on plates containing X-gal and IPTG. With few exceptions as in the case of DNA fragments cloned in frame with *lacZ* gene or introduction of mutations, colonies that contain the desired PCR products are usually white in color (Promega, 1999).

Single white colonies that were suspected to have incorporated the desired RT-PCR product were inoculated into 5 ml of LB broth and incubated overnight with shaking at 200 rpm after which plasmid isolation using Eppendorf's FastPlasmid^{TM} Mini was performed. The plasmid

isolation protocol is summarized as follows: 1.5 ml of fresh bacterial culture was pelleted by centrifugation at 14000 rpm for 1 minute in 2 ml Culture Tube provided by the manufacturer after which the medium is removed by decanting. Each culture tube is added with 400 µl of ice-cold Complete Lysis Solution, and mixed thoroughly by constant vortexing at the highest setting for 30 seconds. The lysate were incubated at room temperature for 3 minutes, transferred into a Spin Column Assembly by pipetting and centrifuged for 60 seconds at 14000 rpm. Following centrifugation, 400 µl of diluted Wash Buffer was added into each Spin Column Assembly which was then subjected to further centrifugation at 14000 rpm for 60 seconds. An additional centrifugation step at 14000 rpm was executed to dry the Spin Column Assemblies before the Spin Columns were transferred into Collection Tube. 50 µl of Elution Buffer was added directly to the center of each Spin Column membrane. The Collection Tubes holding the Spin Columns were centrifuged again at 14000 rpm for 60 second and subsequently, the Spin Columns were removed. 5 µl of the eluted plamid DNA were used for Restriction Digestion analysis while the rest were stored at -20 °C.

Restriction digestion analysis was performed in order to attain confirmation that the desired RT-PCR product has been successfully cloned. The analysis is carried out using *Eco*RI in a 20 µl reaction prepared in the following order: 12 µl of sterile ddH_2O, 2 µl of 10X *Eco*RI buffer (Fermentas, USA), 5 µl of DNA, and 1 µl of *Eco*RI restriction enzyme. The reaction was gently mixed by pipetting and incubated for 1-3 hours at 37°C. Following incubation, inactivation of the enzyme was done by heating at 65 °C for 10 minutes. Five µl of the DNA was screened using agarose gel electrophoresis.

Sequencing of PCR product

Verification of the RT-PCR product was performed using DYEnamic ET Terminator Cycle Sequencing Kit. Cycle sequencing was done as follows: Each 10 µl cycle sequencing reaction made up of 3 µl RT-PCR product, 1 µl of 5 pmol primer, 4 µl of sequencing reagent premix and 2 µl of water was prepared into 0.5 ml tube, mixed by gentle pipetting, and centrifuged briefly to collect the contents at the bottom of the tubes. 25 runs of termal cycling were accomplished at 95 °C for 20 seconds, 50 °C for 15 seconds and 60 °C for 60 seconds.

Upon completion of cycling, 1 µl (1/10 volume) of sodium acetate/EDTA buffer was added into each tube followed by addition of 80 µl of 95% ethanol. The tubes were mixed using a vortex mixer then centrifuged at room temperature in a microcentrifuge for 15 minutes at 12000 rpm. Following centrifugation, the supernatant was aspirated from each tube and the remaining DNA pellets were washed with 70% ethanol. The tubes were briefly centrifuged after which the supernatants were removed by aspiration and air dried for 2-5 minutes. The dried DNA samples were then sent to Amersham Bioscience for subsequent analysis.

RESULTS AND DISCUSSION

Screening of Total RNA

Prior to RT- PCR experiment, screening of the commercial total RNA from normal colon tissue via agarose gel electrophoresis using 2% agarose is necessary for verification of its condition. The gel documentation of commercially available total RNA from normal colon tissue was obtained from others (courtesy of Ivyna Bong Pau Ni of Human Molecular Genetic Lab, UNIMAS) (Figure 1).

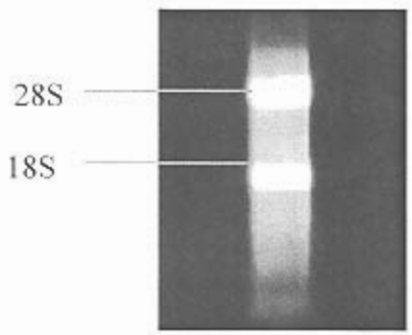

Figure 1: Total RNA from normal colon tissue purchased from BD Biosciences, Clontech Laboratories, Inc. Cat no: 64065-1. Courtesy of Ivyna Bong Pau Ni, Human Molecular Genetic Lab, UNIMAS.

Reverse Transcription PCR

RT- PCR products of the entire 8 normal *APC* gene fragments were successfully yielded using respective primers shown in table 1. Out of the 8 fragments, only five fragments designated *APC* 1, *APC* 2, *APC* 5, *APC* 6 and *APC* 8 consistently produce single PCR product with respective sizes that correspond to the expected values (1172bp, 1132bp, 1169bp, 1248bp and 1290bp). The other three fragments (*APC* 3, *APC* 4, and *APC* 7) yielded multiple products (Figure 2).

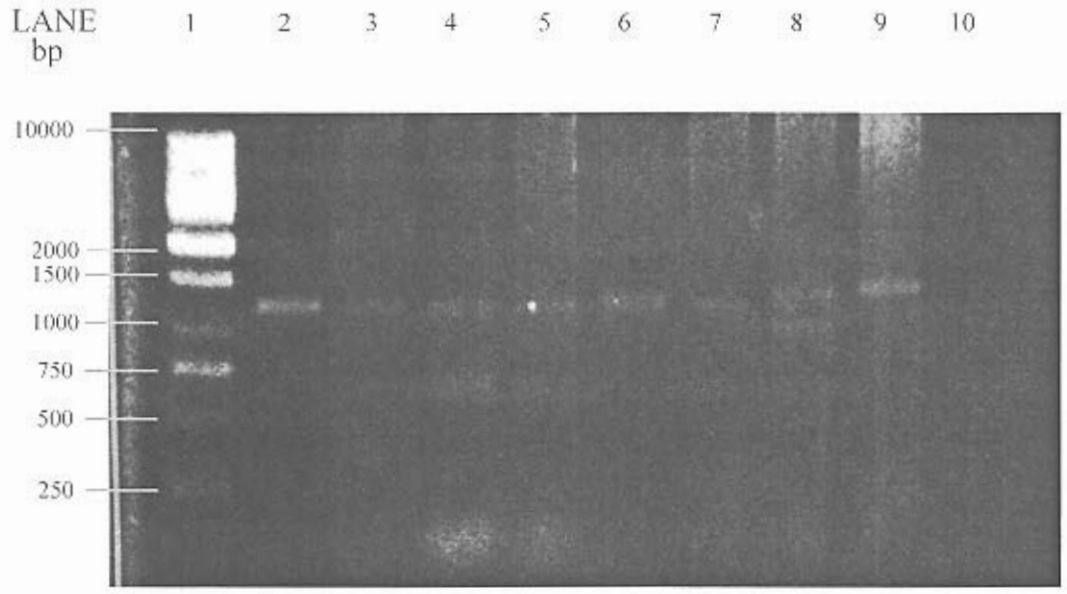

Figure 2: 2% Agarose gel electrophoresis of amplified RT-PCR products. Lane 1 represents the GeneRuler® 1 kb DNA ladder. Lane 2 -9 represent RT-PCR product of *APC* 1-9 at respective annealing temperature of 58°C, 58°C, 56°C, 58°C, 58°C, 54°C, 56°C and 56°C. Lane 10 represents the negative control.

Appropriate annealing temperature is essential to obtain optimized PCR results. Although equation for calculating annealing temperature has been formulated, Newton and Graham (1994) mentioned that calculated annealing temperature serves only as a reference point for initiation of experimentation. The ideal annealing temperature is often 3-12 °C higher than the calculated value. Amplifications of *APC* 5, *APC* 6, and *APC* 8 at respective calculated annealing temperature of 58 °C, 54 °C and 56 °C were successful in producing clear single band upon visualization. PCR amplification of *APC* 1 and *APC* 2 at respective calculated annealing temperature of 53 °C and 55 °C (Figure 3) yielded non-specific PCR products in addition to the DNA fragments of expected sizes. This finding can be explained by the fact that primer specificity tends to decline when the annealing temperature is inappropriate and therefore results in amplification of non specific products other than the desired DNA fragment. Upon increasing the annealing temperature to 58 °C, clear distinct DNA bands of expected size was visualized for both amplified fragments of *APC* 1 and *APC* 2.

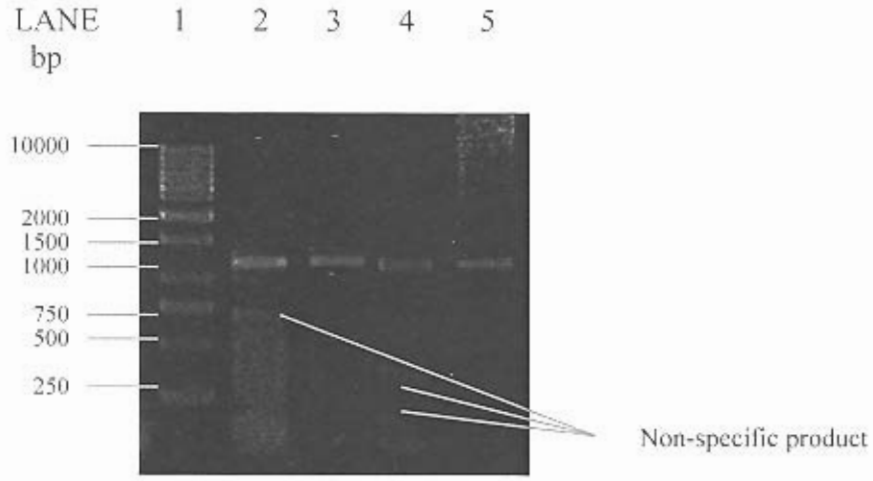

Figure 3: 2% agarose gel electrophoresis of *APC* 1 and *APC* 2 and the presence of non-specific PCR products. Lane 1 represents PCR product of *APC* 1 at the annealing temperature of 53°C. Lane 4 represents the PCR product of *APC* 2 at the annealing temperature of 55°C. Lane 3 and 5 represent PCR products of *APC* 1 and *APC* 2 respectively at the annealing temperature of 58°C.

PCR amplification of, *APC* 3, *APC* 4 and *APC* 7 at calculated temperature of 56 °C, 58 °C and 56 °C also results in production of non-specific products alongside DNA fragment of expected size. Due to insufficient starting material, annealing temperature optimizations for PCR of *APC* 3, *APC* 4 and *APC* 7 were not performed.

RT-PCR products of four DNA fragments namely *APC* 1, *APC* 2, *APC* 5 and *APC* 8 were excised from the agarose gel and purified (Figure 4).

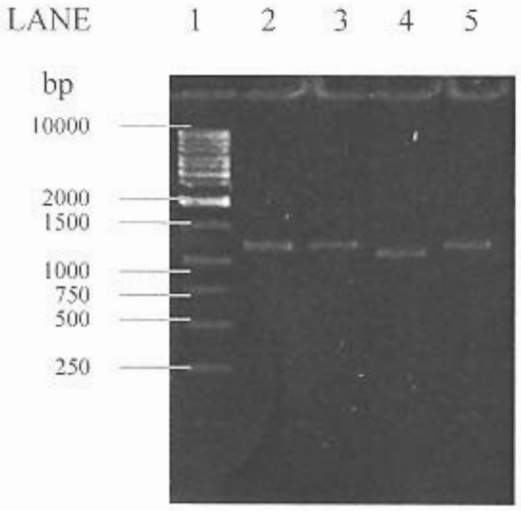

Figure 4: 2% agarose gel electrophoresis of purified PCR product. Lane 1 represents the GeneRuler® 1 kb DNA ladder. Lane 2-5 represent purified PCR product of *APC* 1, 2, 5 and 8 respectively.

Subcloning of RT-PCR product

The pGEM®-T easy vector by Promega, USA is prepared by cutting with *Eco*RV to produce single 3'-T overhangs at the insertion site. This enhances its ligation with Taq-polymerase generated PCR product that is added with a non-template dependent single deoxyadenosine at the 3'-ends (Clark, 1988).

Presumably, all the *APC* gene fragments amplified using Taq Polymerase (Promega, USA) possesses the 3'-A overhang that can ligate efficiently with the pGEM®-T easy vector used.

Cloning of purified RT-PCR products of *APC* 1, *APC* 5 and *APC* 8 were carried out with positive results obtained for *APC* 1 only. Figure 5 represent the gel documentation of restriction endonuclease digested pGEM-T vector that incorporates *APC* 1 fragment in it.

The restriction endonuclease digestion performed using *Eco*RI give rise to three DNA band upon visualization. The band measured 3000 bp corresponds to the linearized vector which has been cleaved with the enzyme. Two other bands of 681bp and 522bp respectively represent the *APC* 1 gene fragment. In this case, a single band of 1203bp was initially expected. Based on the existing Genbank data, the 5'-GAATTC-3' recognition site of *Eco*RI enzyme is present in the *APC* 1 gene itself and thus, cleavage within the initial *APC* 1 sequence results in the two smaller fragments observed.

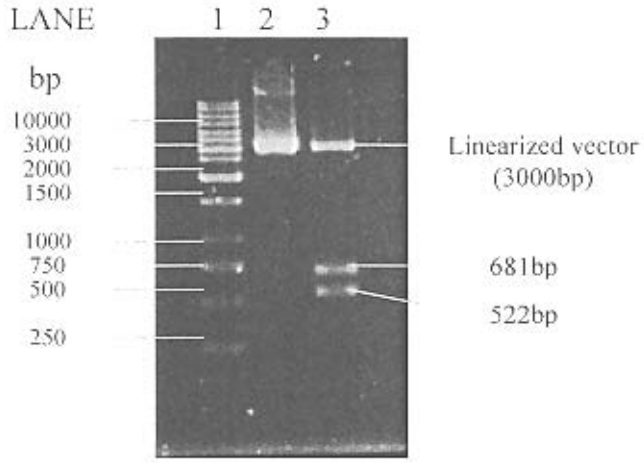

Figure 5: 2% agarose gel electrophoresis of cloned product. Lane 2 represents the negative control: the uncut plasmid incorporated with the cloned PCR product of *APC* 1. Lane 3 represents the DNA fragments obtained following restriction digestion of plasmid incorporating the cloned product of *APC* 1. The band measured 3000 bp is the linearized vector cleaved with EcoRI. Two smaller bands measured 681bp and 522 bp respectively represent the *APC* 1 gene fragment together with flanking regions.

Several problems were encountered during the subcloning process. Particularly for *APC* 5 and 8, ligation process was unsuccessful because there were no white colonies observed on LB+ampicilin+xgal+IPTG plates inoculated with transformed JM109. This could be due to insufficient A-tailing of PCR fragment or suboptimal insert:vector ratio. Ligation of PCR product could also be inhibited due to formation of pyrimidine dimers; a frequent problem that arises following overexposure of DNA to UV light during gel purification (according to the manufacturer).

Sequencing of RT-PCR product

Cloned product obtained for *APC* 1 was sent to Amersham Bioscience for automated sequencing which yielded a negative result. Amersham Bioscience reported that the sequencing failure is probably due to the insufficient of DNA template used or the insufficient of cycles of amplification during cycle sequencing. Left over of sodium acetate salt used during ethanol/ sodium acetate precipitation may also lead to complete inhibition of deoxynucleotide incorporation and hence, a failure in sequencing results (Perbal, 1988).

Restriction Site Characterization

Based on the existing GenBank data (Accession number: NM_000038), a restriction endonuclease sites map of *APC* cDNA is as follows:

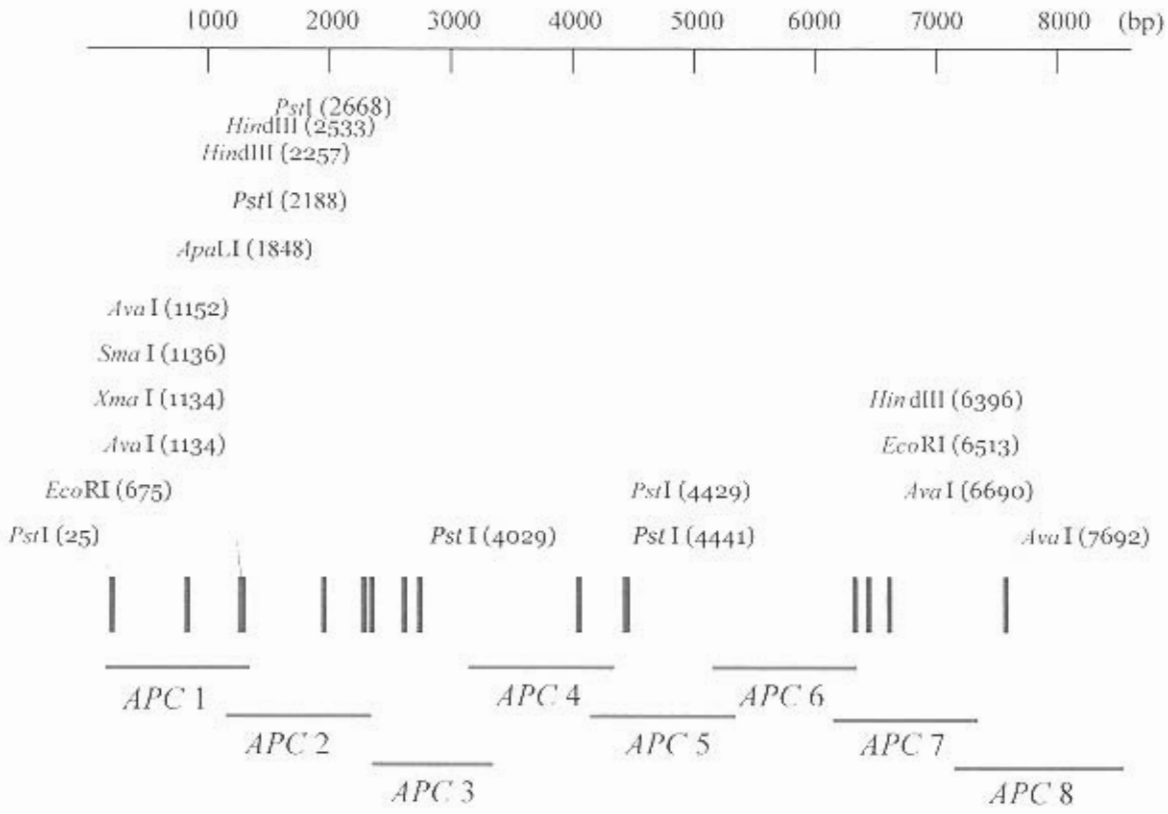

Figure 6: A Vector NTI Suite version 7.1a generated map of unique restriction endonuclease sites on normal *APC* gene (8.5 kb) as deduced from the cDNA sequence from GenBank data. Simulated DNA sequence overlap and alignment of the 8 *APC* fragments are shown below the restriction map.

Since the sequence of *APC* cDNA from experimented samples have not been acquired, it is not possible to construct a restriction map as above. Should the sequence of *APC* cDNA from both normal and tumor colorectal cancer tissue be obtained, it would be possible to construct the map to locate the appropriate flanking restriction enzyme sites of *APC* mutation markers. Nevertheless, a preliminary restriction endonuclease digestion performed on *APC* 1 using *Eco*RI (Figure 5) confirms the presence of the *Eco*RI site at 675 bp as elucidated in the restriction map (Figure 6).

CONCLUSION AND FUTURE WORK

Due to time constraint and inadequacy of total RNA from colon tissue, only one out of eight partial *APC* fragment from normal colon tissue was successfully amplified, cloned and sent for sequencing. Nevertheless, RT-PCR amplification attempted on all the 8 partial fragments has yielded products that match the predicted sizes. This indicates that the previously untested primers actually yield favorable RT-PCR result. Future work should focus on obtaining the sequence of the entire 8 partial *APC* fragment which could then be verified by comparison with the GenBank Database. Subsequently, *APC* gene could be amplified and sequenced using total RNA from local colorectal cancer patients. Differences in the sequence of *APC* cDNA from normal and tumor colorectal tissue can then be elucidated. Based on the restriction site map, combination of restriction enzyme- *APC* mutation marker can be determined. Finding of such novel mutation marker will facilitate in future colorectal cancer diagnostic via highly reproducible restriction enzyme-based method which is also less costly when compared to DNA sequencing.

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