MOLECULAR GENETIC ANALYSIS OF COLORECTAL CARCINOMA: GENE EXPRESSION PROFILING AND ANALYSIS OF THE TUMOUR SUPPRESSOR GENES APC AND DCC

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MOLECULAR GENETIC ANALYSIS OF COLORECTAL CARCINOMA: GENE EXPRESSION PROFILING AND ANALYSIS OF THE TUMOUR SUPPRESSOR GENE, DCC

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ABSTRACT

Colorectal carcinoma (CRC) ranks third among the ten leading causes of cancers in Malaysia (Annual Report of Cancer Incidence in Malaysia, 2002). The CRC tumourigenesis involved the inactivation of tumour suppressor genes, and activation of proto-oncogenes. Generally, colorectal adenoma is initiated by genetic aberrations in adenomatous polyposis coli (APC) gene, which cause the stabilization of β-catenin via activation of Wnt transduction pathway. These events will eventually lead to over-expression of its downstream target genes such as Cyclin D1, e-myc, e-jun and Matrilysin, which function as cell cycle regulators, apoptotic genes and transcription factors.

In this study, we report the simultaneous evaluation of gene expression profiles of Caucasian and Malaysian CRC patients using DNA microarray. The data on gene expression patterns revealed a total of 831 up-regulated genes and 103 down-regulated genes. Among the differentially expressed genes, 12 of them were verified by RT-PCR assay. They are Wnt2, Apo3, OSF-2p1, immunoglobulin lambda heavy chain, immunoglobulin heavy chain, V region, glutathione peroxidase, Tyl, Bbl, Mpvl7, Tsg101, ribosomal protein L7a (surf3) large subunit, ribosomal protein L32 and MLC-2. Most of the differentially expressed transcripts represented novel findings (OSF-2P1, Apo3, TSG101, MPV17, MLC-2 and TYL) while some of them are coincided with existing literature (Wnt, and ribosomal protein L7). Apart from that, analysis of Deleted in Colorectal Carcinoma (DCC) gene, which plays an important role in the initiation and progression of CRC, was performed in this study. Mutational analysis of DCC gene in Asian CRC is still very limited. By using novel DCC exonic primers, mutational analysis of DCC mRNA regions in CRC was successfully carried out and results obtained revealed several mutation sites in the CRC samples examined.
**ABSTRAK**

Di Malaysia, kanser kolon dan rektum (CRC) menduduki tempat ketiga antara sepuluh jenis kanser yang terutama (berpandukan laporan tahunan kejadian kanser di Malaysia, 2002). Faktor-faktor yang menyebabkan ketumbuhan CRC melibatkan ketidakaktifan gen-gen perencat kanser dan pengaktifan proto-onkogen. Secara umum, CRC dicetuskan oleh perubahan genetik dalam gen adenomatous polyposis coli (APC) di mana perubahan ini menyebabkan penstabilan β-catenin melalui perangsangan lintasan Wnt. Kejadian semua ini akan membawa kepada pengekspresan lebihan gen-gen sasaran seperti Cyclin D1, c-myc, c-jun dan Matrilysin yang berfungsi sebagai pengawal, kitaran sel, gen-gen apoptotik dan faktor-faktor transkripsi. Dalam penyelidikan ini, kami melaporkan penilaian kesamaan bagi profil ekspresi gen daripada tiga eksperimen mikroarray (peringkat I, II dan III). Data yang diperolehi daripada profil pengekspresan gen menunjukkan sejumlah 831 atas-regulasi gen dan 103 bawah-regulasi gen. Sebanyak 12 gen di antara gen yang diekspres secara berbeza telah disahkan oleh RT-PCR. Gen-gen ini ialah Wnt2, Apo3, OSF-2p1, immunoglobulin lambda heavy chain, immunoglobulin heavy chain, V region, glutathione peroxidase, TYL, BB1, Mpv17, TSG101, ribosomal protein L7a (surf3) large subunit, ribosomal protein L32 and MLC-2. kebanyakkan transkrip yang diekspres secara berbeza mewakili penemuan baru (OSF-2p1, Apo3, TSG101, MPV17, MLC-2 and TYL) manakala sebahagian gen-gen adalah selaras dengan hasil penyelidikan penyelidik yang lain (Wnt, and ribosomal protein L7). Selain itu, DCC gen analisis yang memainkan peranan penting dalam pencetusan dan perkembangan CRC telah dijalankan dalam penyelidikan ini. Analisis susunan asid nukleik bagi mRNA DCC dalam CRC telah dijalankan dengan menggunakan primer-primer exonik DCC telah berjaya diperolehi, dan keputusan yang dihasilkan telah menunjukkan beberapa tapak mutasi dalam sampel CRC yang dikaji.
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<th>Description</th>
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<td>absorbance at wavelength 260</td>
</tr>
<tr>
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<td>absorbance at wavelength 280</td>
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<td>A or a</td>
<td>adenine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>ABI</td>
<td>Applied Biosystem Inc.</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>C or c</td>
<td>cytosine</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
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<td>colorectal carcinoma</td>
</tr>
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</tr>
<tr>
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<td>cyanine 5</td>
</tr>
<tr>
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<td>deleted in colorectal carcinoma</td>
</tr>
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<td>dinucleotide triphosphate</td>
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<td>dUTP</td>
<td>deoxyuracil triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence taq</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>G or g</td>
<td>guanine</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary non-polyposis colorectal cancer</td>
</tr>
<tr>
<td>hr(s)</td>
<td>hour(s)</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertoni broth</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>min(s)</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biological Information</td>
</tr>
<tr>
<td>NH₄Oac</td>
<td>ammonium acetate</td>
</tr>
<tr>
<td>°C</td>
<td>degree of Celsius</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RBI</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase - PCR</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
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<td>second(s)</td>
</tr>
<tr>
<td>SOB</td>
<td>Salt-optimized broth</td>
</tr>
<tr>
<td>SOC</td>
<td>Salt-optimized medium</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride / sodium citrate</td>
</tr>
<tr>
<td>T or t</td>
<td>thymidine</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>V or v</td>
<td>volts</td>
</tr>
<tr>
<td>Ver</td>
<td>version</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl- β-D-thiogalactoside</td>
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CHAPTER ONE

Introduction

Tumours of the colon and rectum can be divided into adenomas and carcinomas (Corman, 1993). Colorectal adenoma is a benign tumour that is derived from the glandular while colorectal carcinoma (CRC) is a malignant tumour that arises from the epithelium (Corman, 1993). Colorectal adenomas can be divided into tubular, villous and tubulovillous adenomas according to the morphology of the glandular arrangement within the polyps (Jass and Sobin, 1989). CRC has neoplastic potential, whereby the cells would penetrate into the muscularis mucosa layer of the colon and metastasize to the distant organ such as liver if left untreated (Paluszkieiczka et al., 2004).

Colorectal carcinomas can be classified into sporadic and germ-line colorectal carcinomas. More than 70% of cancers of the colorectal are sporadic adenomatous polyps (Hardy et al., 2000). Sporadic CRC is caused by genetic abnormalities in somatic cells, which happens during cell growth and development. Unlike sporadic CRC, germ-line CRC is a result of genetic aberrations, which are inherited from the parents. The examples of germ-line CRC are familial adenomatous polyposis coli (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). HNPCC is caused by deficiency in mismatch repair (MMR) genes, such as MLH1, MSH2, PMS1 and PMS2 (Nicolaides et al., 1994; Vasen et al., 1996; Aarnio et al., 1999) whereas FAP is a disease initiated by mutation in adenomatous polyposis coli (APC) gene. According to Fodde (2002), mutation in APC gene is sufficient to initiate colorectal adenomas, while accumulation of the other genetic disorders are needed to transform the adenomas to carcinoma. In 1996, Vogelstein
and Kinzler proposed a genetic model of colorectal tumourigenesis. This genetic model is applied for FAP and some sporadic colorectal carcinoma cases. In this model, mutation in \textit{APC}, \textit{K-ras}, \textit{SMAD2/4} or \textit{DCC} or \textit{DPC4} and \textit{p53} genes are involved in the transformation of normal epithelial to CRC. Activation of Wnt signaling is thought to cause accumulation of \(\beta\)-catenin in the nucleus and transcriptional activation of Wnt downstream target genes. To date, the mutation cluster region (MCR) of \textit{APC} has been identified but mutations in \textit{DCC}, \textit{K-ras} and \textit{p53} genes are still poorly understood.

Gene expression profiling of colorectal carcinomas revealed genes that are involved in the CRC tumourigenesis pathways, for example, \textit{c-myc} and \textit{Cyclin D1} genes (Erisman \textit{et al.}, 1985 & 1988; Sikora \textit{et al.}, 1987; Finley \textit{et al.}, 1989; Rowley \textit{et al.}, 1990; Hur \textit{et al.}, 2000; Fodde \textit{et al.}, 2001). However, the information obtained from the endogenous gene expression profiling of CRC is still not sufficient to completely delineate the CRC tumourigenesis pathways. More expression studies on CRC are needed to solve this problem.

In our project, we aim at identifying gene expression profiles of colorectal carcinomas in the Malaysian context by using cDNA microarray approach. Apart from that, we will perform mutational analysis of mRNA and genomic regions of the \textit{DCC} gene. We would then link the mutation data with the gene expression pattern. If we have identified mutation(s) in the \textit{DCC} gene, we would investigate whether genes involved in the pathways or other associated pathways show differential expression. By this, we would know whether mutations in these genes have any impact or relationship with the differential expression genes in our CRC cases. This would provide us possible clues on the delineation of CRC tumourigenesis pathways.
CHAPTER TWO

Literature Review

2.1 Structure of Colorectal Tissue

Human colon and rectum are organs of the digestive system. The colon is a tube-shaped, muscular and about four feet long organ. It is twisting and turning from the end of the small bowel to the anus. The colon can be divided into 4 main regions, which are transverse colon, ascending colon, descending colon and sigmoid colon (Corman, 1993) (Figure 1). Cecum and ascending colon play a major role in the water and electrolyte absorption and fermentation of the undigested sugars while the descending colon, sigmoid colon and rectum are predominantly responsible for the storage and evacuation of the faeces.

2.2 Colorectal Organogenesis

The digestive tube of the human embryo consists of fore-gut and hind-gut. Fore-gut is located within the cephalic flexure whereas hind-gut is situated within the caudal flexure.

About the 4th week, the fore-gut of the embryo opens freely into the yolk-sac and the opening is gradually narrowed into the yolk stalk (vitelline duct). The gut undergoes further elongation to form a V-shaped loop that passes from the vitelline duct to the umbilicus. In the 6th week, the diverticulum of the gut is developed behind the vitelline duct. Part of the loop on the distal part of the cecal diverticulum then increases in diameter and forms the ascending and transverse portions
of the colon. After 5 months, the proximal part of the cecal diverticulum expands and forms the cecum.

Apart from fore-gut, the hind-gut is lengthened backwards into the body stalk as a tube of Allantois. The body stalk, with the Allantois is then carried forward to the ventral aspect of the body and forms a bend at the junction of the hind-gut and Allantois. Subsequently, the bend dilates and becomes a pouch that contains the entodermal cloaca. The entodermal cloaca is then divided into dorsal and ventral parts by urorectal septum. The dorsal part of the cloaca finally forms the rectum.

2.3 Functions and Development of Colonic Tissue

The colon itself consists of four layers. Starting from the inner layer to the outer layer, the colon is composed of lumen, mucosa, submucosa, muscularis externa and serosa. Mucosa is built up by a smooth muscle, which consists of 3 layers. They are known as surface epithelium, lamina propria and muscularis mucosae. The surface of epithelium consists of goblet cells and crypts, which are oriented as straight tubular glands that extend down into the muscularis mucosae. Crypts excrete mucus to facilitate the passage of faeces along the colon. Apart from that, lamina propria and muscularis mucosae contain blood and lymph vessels, nerves and cells of the immune system. Hence, when the tumour cells invade muscularis mucosae, they would become more aggressive and metastasize to the distant organs via blood vessels and lymph nodes after a period of time (Kerr, 1999).

The submucosa layer is comprises of fibroelastic loose connective tissue. It contains blood vessels, leukocytes, nerve cells and fat. Besides that, muscularis externa can be further divided into inner and outer layers. The inner layer is formed by circular fibers whereas the outer layer is
formed by longitudinal fibers. There are three longitudinal strips known as teniae colis in the outer layer. Teniae colis functions in the contraction of colon, faecal compaction and distal transport of luminal contents. The last layer, serosa or adventitia layer is a thin loose connective tissue (Kerr, 1999).

2.4 Duke’s Classification of Colorectal Carcinoma

Duke’s staging classifies CRC into 4 different stages, with 90-100%, 75-85%, 30-40% & <5% five years survival rate in stages I, II, III and IV, respectively. Stage I refers to the tumour that is confined to the submucosa or muscularis propria. Tumours that invade the subserosa layer are classified as Stage II. Tumour is classified as Stage III when the cancerous cells invade the lymph nodes, while Stage IV represents malignant tumour that causes distant metastasis.

2.5 Incidence of Colorectal Carcinoma

Colorectal carcinoma is one of the most common cancer-related deaths for men and women worldwide. Globally, CRC is the third leading caused of human cancers for both sexes, it accounted for 10% of new cases and 8.7% of total cancer mortality in 2000 (Shibuya et al., 2002).

In Malaysia, approximately 26,089 cases of cancers were diagnosed in 2002. CRC ranks third after lung and nasopharynx cancers in males while in females, CRC ranks the third with breast and cervix uteri cancers as the leading causes (Figure 2 and Figure 3) (According to the annual report of cancer incidence in Malaysia, 2002). The CRC incidence occurs at all ages with a marked increase from 50 years and 70 years onwards for males and females, respectively.
According to Corman et al. (1979), most of the colonic lesions such as benign tumours or adenomas were detected in the rectum and sigmoid colon. They account for 43% and 25% in rectum and sigmoid colon, respectively. Further, 18% of the colonic lesions were detected in ascending colon, 9% in transverse colon and 5% in descending colon. This finding indicated that most of the colorectal carcinomas were distributed in the distal colon and they arise more frequently in the left side of the colon (ascending) when compared to the right side (descending).
Figure 1: Structure of the human colon and rectum. Colon is divided into 4 regions. They are transverse colon, ascending colon, descending colon and sigmoid colon (Adapted from Corman, 1993).