UNIVERSITI PUTRA MALAYSIA

ELUCIDATION OF THE WNT & AKT/PHOSPHOINOSITIDE-3-KINASE PATHWAYS IN COLORECTAL CARCINOMA

KHOR TIN OO

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ELUCIDATION OF THE WNT & AKT/PHOSPHOINOSITIDE-3-KINASE PATHWAYS IN COLORECTAL CARCINOMA

By

KHOR TIN OO

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Degree of Doctor of Philosophy

April 2004
Specially dedicated to,

My beloved wife, son (Hong Ze), parents and sister

The memory of,

My grandma and mother-in-law

For their invaluable love, understanding, patience, support and constant faith.
Colorectal cancer (CRC) is the third most common cancer in Malaysia and is currently the commonest cancer in males. Genetics, experimental and epidemiological data suggest that CRC develops from complex interaction between inherited susceptibility and environmental factors. Accumulating evidence suggests that the Wnt and PI3K (phosphoinositide-3-kinase)/Akt signalling pathways play a causative role in tumorigenesis of colorectal cancer.

By employing immunohistochemical method, the expression and correlation of several key regulators or related biomolecules of the Wnt and PI3K/Akt signalling pathways in 47 archival formalin fixed, paraffin embedded tissues of surgically resected colorectal cancer (CRC) specimens performed at Kuala Lumpur Hospital (KLH) between 1999 and 2000, were studied. Laser captured microdissection
technique, polymerase chain reaction and direct sequencing were used to investigate mutations in exon 3 of the \(\beta\)-catenin gene. Mutations in the mutation cluster region (MCR) of adenomatous polyposis coli (APC) gene were also investigated. The expressions of Wnt-1, WISP-1 and FRAT-1 mRNA were determined by reverse-transcription and real-time polymerase chain reaction method.

The results showed that: The expressions of Wnt-1, FRAT-1, APC, nuclear \(\beta\)-catenin, cytoplasmic \(\beta\)-catenin, membrane \(\beta\)-catenin, membrane E-cadherin, cytoplasmic E-cadherin, WISP-1, cyclin-D1, p-Akt1 (Ser473), p-Akt1/2/3 (Thr308), p-BAD (Ser136), p-GSK 3\(\beta\)(Ser9) and survivin were found in 55.3%, 36.2%, 51.1% 44.6%, 95.7%, 30.6%, 46.8%, 95.7%, 31.9%, 10.6%, 34%, 44.7%, 57.4% 44.7% and 59.6% of CRC tissues, respectively and 17.5%, 5% 100%, 0%, 75%, 100%, 100%, 50%, 12.5%, 0%, 5%, 12.5%, 22.5%, 22.5% and 32.5% of apparently normal adjacent tissues, respectively. The sum of scores for all biomolecules except APC, membrane \(\beta\)-catenin and membrane E-cadherin staining was significantly higher in CRC tissues in comparison to apparently normal adjacent tissues (p < 0.05). The sum of score for APC, membrane \(\beta\)-catenin and membrane E-cadherin staining was significantly lower in CRC tissues in comparison to apparently normal adjacent tissues (p < 0.05). The expression of Wnt and PI3K/Akt signalling pathway-related biomolecules was interrelated. The results of nucleotide sequencing showed that no mutations at exon-3 of \(\beta\)-catenin were found. However, point mutations in the mutation cluster region of the APC gene leading to the formation of truncated APC protein, were found in four
out eleven CRC tissues examined. A 1.43 to 21.26-fold and 1.11 to 109.14-fold increase in the level of expression of Wnt-1 and FRAT-1 mRNA was found in eight out of eleven CRC tissues relative to apparently normal adjacent tissues. On the other hand, a 1.94 to 46.69-fold increase in the level of WISP-1 mRNA was found in all the CRC tissues.

This study has provided important information for researchers and clinicians in terms of clinical evidence of the involvement of the Wnt signalling pathway and PI3K/Akt signalling pathway in colorectal tumorigenesis. In addition, the present study also provided crucial information on the elucidation of the relationship between the biomolecules of these signalling pathways towards understanding their roles in colorectal tumourigenesis and the identification of potential targets for advance therapeutic intervention of CRC. Based on our current results, we propose that Wnt-1, FRAT-1 and WISP-1 could be served as potent therapeutic target for the treatment of CRC.

On the basis of our present study, we conclude that the Wnt and PI3K/Akt signalling pathways are involved in tumourigenesis of CRC in Malaysia. These pathways are interrelated although they might also act independently in promoting tumour growth and inhibition of apoptosis. This study has also provided useful information for the search or design of better antitumour interventions.
Abstrak tesis yang dikenalkan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENJELASAN LINTASAN WNT DAN AKT/PHOSPHOINOSITIDE-3-KINASE DALAM KARSINOMA KOLOREKTAL

Oleh

KHOR TIN OO

April 2004

Pengerusi: Profesor Dr Seow Heng Fong, Ph.D.

Fakulti: Perubatan dan Sains Kesihatan

Barah kolorektal (CRC) merupakan barah yang ketiga paling kerap di Malaysia dan pada ketika ini, ia merupakan barah yang paling kerap di kalangan lelaki. Data genetik, eksperimental dan data epidemiologi menyarankan bahawa CRC berkembang hasil interaksi antara faktor persekitaran dan faktor keturunan. Bukti-bukti telah menyarankan bahawa lintasan isyarat PI3K (phosphoinositide-3-kinase) /Akt dan Wnt memainkan peranan yang penting dalam perkembangan barah kolorektal.

Dengan menggunakan kaedah immunohistokimia, ekspresi dan hubungan antara beberapa pengawal-atur atau biomolekul yang berkaitan dengan lintasan isyarat

Keputusan kami menunjukkan bahawa ekspresi Wnt-1, FRAT-1, APC, β-katenin nukleus, β-katenin sitoplasma, β-katenin membran, E-cadherin membran, E-cadherin sitoplasma, WISP-1, cyclin-D1, p-Akt1 (Ser473), p-Akt1/2/3 (Thr308), p-BAD (Ser136), p-GSK 3β(Ser9) dan survivin telah dikesan di 55.3%, 36.2%, 51.1% 44.6%, 95.7%, 30.6%, 46.8%, 95.7%, 31.9%, 10.6%, 34%, 44.7%, 57.4% 44.7% dan 59.6% tisu CRC, masing-masing dan 17.5%, 5% 100%, 0%, 75%, 100%, 100%, 50%, 12.5%, 0%, 5%, 12.5%, 22.5%, 22.5% dan 32.5% tisu sekeliling yang kelihatan biasa, masing-masing. Jumlah skor untuk semua biomolekul kecuali APC, β-katenin membran and E-cadherin membran adalah lebih tinggi dalam tisu CRC berbanding dengan tisu sekeliling yang kelihatan biasa (p < 0.05). Jumlah skor untuk APC, β-katenin membran and E-cadherin membran adalah lebih rendah dalam tisu CRC berbanding tisu sekeliling yang kelihatan biasa (p < 0.05). Ekspresi biomolekul yang berkaitan dengan lintasan isyarat Wnt dan PI3K/Akt adalah saling berhubungan. Keputusan penjujukan menunjukkan bahawa tidak ada mutasi berlaku di ekson-3,

Kajian ini telah menghasilkan maklumat yang penting kepada para penyelidik dan perubatan dari segi bukti klinikal bagi pembabitan lintasan isyarat Wnt dan PI3K/Akt dalam tumorigenesis kolorektal. Kajian kami juga memberi maklumat penting dalam penjelasan hubungan antara biomolekul bagi lintasan isyarat yang berkenaan, menuju pemahaman peranan mereka di dalam tumorigenesis kolorektal dan pengenalan sasaran-potensi bagi intervensi CRC therapi maju. Berdasarkan keputusan ini, kami mencadangkan bahawa Wnt-1, FRAT-1 dan WISP-1 boleh dianggap sebagai sasaran terapeutik yang berpotensi untuk rawatan CRC.

Berdasarkan keputusan yang diperolehi, kami membuat kesimpulan bahawa lintasan isyarat Wnt dan PI3K/Akt adalah berkait dengan tumourigenesis CRC di Malaysia. Lintasan isyarat ini adalah saling berhubungan walaupun mereka juga boleh bertindak secara bersendirian untuk menggalakkan pertumbuhan barah dan perencatan apoptosis. Kajian ini telah memberi maklumat yang berguna kepada para...
penyelidik dan doctor perubatan dalam penemuan dan penerokaan intervensi anti-barah yang lebih baik.
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I certify that an Examination Committee met on 14th April 2004 to conduct the final examination of Khor Tin Oo on his Doctor of Philosophy thesis entitled “Elucidation of the Wnt and Akt/Phosphoinositide-3-Kinase Pathways in Colorectal Carcinoma” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 10 SEP 2004
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

KHOR TIN OO

Date: 15/07/04
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<td>4.8</td>
<td>Representative slides showing the immunohistochemical staining of E-cadherin</td>
<td>94</td>
</tr>
<tr>
<td>4.9</td>
<td>Representative slides showing the immunohistochemical staining of FRAT-1</td>
<td>95</td>
</tr>
<tr>
<td>4.10</td>
<td>Representative slides showing the immunohistochemical staining of WISP-1</td>
<td>96</td>
</tr>
<tr>
<td>4.11</td>
<td>Representative slides showing the immunohistochemical staining of cyclin-D1</td>
<td>97</td>
</tr>
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</table>
4.12 Representative slides showing the immunohistochemical staining of survivin

4.13 The purity of RNA isolated from the fresh CRC and adjacent normal tissues

4.14 Amplification of WISP-1 mRNA

4.15 Amplification of Wnt-1 mRNA

4.16 Amplification of FRAT-1 mRNA

4.17 Amplification of 18S gene

4.18 Semi-quantitative method for comparison of Wnt-1, FRAT-1 and WISP-1 mRNA in CRC vs normal adjacent tissues

4.19 Representative real-time PCR cycling profile of Wnt-1, FRAT-1 and WISP-1 amplification.

4.20 Representative melting curve for Wnt-1, FRAT-1 and WISP-1 gene.

4.21 Relative quantification of Wnt-1, FRAT-1 and WISP-1.

4.22 The outlines of results from (A) previous studies in comparison to the (B) present study on the relationship between the biomolecules in the Wnt signalling pathway

5.1 Representative slides showing the immunohistochemical staining of p-Akt1 (Ser473)

5.2 Representative slides showing the immunohistochemical staining of p-Akt1/2/3 (Thr308)

5.3 Representative slides showing the immunohistochemical staining of p-GSK 3β (Ser9)

5.4 Representative slides showing the immunohistochemical staining of p-BAD (Ser136)

5.5 The outlines of results from (A) previous studies in comparison to the (B) present study on the relationship between the biomolecules in the PI3K/Akt signalling pathway

6.1 The cross-talk between the Wnt signalling pathway and PI3K/Akt signalling pathway
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AAPC</td>
<td>Attenuated adenomatous polyposis coli</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CKIC</td>
<td>Casein kinase I epsilon</td>
</tr>
<tr>
<td>Cox</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal carcinoma/cancer</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer</td>
</tr>
<tr>
<td>Dkk</td>
<td>Dickkopf</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Dideoxynucleotide triphosphates</td>
</tr>
<tr>
<td>Dsh/Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FOBT</td>
<td>Faecal occult blood test</td>
</tr>
<tr>
<td>FRAT</td>
<td>Frequently rearranged in advance T-cell lymphocytes</td>
</tr>
<tr>
<td>FrzB</td>
<td>Frizzled-related protein</td>
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<tr>
<td>Fz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GBP</td>
<td>Glycogen synthase kinase binding protein</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
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<tr>
<td>HNPPC</td>
<td>Hereditary non-polyposis colorectal cancer</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>JNK</td>
<td>Jun kinase</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pair</td>
</tr>
<tr>
<td>LCM</td>
<td>Laser capture microdissection</td>
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<tr>
<td>LEF</td>
<td>Lymphoid enhancer factor</td>
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<tr>
<td>LRP</td>
<td>Low density lipoprotein-receptor-related protein</td>
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<tr>
<td>mA</td>
<td>Milliamperre</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
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<td>Magnesium Chloride</td>
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<tr>
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<td>messenger Ribonucleic acid</td>
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<td>Mucin antigen 1</td>
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<tr>
<td>nM</td>
<td>Nano molar</td>
</tr>
<tr>
<td>NHS</td>
<td>Nurses' health study</td>
</tr>
<tr>
<td>Nkd</td>
<td>Naked cuticle</td>
</tr>
<tr>
<td>p-Akt</td>
<td>Phosphorylated Akt</td>
</tr>
<tr>
<td>p-BAD</td>
<td>Phosphorylated BAD</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
</tbody>
</table>