UNIVERSITI PUTRA MALAYSIA

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

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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.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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

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April 2004

Chairman: Professor Seow Heng Fong, Ph.D.

Faculty: Medicine and Health Sciences

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 β-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 β-catenin, cytoplasmic β-catenin, membrane β-catenin, membrane E-cadherin, cytoplasmic E-cadherin, WISP-1, cyclin-D1, p-Akt1 (Ser473), p-Akt1/2/3 (Thr308), p-BAD (Ser136), p-GSK 3β(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 β-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 β-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 β-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 dikemukakan 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

PI3K/Akt dan Wnt telah dikaji dalam 47 sampel tisu yang diperolehi daripada pesakit CRC yang menjalani pembedahan di Hospital Kuala Lumpur antara 1999 dan 2000 dan telah diblokkan di dalam paraffin. Kaedah "laser captured microdissection", " polymerase chain reaction (PCR)" dan "direct-sequencing" telah digunakan untuk mengkaji mutasi yang berlaku pada ekson 3, gen β-katenin. Mutasi pada "mutation cluster region (MCR)", gen APC juga dikaji. Ekspresi mRNA Wnt-1, WISP-1 dan FRAT-1 juga dikenalpasti dengan menggunakan kaedah "real-time"-PCR.

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,

gen β-katenin. Walau bagaimanapun, mutasi pada MCR, gen APC yang menyebabkan pembentukan protein APC yang "truncated" telah dikesan pada empat daripada sebelas spesimen CRC yang dikaji. Peningkatan ekspresi mRNA Wnt-1 dan FRAT-1 sebanyak 1.43 ke 21.26-kali dan 1.11 ke 109.14-kali masing-masing telah didapati dalam lapan daripada sebelas tisu CRC berbanding tisu sekeliling yang kelihatan biasa. Sebaliknya, peningkatan sebanyak 1.94 ke 46.69-kali mRNA WISP-1 telah berjaya dikesan di kesemua tisu CRC yang dikaji.

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 intenvensi CRC therapi maju. Berdasarkan keputusan ini, kami mencadangkan bahawa Wnt-1, FRAT-1 dan WISP-1 boleh dianggap sebagai sasaran teraputik 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

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Finally, I would like to express my deepest gratitude to my wife, parents and sister for their continual support and encouragement for me to proceed to completion of this project.

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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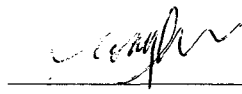
DECLARATION

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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µg	Microgram
µl	Microlitre
5-FU	5-Fluorouracil
AAPC	Attenuated adenomatous polyposis coli
APC	Adenomatous polyposis coli
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CKIЄ	Casein kinase I epsilon
Cox	Cyclooxgenase
CRC	Colorectal carcinoma/cancer
DCC	Deleted in colorectal cancer
Dkk	Dickkopf
DNA	Deoxyribonucleic acid
dNTPs	Dideoxynucleotide triphosphates
Dsh/Dvl	Dishevelled
EGF	Epidermal growth factor
FAP	Familial adenomatous polyposis
FOBT	Faecal occult blood test
FRAT	Frequently rearranged in advance T-cell lymphocytes
FrzB	Frizzled-related protein
Fz	Frizzled
g	Gram
GBP	Glycogen synthase kinase binding protein
GSK3β	Glycogen synthase kinase 3β
HNPCC	Hereditary non-polyposis colorectal cancer
IAP	Inhibitor of apoptosis protein
IGF	Insulin-like growth factor
JNK	Jun kinase
kb	Kilobase pair
LCM	Laser capture microdissection
LEF	Lymphoid enhancer factor
LRP	Low density lipoprotein-receptor-related protein
mA	Milliampere
mg	Milligram
$MgCl_2$	Magnesium Chloride
mRNA	messenger Ribonucleic acid
MUC1	Mucin antigen 1
nM	Nano molar
NHS	Nurses' health study
Nkd	Naked cuticle
p-Akt	Phosphorylated Akt
p-BAD	Phosphorylated BAD
PBS	Phosphate buffer saline