UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION OF INDUCED RAT MAMMARY GLAND TUMOR AND THE ANTITUMOR EFFECT OF RECOMBINANT HUMAN ERYTHROPOIETIN AND TAMOXIFEN

SAIRAH ABDUL KARIM

IB 2009 12

CHARACTERISATION OF INDUCED RAT MAMMARY GLAND TUMOR AND THE ANTITUMOR EFFECT OF RECOMBINANT HUMAN ERYTHROPOIETIN AND TAMOXIFEN

SAIRAH ABDUL KARIM

DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA

2009

CHARACTERISATION OF INDUCED RAT MAMMARY GLAND TUMOR AND THE ANTITUMOR EFFECT OF RECOMBINANT HUMAN ERYTHROPOIETIN AND TAMOXIFEN

By

SAIRAH ABDUL KARIM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfillment of the Requirements for the Degree of Doctor of Philosophy

2009

DEDICATION

To my parents, Mr. Abdul Karim Jabar and Mrs. Noli Othman……..

To my beloved husband Mohd. Fadhil Ahmad and my lovely children, Faiz Isqandar and Aleya Yasmin who bring me great happiness…….

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

CHARACTERISATION OF INDUCED RAT MAMMARY GLAND TUMOR AND THE ANTITUMOR EFFECT OF RECOMBINANT HUMAN ERYHTROPOIETIN AND TAMOXIFEN

By

SAIRAH ABDUL KARIM

2008

Chairman : Professor Rasedee Abdullah, PhD

Faculty : Institute of Bioscience

Breast cancer is the most common cancer and the incidence and mortality rate had remained high. In Malaysia alone, breast cancers accounted for 31% of all new cancer cases and are among the most fatal cancers. Since breast cancers are complex diseases, there is no single marker that is both sensitive and specific for early detection of the disease. The present study was undertaken to characterize rat mammary gland tumors as a model for breast cancers and to determine parameters that could be used as early tumor markers. The study also undertook to determine the effect of recombinant human erythropoietin (rHuEPO) and Tamoxifen on the rat mammary gland tumor.

In the first part of the study serum biochemical parameters, angiogenic factors and tumor markers, tumor histopathology and ultrastructure and expression of estrogen (ER) and erythropoietin receptors (EPOR) were determined. Twenty female Sprague-Dawley rats, aged six to seven weeks were divided into two

groups of 10 rats per group. The rats were treated intragastrically, the first group with 20 mg 7,12-dimethylbenz(a)anthracene (DMBA) per rat to induce mammary tumor development and the second group with 1 mL 0.9% normal saline and served as the control. The animals were palpated weekly for tumor mass and sacrificed two weeks after tumor occurrence. Blood was withdrawn through cardiac puncture before tumor induction and weekly thereafter. Serum biochemical parameters analysed were alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine transferase (ALT), lactate dehydrogenase (LDH), creatinine kinase (CK), glucose, blood urea nitrogen (BUN) and creatinine by a chemistry analyser using standard diagnostic kits. Serum tumor markers,namely α-fetoprotein (AFP) and CA15-3 were analysed using automated immunoassay analyser, while the angiogenic factors, matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF) were determined by the ELISA technique. Tumor tissues excised from sacrificed animals were subjected to histopathological analysis and ER-α and EPOR determination through immunohistochemistry (IHC). Tumor ultrastructure was examined by transmission electron microscopy (TEM).

The results showed higher ALT, ALP and AST concentrations in the DMBA-treated than the control group reflecting abnormal liver function. Pronounced increases in serum LDH were also observed in the DMBA-treated group. Angiogenic factor estimations showed that serum MMP-2 levels remained high throughout the study and seemed to have played a greater role than VEGF in

early stage tumorigenesis. Serum tumor markers, AFP and CA 15.3 were not detected in either the treated or control rats. Histopathological analysis showed features typical of neoplastic cells which were enlarged nuclei, conspicuous nucleoli nuclear pleomorphism, high nuclear to cytoplasma ratio, hyperchromasia, and epithelial cell and stroma hyperplasia. These features are similar to that found in breast cancers. Immunohistochemical analysis showed that ER-α and EPOR were present in the DMBA-induced rat mammary tumor, which also resemble human breast cancers. Transmission electron microscopy analysis of the tumor demonstrated the co-existence of apoptosis, necrosis and aponecrosis which may be used in the determination of mammary gland tumor and breast cancer development in the early stages.

In conclusion, the combination of serum liver-related enzymes, serum MMP-2 and histological changes, ER-α and EPOR expression, evidences of apoptosis, necrosis and aponecrosis may form the panel for screening and determination of early mammary gland tumors in high risk cancer patients.

The second phase of the study involved the development of a xenograft-induced mammary gland tumor model in rats as a substitute for the conventional drug-induced method. The xenograft-induced mammary gland tumor seems to be reliable and can produce tumors within a short period. The xenograft model was then used to evaluate the effects of rHuEPO, Tamoxifen and Tamoxifen-rHuEPO combination on mammary gland tumor growth and

angiogenesis. In this study, 24 rats were divided into four groups of six rats each. Each rat was treated orally: Group 1 with 60 IU rHuEPO; Group 2 with 20 mg Tamoxifen; Group 3 with a combination of 20 mg Tamoxifen and 60 IU rHuEPO; Group 4 with 1 mL 0.9% normal saline and served as the control. The results showed that rHuEPO did not promote mammary tumor growth and in fact may enhance the cytotoxicity of Tamoxifen through the stimulation of proapoptotic and antiproliferative effects. This study suggests that rHuEPO treatment in cancer patients may not only be beneficial for the alleviation of anemia due to the disease, but also augments the effect of certain chemotherapeutic drugs used in the treatment of cancers.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENCIRIAN TUMOR KELENJAR MAMA TIKUS TERARUH DAN KESAN ANTITUMOR ERITROPOIETIN MANUSIA REKOMBINAN DAN TAMOXIFEN

Oleh

SAIRAH ABDUL KARIM

2008

Pengerusi : Profesor Rasedee Abdullah, PhD

Fakulti : Institut Biosains

Kanser payudara merupakan kanser yang sangat biasa berlaku dan kadar insidens dan kematiannya kekal tinggi. Di Malaysia sahaja, kanser payudara menyumbang sebanyak 31% kepada kes baru kanser dan merupakan kanser yang paling banyak menyebabkan kematian. Memandangkan kanser payudara adalah penyakit yang kompleks, tiada satu petanda tunggal yang peka dan khusus untuk pengesanan awal penyakit ini. Kajian ini telah diambil untuk mencirikan tumor kelenjar mama tikus sebagai model untuk kanser payudara and menentukan parameter yang boleh digunakan sebagai petanda awal tumor. Kajian ini juga telah dijalankan bagi menentukan kesan eritropoietin manusia rekombinan (rHuEPO) dan Tamoxifen terhadap tumor kelenjar mama tikus.

Di dalam bahagian pertama kajian, parameter biokimia serum, faktor angiogenesis dan petanda tumor, histopatologi tumor dan ultrastruktur dan pernyataan reseptor estrogen (ER) dan eritropoietin (EPOR) telah ditentukan. Di dalam kajian ini, 20 ekor tikus betina Sprague-Dawley, berumur 6 hingga 7 minggu telah dibahagikan kepada dua kumpulan terdiri daripada 10 ekor tikus setiap kumpulan. Tikus-tikus ini diperlakukan secara intragaster, kumpulan pertama dengan 20 mg 7,12-dimetilbenz(a)antrasin (DMBA) setiap ekor untuk mengaruhkan pembentukan tumor dan kumpulan kedua diberi 1 mL 0.9% larutan salina normal dan bertindak sebagai kumpulan kawalan. Haiwan dipalpatkan setiap minggu untuk pengesanan tumor dan dimusnahkan dua

minggu selepas kejadian tumor. Darah diambil secara tusukan jantung sebelum pembentukan tumor diaruhkan dan setiap minggu seterusnya. Parameter biokimia serum yang dianalisis ialah alkalin fosfatase (ALP), aspartat aminotransefase (AST), alanin transferase (ALT), laktat dehidrogenase (LDH), glukosa, urea nitrogen darah (BUN) dan kreatinin secara penganalisis kimia dan kit diagnostik standard. Petanda tumor serum, α-fetoprotein (AFP) dan CA 15.3 telah dianalisis menggunakan penganalisis immunoassai automatik, sementara faktor angiogenesis, matrik metalloproteinase-2 (MMP-2) dan faktor pertumbuhan endotelium vesel (VEGF) telah ditentukan menggunakan teknik ELISA. Pada tisu tumor yang diambil dari haiwan yang dimusnahkan, dilakukan analisis histopatologi dan penentuan reseptor ER-α dan EPOR secara immunohistokimia (IHC). Ultrastruktur tumor telah diperiksa menggunakan mikroskop elektron transmisi (TEM).

Keputusan kajian menunjukkan kepekatan ALT, ALP dan AST lebih tinggi dalam tikus kumpulan DMBA-diperlaku berbanding kumpulan kawalan, mencerminkan keabnormalan fungsi hati. Peningkatan nyata LDH serum diperhatikan dalam kumpulan DMBA-diperlaku. Penganggaran faktor angiogenesis menunjukkan bahawa paras MMP-2 serum kekal tinggi sepanjang kajian dan nampaknya memain peranan lebih besar daripada VEGF pada peringkat awal tumorigenesis. Petanda tumor serum, AFP dan CA15.3 tidak dikesan samada dalam tikus tumor kelenjar mama DMBA-teraruh atau kawalan. Analisis histopatologi menunjukkan sifat yang tipikal untuk sel neoplasia seperti nukleus besar, pleomorfisma nukleus ketara, nisbah nuklear

kepada sitoplasma tinggi, hiperkromasia dan hiperplasia sel epithelium dan stroma. Semua sifat ini menyerupai apa yang terdapat pada kanser payudara. Analisis immunohistokimia menunjukkan kehadiran ER-α dan EPOR pada tumor kelenjar mama DMBA-teraruh yang juga menyerupai kanser payudara manusia. Analisis elektron mikroskop transmisi terhadap tumor menunjukkan pewujudan bersama apoptosis, nekrosis dan aponekrosis yang mungkin boleh diguna dalam penentuan perkembangan tumor kelenjar mama dan kanser payudara peringkat awal.

Sebagai kesimpulan, gabungan enzim berkaitan hati, MMP-2 serum dan perubahan histologi, pernyataan ER-α dan EPOR, bukti apoptosis, nekrosis dan aponekrosis mungkin boleh jadi panel bagi penyaringan dan penentuan untuk peringkat awal tumor kelenjar mama bagi pesakit-pesakit berisiko tinggi untuk kanser.

Fasa kedua kajian ini melibatkan pembentukan tumor kelenjar mama xenograf-teraruh pada tikus sebagai ganti kepada kaedah drug-teraruh biasa. Tumor kelenjar mama xenograf-teraruh adalah stabil dan penghasilan tumor berlaku dalam masa yang singkat. Model xenograf ini seterusnya digunakan untuk menilai kesan rHuEPO, Tamoxifen dan gabungan Tamoxifen-rHuEPO terhadap pertumbuhan tumor kelenjar mama dan angiogenesis. Dalam kajian ini, 24 ekor tikus dibahagikan kepada empat kumpulan terdiri daripada enam ekor setiap kumpulan. Setiap ekor tikus diperlakukan secara oral: Kumpulan 1 diberi 60 IU

rHuEPO; Kumpulan 2 diberi 20 mg Tamoxifen; Kumpulan 3 diberi gabungan 20 mg Tamoxifen dan 60 IU rHuEPO; Kumpulan 4 diberi 1 mL 0.9% larutan salina normal dan bertindak sebagai kawalan. Keputusan kajian menunjukkan rHuEPO tidak menggalakan pertumbuhan tumor kelenjar mama malah mungkin meningkatkan lagi kesitotoksikan Tamoxifen melalui perangsangan kesan proapoptosis dan antiproliferatif. Kajian ini mencadangkan rawatan rHuEPO bagi pesakit kanser bukan hanya berfaedah untuk meringankan anemia disebabkan penyakit tersebut, tetapi juga memperkuatkan kesan kesitotoksikan beberapa drug kemoterapi tertentu yang diguna dalam rawatan kanser.

ACKNOWLEDGEMENTS

I would sincerely like to extend my greatest and deepest appreciation to my main supervisor, Prof. Dr. Rasedee Abdullah for his guidance, advice and encouragement which he unselfishly gave throughout the course of this study. I would also like to thank to my co-supervisors, Prof. Dato Dr. Sheikh Omar Abdul Rahman and Assoc. Prof. Dr. Rozita Rosli for their suggestions and support.

Sincere gratitude and appreciation are forwarded to Dr. Hazilawati Hamzah, Mrs. Fauziah Nordin, Mr. Halmi Othman, Mr. Abdullah Misron, staff in Hematology Laboratory of Faculty of Veterinary Medicine for their technical assistance during this study. Thanks also forwarded to staff in Electron Microscopy and Imaging Unit, Institute of Bioscience (IBS) and to all my friends in UPM.

Last but not least, I would like to extend my extreme gratitude to my sporting and understanding loving husband, Mr. Mohd. Fadhil Ahmad, my inspired children, Faiz Isqandar and Aleya Yasmin, my supportive parents and family members for their constant support and encouragement.

I certify that an Examination Committee has met on **13 August 2009** to conduct the final examination of **Sairah Abdul Karim** on her **Doctor of Philosophy** thesis entitled Characterisation of Induced Rat Mammary Gland Tumor and the Antitumor Effect of Recombinant Human Erythropoietin and Tamoxifen 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 Doctor of Philosophy.

Members of the Examination Committee are as follows:

Mohd Hair Mohd Bejo, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rani Bahaman, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Examiner 1)

Mohd Ali Rajion, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Examiner 2)

Yasmin Hanum, PhD
Associate Professor,
Universiti Kebangsaan Malaysia
External Examiner,

_____
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Rasedee Abdullah, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Dato' Sheikh Omar Abdul Rahman
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Rozita Rosli, PhD
Associate Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledge. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

_____

SAIRAH ABDUL KARIM

Date: 25 November 2008

TABLE OF CONTENTS

LIST OF FIGURES

13	Mammary tumour section from a female Sprague-Dawley rat with mild hyperplasia (H&E, x400)	
14	Mammary tumour section from a female Sprague-Dawley rat with focal necrosis (H&E, x200)	
15	Normal mammary gland tissue of Sprague-Dawley rat showing single layer of epithelial cells surrounding each tubules (H&E, x200)	
16	Immunohistochemical staining of ER-α in normal mammary gland tissue shows negative immunostaining in the epithelial cells of the tubules (DAB)	
17	Immunohistochemical staining of ER-α in the mammary tumor from a female Sprague-Dawley rat showing strong intensity immunostaining (brown) (DAB)	
18	Immunohistochemical staining of ER-α in the mammary tumor from a female Sprague-Dawley rat showing medium intensity immunostaining (brown) (DAB)	
19	Immunohistochemical staining of EPOR in the normal mammary gland tissue from a female Sprague-Dawley rat showing nuclear staining in several cells (arrows) and classified as level 1 (DAB, x400)	
20	Immunohistochemical staining of EPOR in the mammary tumor from a female Sprague-Dawley rat showing homogenous nuclear staining (DAB)	
21	Immunohistochemical staining of EPOR in the mammary tumor from a female Sprague-Dawley rat showing strong nuclear staining of tumor cells with granular appearance (arrow) (DAB)	
22	Immunohistochemical staining of EPOR in the mammary tumor from a female Sprague-Dawley rat showing cell with cytoplasmic EPOR staining (arrow) and granular or non-homogenous appearance in most of the tumor cells (DAB)	

23	Mammary tumor tissue from female rat showing cells pleomorphism with perinuclear chromatin condensation suggesting the cell undergoing apoptosis and the presence of multiple nucleoli. The amorphous dense masses (arrows) are necrotic cells.	
24	Mammary tumor tissue from female rat showing aponecrotic degeneration (red arrow). The cytoplasm is scanty and contained very few organelles, variable-sized vacuoles (yellow arrow) and disrupted mitochondria. The nucleus still intact and the nuclear envelope convoluted and irregular. Heterochromatin is prominent at the perinuclear region (green arrow).	
25	Mammary tumor tissue section from female rat shows swelling cell with relatively large nucleus and a prominent nucleolus (N). The cytoplasm contains numerous variable sized of vacuoles and defective cristae of mitochondria are noted (arrows).	
26	Mammary tumor tissue from female rat showing bizarre nuclear shape with an indentation and the cytoplasm contains numerous cisternae rough endoplasmic reticulum (rER) (arrows).	
27	Mammary tumor tissue from female rat showing a cell with very distinctive multiple nucleoli (N) and indented nuclear membrane (arrows).	
28	Mammary tumor tissue from female rat showing transverse section of eosinophil which can be recognized by the presence electron dense-stripe crystals with cytoplasmic projection (arrows) or pseudopodias.	
29	Mammary tumor tissue from female rat showing a plasma cell (centre) with eccentrically-positioned nucleus and very extensive rough endoplasmic reticulum (rER) in the cytoplasm. Mast cell (left) is characterised by the presence of large dense granules.	
30	A female Sprague-Dawley rat with mammary tumor induced by xenograft technique. Note the tumor is localized at the thoracic region (arrow)	

LIST OF PLATES