



UNIVERSITI PUTRA MALAYSIA

**FUNCTIONAL CHARACTERIZATION OF CALRETICULIN IN INVASIVE
HUMAN BREAST CANCER**

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HUMAN BREAST CANCER**

By

MOHAMMADREZA ZAMANIAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
in Partial Fulfilment of the Requirements for the Degree of PhD**

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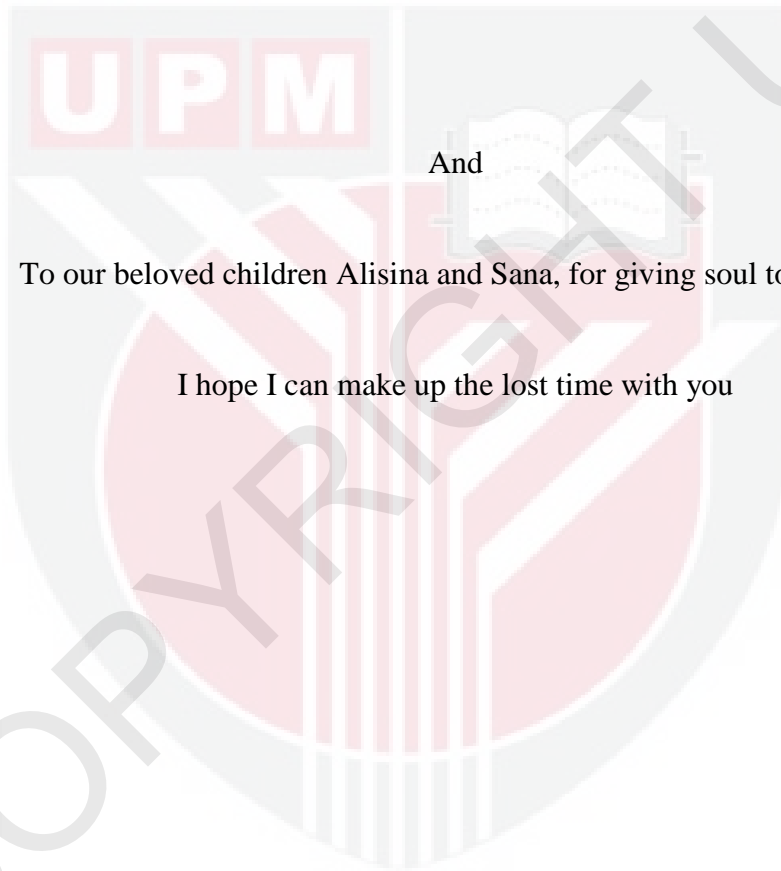
In dedication to:

My beloved wife Zeinab, who has supported me in all of my life events, particularly
in raising the decision to change our future

And

To our beloved children Alisina and Sana, for giving soul to our life

I hope I can make up the lost time with you



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in partial fulfilment of the requirement for the degree of PhD

FUNCTIONAL CHARACTERIZATION OF CALRETICULIN IN INVASIVE HUMAN BREAST CANCER

By

MOHAMMADREZA ZAMANIAN

October 2011

Chairperson : Professor Rozita Rosli, PhD

Faculty : Medicine and Health Sciences

Breast cancer is the second leading cause of cancer deaths in women. Invasion is an important hallmark of cancer that confers the ability of cells to metastasize and spread to other sites of the body. Thus, finding novel molecular biomarkers of invasion and metastasis is integral for the advancement of breast cancer management. Calreticulin (CRT) is a multifunctional endoplasmic reticulum protein. CRT is a major player in intracellular calcium storage and transfer. It also plays a role in other cellular functions such as chaperoning and adhesion. Previous studies have tried to correlate CRT overexpression with tumorigenesis. For example, CRT has been postulated to be a contributing factor in the role of thrombospondin-1 in invasion. CRT has also been shown to interact with Estrogen Receptor- α and revert hormone-independent inhibition of breast cancer cell invasion.

The main objective of this study was to functionally characterize the possible role(s) of CRT in breast cancer cell invasion phenotype. The specific objectives of the study were to (i) compare CRT protein expression in different invasive states of human breast cancer tissues, (ii) determine CRT-dependent migratory and invasive

potentials of breast cancer cells and (iii) establish the CRT-dependent gene regulatory pathways.

To confirm the CRT-associated breast cancer pattern of expression, immunohistochemistry analysis of formalin fixed paraffin-embedded breast cancer tissues was conducted. Using siRNA technology, we developed a CRT-knockdown model of MCF7 breast cancer cell line. We evaluated the CRT genotype-phenotype correlations using migration and transwell invasion assays as well as cell-cycle analysis. Western blotting and quantitative-real time polymerase chain reaction (qRT-PCR) assays were used to determine the level of CRT expression, while β -actin was used as the housekeeping gene. Finally, microarray-based global gene expression profiling was conducted to dissect the possible CRT pro-invasive regulatory pathways.

Meta-analysis of the immunohistochemical results confirmed that the expression of CRT was significantly higher ($p < 0.05$) in the stromal compartments of malignant tissues as compared to those from non-malignant samples. Subsequently, successful siRNA-mediated CRT gene silencing in MCF7 cells was achieved and this was confirmed both at mRNA and protein levels by qRT-PCR and western blot, respectively. Consequently, using migration and transwell invasion assays, the migratory and invasive potentials of CRT-deficient cells were compared to CRT-expressing cells. A significant loss in the migratory and invasive potentials was evident in CRT-deficient cells ($p < 0.05$). Finally, global gene expression profiling successfully identified various gene networks involved in CRT breast cancer signaling.

Hence, besides confirming CRT overexpression in invasive breast cancer tissues, we demonstrated the CRT-dependent pro-invasive potential and signaling pathways *in vitro*. Future work will focus on defining the mechanistic role of invasion and characterizing the possible CRT-dependent molecular targets as diagnostic, prognostic and therapeutic biomarkers of breast cancer.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah PhD

**PENCIRIAN FUNGSI CALRETICULIN DALAM KANSER PAYUDARA
INVASIF MANUSIA**

Oleh

MOHAMMADREZA ZAMANIAN

Oktober 2011

Pengerusi : Profesor Rozita Rosli, PhD

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Kanser payudara ialah punca kematian kedua yang utama di kalangan wanita. Pencerobohan merupakan suatu ciri penting kanser yang membolehkan sel-sel membesar dan merebak ke bahagian badan yang lain. Oleh sebab itu, pencarian penanda bio molekular yang baru bagi pencerobohan dan perebakan penting dilakukan demi kemajuan dalam rawatan kanser payudara. Calreticulin (CRT) ialah protein retikulum endoplasma yang mempunyai pelbagai fungsi. CRT berperanan utama dalam penyimpanan dan perpindahan kalsium intrasel. CRT juga memainkan peranan dalam fungsi-fungsi sel yang lain seperti pengirangan dan pelekatan. Kajian-kajian yang lepas telah cuba untuk menghubungkan antara ekspresi berlebihan CRT dengan tumorigenesis. Sebagai contoh, CRT telah dipostulat sebagai faktor penyumbang kepada peranan thrombospondin-1 dalam pencerobohan sel. CRT juga telah dibuktikan berinteraksi dengan reseptor estrogen- α dan membalikkan perencatan yang tidak bergantung kepada hormon dalam pencerobohan sel kanser payudara.

Objektif utama kajian ini adalah untuk mencirikan fungsi peranan-peranan CRT yang mungkin dalam fenotip pencerobohan sel kanser payudara. Objektif spesifik kajian ini adalah untuk (i) membandingkan ekspresi protein CRT pada peringkat yang berbeza dalam pencerobohan tisu kanser payudara, (ii) menentukan potensi perpindahan dan pencerobohan sel-sel kanser payudara yang bergantung pada CRT dan (iii) menentukan laluan pengawalan gen yang bergantung pada CRT.

Bagi memastikan ekspresi kanser payudara yang berkait dengan CRT, analisis imunohistokimia telah dijalankan pada tisu kanser payudara yang diawet dengan formalin dan dibenamkan dalam parafin. Dengan menggunakan teknologi siRNA, kami telah membentuk model knockdown CRT bagi titisan sel kanser payudara MCF7. Kami telah menilai hubungkait genotip-fenotip CRT dengan menggunakan ujian perpindahan dan pencerobohan transwell dan juga analisis kitaran sel. Ujian mendapan Western dan tindak balas berantai polimerase masa nyata kuantitatif (qRT-PCR) telah dilakukan untuk menentukan tahap ekspresi CRT dan β -aktin telah digunakan sebagai gen pengawal atur. Akhir sekali, pemprofilan ekspresi gen secara global berasaskan jujukan mikro telah dijalankan untuk menentukan laluan pengawalan CRT berkemungkinan yang cenderung mencerooboh.

Analisis meta imunohistokimia telah mengesahkan bahawa ekspresi CRT adalah lebih tinggi secara signifikan ($p < 0.05$) di dalam bahagian stroma tisu malignan berbanding dengan sampel yang bukan malignan. Seterusnya, penyenyapan gen CRT melalui pengantaraan siRNA telah berjaya dilakukan di dalam sel MCF7 dan ini telah dipastikan pada peringkat mRNA dan protein masing-masing melalui qRT-PCR dan mendapan Western. Berikutan itu, potensi perpindahan dan pencerobohan sel-sel

yang kekurangan CRT dibandingkan dengan sel-sel yang mengekspresikan CRT dengan menjalankan ujian perpindahan dan pencerobohan transwell. Penurunan potensi perpindahan dan pencerobohan pada sel-sel yang kekurangan CRT adalah signifikan ($p < 0.05$). Akhir sekali, pemprofilan ekspresi gen global telah mengenalpasti pelbagai jaringan gen yang terlibat dalam pengisyaratan CRT kanser payudara.

Dengan itu, selain mengesahkan ekspresi CRT yang tinggi di dalam tisu kanser payudara invasif, kami juga telah menunjukkan potensi dan laluan secara in vitro yang bergantung pada CRT yang cenderung invasif. Penyelidikan pada masa hadapan akan lebih ditumpukan dalam pentakrifan peranan mekanistik proses pencerobohan dan pencirian sasaran molekular yang bergantung pada CRT yang mungkin sebagai penanda bio kanser payudara yang diagnostik, prognostik dan terapeutik.

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I certify that a Thesis Examination Committee has met on 31st October 2011 to conduct the final examination of Mohammadreza Zamanian on his thesis entitled “Identification and evaluation of calreticulin in invasive human breast cancer” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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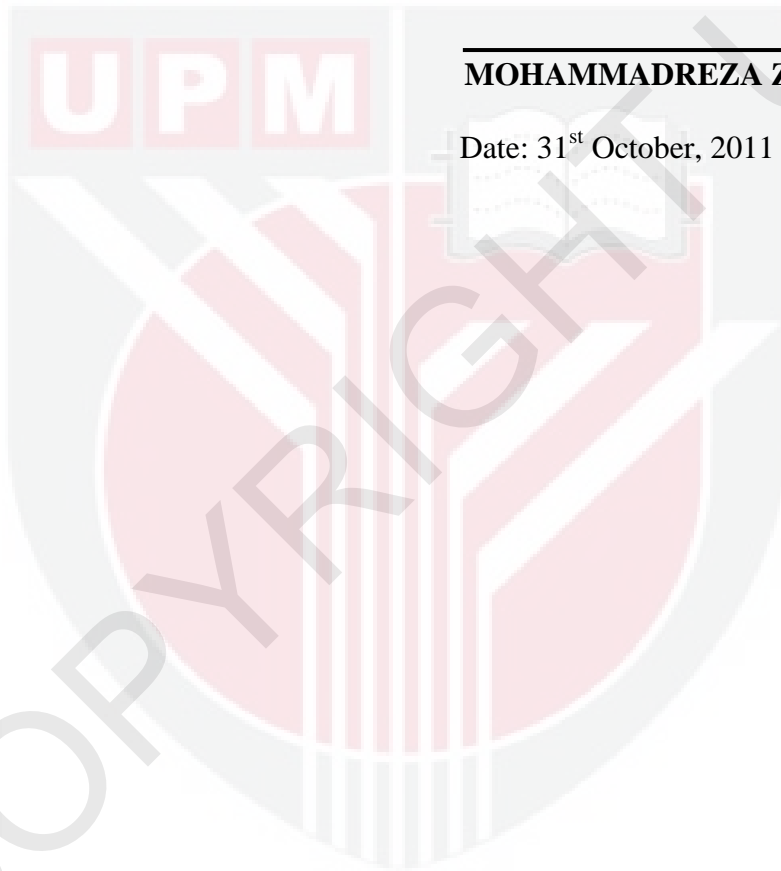
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DECLARATION

I declare that the thesis is 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 Universiti Putra Malaysia or other institutions.



MOHAMMADREZA ZAMANIAN

Date: 31st October, 2011

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