ORIGINAL ARTICLE

Detection of Calreticulin as a Candidate Prognostic Biomarker in Invasive Breast Carcinoma from a Biological Scaffold-Based 3D Co-culture System

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ABSTRACT

Introduction: Breast cancer is the most common cancer in women and the world's second leading cause of death in women, after lung cancer. Calreticulin (CRT), an endoplasmic reticulum (ER) multipurpose protein, has been proposed as a potential biomarker for breast cancer. However, reports on the correlation between CRT expression and cell invasiveness in breast cancer micro-tissues are scarce. Thus, in the current study, we analyzed the potential correlation between CRT and invasiveness of breast cancer in a biological scaffold-based 3D co-culture system. **Methods:** MCF7, MDA-MB-231 and MCF-10A breast cell lines were co-cultured in a 3-dimensional (3D) system with MRC-5 lung fibroblast cell line in the cell density ratio of 3:1. Thereafter, calreticulin gene and protein expression levels were determined based on quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and immunohistochemistry, respectively. Moreover, via RT-qPCR analysis, the gene expression levels of calreticulin-related candidate metastasis genes in breast cancer micro-tissues were carried out. **Results:** The results showed occasional foci of lumen-like morphology in the non-cancerous breast micro-tissues and the formation of solid clusters for breast cancer micro-tissues. Moreover, immunohistochemistry results revealed protein expression of calreticulin in non-cancerous and cancerous breast micro-tissues with cytoplasmic and nucleic acid localizations. It was found that PCMT1 and ER-α genes were significantly downregulated ($p < 0.01$) in invasive breast cancer micro-tissues. **Conclusion:** This study suggests that CRT and CRT-related candidate metastasis genes may potentially serve as prognostic biomarkers in invasive breast carcinoma.
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INTRODUCTION

Breast cancer remains a significant problem for women, and every year more than one million are affected by this disease worldwide (1). With the advancement in precision medicine and the availability of new preclinical models that simulate *in vivo* biology and microenvironmental factors, more prospective cancer biomarkers and highly precise drug targets have been recently discovered (2). Even though these standard prognostic markers are able to precisely predict the prognosis of approximately 30%, out of the remaining 70% of patients, approximately 30% still develop metastases. Therefore, new prognostic markers are urgently required to identify patients that are more likely to develop metastasis in order to expedite the adaptation of treatment strategies by oncologists.

Calreticulin (CRT), was reported to be overexpressed in breast ductal adenocarcinoma and linked to breast cancer invasiveness and metastasis in clinical studies (3, 4, 5). A previous clinical study conducted in our laboratory also revealed that CRT protein was overexpressed in breast tumour tissues compared to adjacent healthy tissues. Subsequently, the results on whole-genome sequencing of CRT-knockdown samples indicated several CRT-related candidate genes as possible contributors to breast cancer's emerging invasive phenotype (3). Therefore, in this study, CRT and its related candidate genes are proposed as potential prognostic biomarkers of invasive breast cancer (6). In particular, the potential of CRT and its related candidate genes as prognostic biomarkers was determined using a three-dimensional (3D) co-culture

system that mimics important tissue variables *in vivo*, which cannot be observed in the two-dimensional (2D) cell culture model (2). Indeed, based on several tumour organoids and matrix-based cell culture models, cancer cells in 3D settings are more phenotypically stable and similar with their parent cell lines than cancer cells grown in a 2D or monolayer setting (7). Furthermore, by taking tumour micro-environment into consideration, drug resistance in cancer cells grown in 3D co-culture and *in vivo* settings are also similar. Therefore, it is necessary to investigate the potential of calreticulin and its related candidate genes as prognostic biomarkers of breast cancer in a 3D co-culture system to provide a more clinically relevant outcome.

MATERIALS AND METHODS

Cell Lines

In this study, human breast cancer (MCF7 and MDA-MB-231), human mammary epithelial (MCF-10A) and human fetal lung fibroblast (MRC-5) cell lines were obtained from the ATCC (American Type Culture Collection). MDA-MB-231, MCF7, and MRC-5 cell lines were grown in Dulbecco's modified eagle's medium (DMEM) with high glucose; 4500mg/L (Biosera, France) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biosera, France) and 1% (v/v) penicillin/streptomycin (Fisher Scientific). MCF-10A cell line was grown in DMEM/F12 with high glucose (Biosera, France) supplemented with 5% (v/v) horse serum (Biosera, France), 20 ng/mL epidermal growth factor (Thermo Fisher Scientific), 0.5 mg/mL hydrocortisone, 10 µg/mL insulin (Merck, USA) and 1% penicillin/streptomycin.

3D Co-culture System

MCF-7, MDA-MB-231, and MCF-10A cell lines were each co-cultured with MRC-5 cell line in a cell density ratio of 3:1 further seeding equally in 4-well plates pre-coated matrigel (Becton Dickson, USA). Prior to cell seeding, the well was coated with 170 µL of 50% matrigel and incubated at 37°C for 45 minutes to allow gel formation. Afterward, the cells were transferred to the wells with 5% matrigel-medium mixture. The cultured cells were seeded on Matrigel-coated wells for ten days, with daily replacements of the matrigel-medium mixture. This period of 3D cell culture maintenance was chosen considering the limited capacity of the 4-well plate in which cell confluency is achieved at a cell density of 1 x 10^6 cells per well. The growth and morphology of cells cultured in matrigel was observed every day and photographed using an inverted microscope (Zeiss, Germany) at day three and day ten of development. At this stage, the co-cultured cells in a 3D setting are called as micro-tissues.

Sectioning and Slide Preparation

After ten days, the developed micro-tissues were rinsed in phosphate-buffered saline (PBS) and then immediately fixed by immersion in cold 4% paraformaldehyde (PFA) in PBS, for four hours at room temperature. Then, the micro-tissues were rinsed in PBS again and transferred to 1 mL sterile 1.5% (w/v) agarose. Excess agarose was removed, and the agarose blocks were further dehydrated in 10% PFA. The samples were sent to the histology laboratory at the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) for embedding in paraffin wax and sectioning. For the sectioning, Reichert-Jung 2045 Multicut Rotary Microtome instrument was used to prepare serial sections of 4 µm thickness.

Histopathological and Immunohistological Assays

Two sets of all samples (2 replicates x 3 samples) were subjected to histopathological and immunohistochemical analyses. For histopathological analysis, the micro-tissue sections were stained with hematoxylin and eosin, and the patterns of developed breast micro-tissues were examined. Next, to determine the protein expression of CRT in the micro-tissues, the sections were subjected to immunohistochemistry (IHC) using a monoclonal antibody PAB486Hu02 (Cloud-Clone, USA) against calreticulin. To eliminate the presence of non-specific binding and false-positive results, 3D co-culture micro-tissues were used for preparing the negative control and followed the same IHC staining protocol except without the addition of the CRT antibody. On the other hand, kidney tissue slides were used as positive control, as suggested in the antibody datasheet. The positive and negative controls were prepared for each run of IHC staining.

Gene Expression Profiling

In the present study, RT-qPCR gene expression profiling was used to ascertain the expression level of CRT and CRT-related candidate metastasis genes in 3D co-culture samples. The matrigel scaffold of developed 3D co-culture samples was digested with 360 µL/well of CellSperse™ (Cultrex®, USA) solution followed by incubation at 37°C for up to two hours for maximum dissolution. Subsequently, 2µL of 10 mM Ethylenediaminetetraacetic acid (EDTA) was added to each sample to stop CellSperse™ reaction. After centrifugation at 1000 rpm, the cells were washed with PBS and then subjected to total RNA extraction using the Analytik Jena innuPREP RNA mini kit (Fermentas, Germany) as per manufacturer's instructions. RNA samples were then reverse transcribed into cDNA using the SensiFAST™ cDNA synthesis kit (Bioline, USA). The reactions were prepared according to the manufacturer,s protocol, gently mixed and briefly centrifuged. For cDNA synthesis, the reactions were incubated for 10 minutes at 25°C, followed by 15 minutes at 42°C, and was terminated by heating at 85°C for 5 minutes in a thermal cycle (Bio-Rad, USA). The synthesized cDNA were subjected to quantitative PCR (Instrument) using the following settings: PCR was performed using nine sets of primers (IDT, USA) for CRT and its related candidate genes. As for the reference gene (GAPDH), the primer was commercially obtained from Genecopoeia, USA.

Following qPCR, relative gene expression of nine genes, with normalization to GAPDH, was determined via the ΔΔCt value calculation.

Statistical Analysis

Data analysis in this study was performed using IBM®Statistical Package for the Social Sciences (SPSS) software version 22. For determining statistically significant difference between samples and genes of interest, one-way analysis of variance (ANOVA) was used. In addition, to determine the correlation between CRT and its related candidate genes, two-tailed Pearson correlation was employed. The p-values of less than 0.01 were considered as statistically significant.

RESULTS

Morphological Examination of Breast Micro-Tissues

In the present study, tumorigenic and non-tumorigenic breast cells were co-cultured with fibroblast cells in a 3D setting to mimic the breast microenvironment in an *in vivo*-like condition. To observe the growth of cells, the images of the samples were captured on the third and tenth days where the third day was considered the beginning of the development, and the tenth day is the end of the time point of this experiment.

Based on Figure 1, circular micro-tissues were successfully developed for each breast cell line (MCF7, MDA-MB-231 and MCF-10A) co-cultured with MRC-5 cells. At both day 3 and 10, the sizes of MCF-10A/MRC-5 (Figure 1(a) and 1(b)), MCF7/MRC-5 (Figure 1(c) and 1(d)), and MDA-MB-231/MRC-5 (Figure 1(e) and 1(f)) circular micro-tissues increased in ascending order. Due to the larger size of MDA-MB-231/MRC-5 micro-tissues, the micro-tissues was concentrated on a specific region of the matrigel scaffold (Figure 1(e) and 1(f)).

Histological Examination of Breast Micro-Tissues

Haematoxylin and eosin staining was performed to observe the morphology of spheroidal breast micro-tissues. MCF-10A/MRC-5 micro-tissues developed hollow, lumen-like formations (Figure 2(a)) whereas both MCF7/MRC-5 and MDA-MB-231/MRC-5 micro-tissues adopted clusters or masses of cells. In Figure 2(b), it is apparent that lumen-like formation was lost as MCF7/MRC-5 cells form solid and tightly packed clusters. On the other hand, lumen-like formations in MDA-MB-231/MRC-5 micro-tissues was also evident, but to a lesser extent compared to MCF-10A/MRC-5 micro-tissues (Figure 2(c)).

CRT Protein Expression

Via immunohistochemistry analysis, CRT protein was found to be expressed in all non-cancerous and cancerous breast micro-tissues (Figure 3). In addition, CRT protein was expressed in the cytoplasm as well

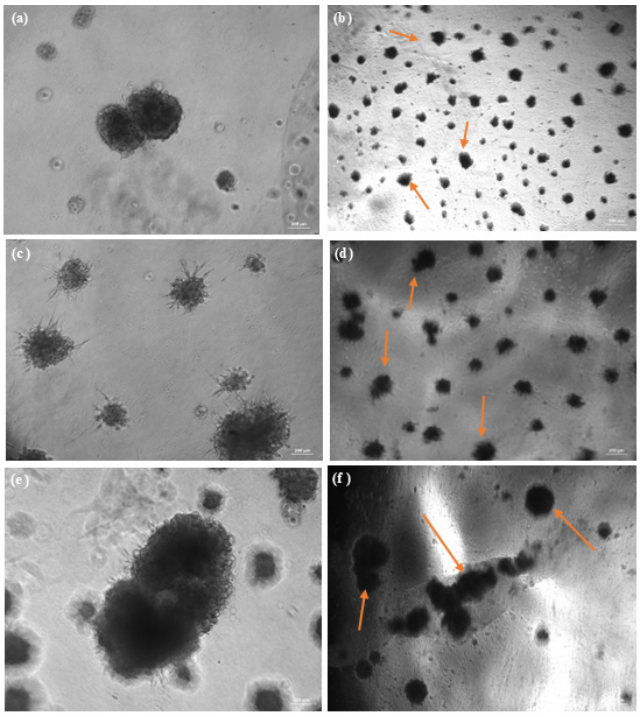

Figure 1: The structure of 3D co-cultured micro-tissues during day 3 and day 10. Panels (a) and (b) show MCF-10A/MRC-5 3D co-cultured micro-tissues. Panels (c) and (d) illustrate MCF7/MRC-5 3D co-culture micro-tissues. Panel (e) and (f) portray MDA-MB 231/MRC-5 3D co-culture cells. The orange arrows indicate some of the developed micro-tissues in the Matrigel scaffold. Day 3 panels, 2(a), 2(c) and 2(e) were captured at 200X magnification while day 10 panels, 2(b), 2(d) and 2(f) were captured at 100X magnification.

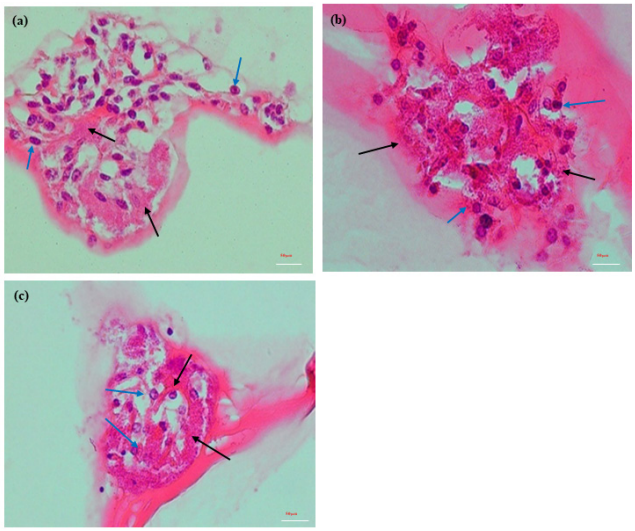

Figure 2: H&E staining results of breast micro-tissues. Panel (a) illustrates the MCF-10A/MRC-5 co-cultured sample, Panel (b) shows MCF-7/MRC-5, and panel (c) portrays MDA-MB 231/MRC-5 3D co-culture sample, Magnification 400X. The blue arrows indicate the nuclei whereas the black arrows show the cytoplasm. The MCF-10A co-cultured micro-tissues (a) has more lumen-like morphology compared to the breast cancer micro-tissues ((b) and (c)).

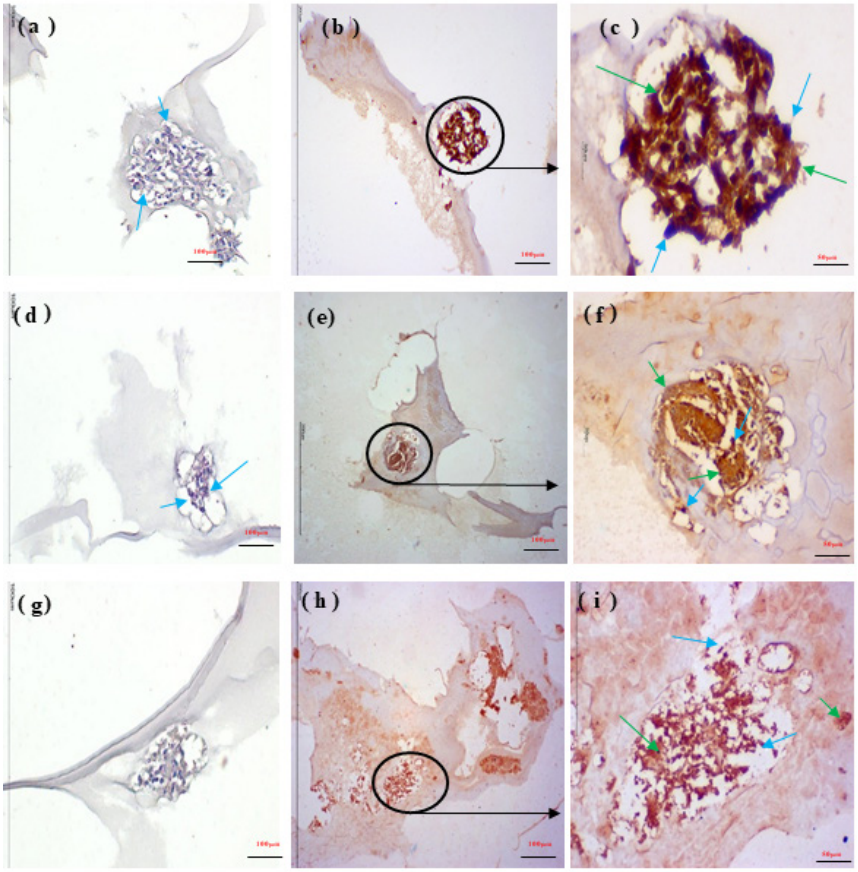

Figure 3: Immunohistochemistry results for CRT expression in 3D co-cultured micro-tissue samples. Panels (a), (d), and (g) depict the negative control for MCF-10A/MRC-5, MCF7/MRC-5 and MDA-MB 231/MRC-5 respectively which demonstrated the positive nuclei stained in proper shape of the micro-tissue sample, Magnification 100X. Panels (b), (e), and (h) show MCF-10A/MRC-5, MCF-7/MRC-5 and MDA-MB 231/MRC-5 IHC stained 3D co-culture micro-tissues, Magnification 100X. Panels (c), (f), and (i) represent panels (b), € and (h), respectively with magnification of 400x. The blue arrows indicate irregular distributed nuclei and the green arrows show higher expression of CRT protein in developed micro-tissue samples. CRT protein was expressed in all breast micro-tissues with cytoplasmic and nuclei localizations. Based on the intensity of the staining, CRT protein expression was higher in the MCF10A/MRC-5 co-cultured micro-tissues.

as the nuclei. The detection of strong brown staining indicates a higher expression level of CRT protein. Therefore, based on the intensity of the staining, in general, the protein expression of CRT was higher in MCF-10A/MRC-5 micro-tissues than in the breast cancer microtissues (MCF7/MRC-5 and MDA-MB-231/MRC-5). However, since scoring of the slides was not performed, a direct comparison of patterns and intensity of CRT protein expression between non-cancerous and cancerous micro-tissues was not possible.

Gene Expression Profiling

As shown in Figure 4, compared to the non-cancerous breast micro-tissues (MCF-10A), the relative gene expression levels of CRT and eight candidate genes were lower in breast cancer micro-tissues (MCF7 and MDA-MB-231). Out of eight genes, the differential expression levels between non-cancerous (MCF-10A) and invasive (MDA-MB-231) breast micro-tissues were only statistically significant for PCMT1 and ER-Alpha ($p < 0.01$). The relative gene expression levels of *PCMT1, FABP3, SERPINB5, PSMD10, RNASE1* and *ER-Alpha* were lower in invasive breast (MDA-MB-231) than non-invasive breast (MCF7) micro-tissues. Vice versa, the relative expression levels of *CRT, MINA* and *NUDT6* genes were higher in the invasive breast micro-tissues However, the differential expression levels between

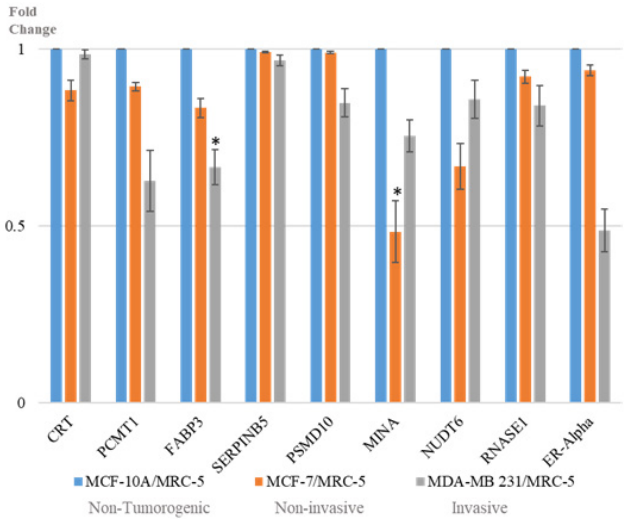

Figure 4: Expression of CRT and CRT related genes of MCF10A/MRC-5, MCF-7/MRC-5 and MDA-MB231/MRC-5 co-cultured cells. Relative expression of CRT gene and CRT-related candidate metastasis genes in tumorigenic and non-tumorigenic co-culture cells (* = $p < 0.01$ as analyzed by independent t-test) and the difference of fold changes between two groups of invasive and non-invasive 3D co-culture breast cancer cells portray in this chart (* = $p < 0.01$ as analyzed by one-way ANOVA). (n=3 of 2 independent biological replicates).

non-invasive (MCF7) and invasive (MDA-MB-231) breast cancer micro-tissues were only statistically significant for *MINA, FABP3* and *ER-alpha* genes.

Pearson's correlation coefficient analysis was used in this study to determine the correlation between the gene expression levels of CRT and its related candidate metastasis genes. CRT gene expression was strongly correlated with candidate metastasis genes in developed 3D co-culture breast cancer cells with Pearson correlation values of +1.00.

DISCUSSION

In this study, gene and protein expression levels of calreticulin (CRT) in non-cancerous, non-invasive and invasive breast micro-tissues were determined, together with the expression levels of eight candidate metastasis genes. The main aim of current study was to determine whether the expression levels of calreticulin in a 3-D co-culture system were similar with the clinical settings, as previously reported. In this study, to mimic the *in vivo* tumour microenvironment of breast cancer, a heterotypic 3D culture model was utilized. In particular, epithelial non-cancer or breast cancer cells were co-cultured with MRC-5 non-cancerous lung fibroblast cells with Matrigel. Matrigel is composed of the ECM proteins that are normally present in the basement membrane (i.e., laminin, collagen IV, heparin sulfate proteoglycan, and nidogen/entactin). Importantly, the 3D culture of breast cancer spheres in Matrigel reflects the established tumour molecular subtype classification. For an instance, in Matrigel-based 3D cultures, triple-negative cell lines including MDA-MB-231 cells generally develop invasive network structures, while luminal A subtypes such as MCF7 cells form round or grape-like colonies (8, 9). These *in vitro* morphological characteristics of breast cancer cells resemble the breast cancer tissues which used clinical samples of breast cancer patients, reassuring that Matrigel is aptly suitable to be used to represent the complex *in vivo* tumour microenvironment of the breast.

MCF-10A/MRC-5 micro-tissues developed lumen-like, hollow formations as previously reported by Zamanian (3) as well as Pinto, et al. (10). Therefore, this finding reflects the morphology of the normal mammary gland. On the other hand, MCF7 cell spheroids were reported to adopt the formation of solid and dense clusters or masses (11). Indeed, in our study, hollow formations in MCF7/MRC-5 micro-tissues were considerably lesser than the non-cancerous micro-tissues (MCF-10A). Moreover, as indicated by the intensity of H&E staining, the MCF7/MRC-5 micro-tissues formed dense clusters.

As for invasive breast cancer, Zamanian (3) reported that morphological features of invasive ductal breast carcinoma based on histological examinations were highly variable between clinical specimens. In particular, invasive breast cancer cells were arranged in different patterns such as cords, clusters and trabeculaes. Likewise, there was a lack of consistency in stromal invasion and lumen-like formations. However, this is not surprising since invasive and metastastic breast cancer is often heterogenous. In the current study, the presence of central hollow formations in MDA-MB-231/MRC-5 micro-tissues was observed but to a lesser extent than the non-cancerous breast micro-tissues (MCF-10A). Vice versa, the micro-tissues also had the tendency to form solid clusters but to a lesser extend than the non-invasive breast cancer micro-tissues (MCF7). Regardless, in general, based on histological examinations of the micro-tissues, the *in vitro* morphological characteristics matched those of *in vivo* and clinical breast specimens and according to the subtypes of breast cancer.

Following the histological examinations, the protein expression of CRT in the breast micro-tissues was determined. Early studies detected the expression of CRT in the extracellular matrix of developing rat teeth in which CRT plays an essential role in mineralization. Moreover, in pancreatic tumours as well as invasive breast tumours, CRT expression was consistently localized to the cytoplasm (3, 12). Zamanian (3) also reported that in non-cancerous breast tissues, CRT was expressed in the glandular and stromal areas. Although CRT was mostly expressed in the cytoplasm, CRT was also present in the nuclei and cell membrane. Interestingly, similar pattern was also observed in invasive breast cancer tissues. However, in the stromal area, CRT expression was significantly lower in the non-cancerous breast lesions. On the other hand, there was no significant difference of CRT expression between different stages and grades of invasive breast cancer lesions.

Based on the IHC examination, although the staining was more intense in the non-cancerous breast micro-tissues (MCF-10A), direct comparison between the micro-tissues was not possible since scoring of the slides was not made. Moreover, there were challenges in the current study particularly in the histological and immunohistochemistry examinations. First, the fragility of the 3D co-culture micro-tissues made slicing and preparing of the micro-tissue sections more challenging. Second, there was no protocol available for semi-quantitation of IHC results using 3D co-culture micro-tissue samples. Third, compared to MCF10A/MRC-5 and MCF7/MRC-5 co-culture cells, MDA-MB- 231/MRC-5 co-culture cells required a longer growth duration with fewer micro-tissues in amount. Finally, with 3D cell culture models, it is difficult to obtain spheroids of similar size, which may possibly lead to high variability of IHC results.

Aside from the CRT protein expression, the gene expression levels of CRT in the micro-tissues were also determined. CRT gene expression was upregulated in various cancers including hepatocellular carcinoma

and colon adenocarcinoma (13,14). Furthermore, few studies have confirmed that the presence of CRT induced invasive and metastatic activities in several cancers such as breast adenocarcinoma (15), gastric cancer (16) and oesophageal squamous cell carcinoma (17). Therefore, it is not surprising that CRT has been numerously proposed as a prognostic biomarker. However, in the present study, the gene expression levels of CRT in the micro-tissues were similar with its protein expression. In particular, the gene expression levels of CRT in the cancerous breast micro-tissues (MCF7 and MDA-MB-231) were lower than non-cancerous breast micro-tissues (MCF10A), but the difference was not significant. Previous studies that reported the gene expression of CRT in 3D and 3D co-culture of breast cell lines were extremely limited, therefore it is not certain if the obtained results were as expected.

However, according to Zamanian (3), silencing of CRT in MCF7 cell line revealed that several candidate genes were differently expressed, and thus may be associated or correlated with CRT. In this study, we narrowed down into eight candidate genes that are involved in cancer metastasis. This is because, in the previous study, whole genome profiling was conducted following migration and invasion analyses of the CRT-silenced MCF7 cells, in which enhanced cell migration and invasion are the hallmarks of cancer metastasis.

In the current research, *PCMT1* and *ER-alpha* were significantly downregulated in the invasive breast cancer micro-tissues (MDA-MB-231) compared to the non-cancerous breast micro-tissues (MCF-10A). This finding was not expected since high expression of *PCMT1* was reportedly involved in tumour invasion and progression (18). Indeed, Zamanian (3) also reported that *PCMT1* was downregulated following knockdown of CRT in MCF7 cells. On the other hand, the downregulation of *ER-Alpha* gene in the invasive breast cancer micro-tissues was expected since *ER-Alpha* gene serves as a tumour suppressor. Most types of cancer displayed downregulations of *ER-Alpha* gene such as breast cancer (19), cervical cancer (20) and malignant prostatic epithelial cells (21). In this study, the gene expression profiling of *ER-Alpha* showed similar results with previous reports.

Similar to *ER-alpha, FABP3* gene is known as one of the vital tumour suppressors in breast cancer (22). In this study, the *FABP3* gene showed a tumour suppressor function by its significant decrease in invasive 3D co-culture breast cancer cells compared to non-invasive breast cancer cells. Although up-regulation of *FABP3* in MDA-MB 231 compared with MCF-10A was reported (23), the results of *FABP3* profiling in the present study are in agreement with most of the previous studies, including Zamanian (3).

Likewise, in the present study, the expression of the *MINA* gene, also known as *RIOX2*, was significantly lower in non-invasive breast cancer cells and showed an oncogene function in 3D co-culture breast cancer samples. *MINA* gene was reported to potentially be an oncogene, based on its overexpression, for various types of cancers (24). Thus, higher expression of the *MINA* gene in MDA-MB 231/MRC-5 co-culture cells might indirectly influence the malignancy of the cells. However, Zamanian (3) reported downregulation of *MINA* following knockdown of CRT in MCF7 cells. This made sense since calreticulin gene expression was also found to be lower in non-invasive breast cancer micro-tissues (MCF7), suggesting the correlation between CRT and *MINA*. Therefore, *MINA* (*RIOX2*) could have a prognostic value as a biomarker in different kinds of cancers, as previously suggested (25). Several candidate genes had significant differential expressions between non-cancer and cancerous breast micro-tissues and were in correlation with calreticulin, However, a better understanding of the related functions of the CRT gene and CRT-related candidate metastasis genes as prognostic markers of invasive breast cancer is required. To achieve this, future studies will require a larger number of samples to confirm these genes as reliable prognostic indicators in invasive breast cancer. The studies should also focus on revealing the mechanisms behind these correlations and their potential benefits in the area of breast cancer management, such as personalized medicine and targeted therapies. In terms of 3D culture, the alternative use of polymer scaffolds instead of fragile matrigel scaffolds can provide a more stable environment for developing the 3D culture micro-tissues. This will allow better differentiation in the morphology and expression of CRT protein in invasive and non-invasive breast cancer micro-tissues for both H&E staining and IHC procedures. Western blot analysis can also be used to validate the expression of the CRT protein in non-tumorigenic and tumorigenic 3D co-culture cells. Finally, to further define the expression of CRT protein in 3D cultivated micro-tissues, the optimum growth time period for the various cell lines used should be determined.

CONCLUSION

To our knowledge, this is the first reported study to identify CRT as a prognostic biomarker for breast cancer progression from a biological scaffold-based 3D co-culture system. The data presented suggest that CRT and CRT-related candidate metastasis genes may be novel targets for therapeutic intervention for invasive breast cancer patients. However, further optimization is needed to recapitulate a better CRT expression in 3D culture.

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