CMBES/SCGB

2016 CMBEC39 Conference
Calgary AB
May 24–27, 2016

FLUID FLOW STIMULATION UPREGULATES EXPRESSION OF S100 GENES DURING BREAST CANCER DEVELOPMENT AND PROGRESSION

Kenneth Fuh[1,2,4], Jessica Withell[2], Robert Shepherd[2,3], Kristina Rinker[2,3,4,5,6,7]
[1]Biomedical Engineering Graduate Program; [2]Cellular & Molecular Bioengineering Research Lab, [3]Department of Chemical & Petroleum Engineering; [4]Arnie Charbonneau Cancer Institute, [5]Department of Physiology & Pharmacology; [6]Libin Cardiovascular Institute of Canada; [7]Centre for Bioengineering Research & Education; University of Calgary, Calgary, Alberta. T2N 1N4. Canada.

ABSTRACT

S100 proteins are intracellular calcium ion sensors that participate in a wide range of tumorigenic processes. In spite of several S100 genes being overexpressed in breast cancer, their roles during disease development remain elusive. Human mammary epithelial cells (HMECs) can be exposed to fluid shear stresses which likely alter gene expression profiles. The implications of these interactions to normal tissue function and potential breast cancer development have not been previously studied.

The goal of this study was to analyze expression profiles of S100 genes upon exposing HMECs to fluid flow, with significant findings examined in clinical datasets. S100 genes were among the most upregulated genes with flow exposure. Overexpression of S100 genes were also observed in tissue from patients with early stage breast cancer, and in most breast cancer patients suggesting roles during disease development. Survival analyses revealed reduced survival times for patients with elevated expression of S100P and S100A7.

This study shows that exposing HMECs to fluid flow upregulates genes identified clinically to be overexpressed during breast cancer development and progression, including S100A7 and S100P. These findings show S100 genes are flow-responsive and participate in a fundamental adaptation pathway in normal tissue that is also active in breast cancer.

INTRODUCTION

The S100 gene family in humans consists of 21 members that regulate cellular responses by acting both as intracellular calcium ion sensors and as extracellular factors[1,2]. Overexpression of S100 genes have been implicated in a wide range of tumorigenic processes, including cell proliferation, angiogenesis, metastasis and immune invasion[1,3]. Overexpression of S100 genes, particularly S100P and S100A7, has been observed in several types of cancers, including breast carcinomas[4,5]. However, their roles during breast cancer development and progression have not been well defined[1].

Human mammary epithelial cells (HMECs) can be exposed to fluid shear stresses resulting from blood flow and lymphatic drainage, which potentially alter gene expression patterns and phenotypic profiles. The objective of this study was to analyze changes in expression profiles of S100 genes upon exposing HMECs to fluid flow. Differentially expressed genes were mapped onto a human interactome on the Cytoscape software platform, and used to extract pronounced interactions between static and flow-exposed cells. Finally, the prognostic values of several overexpressed genes were evaluated in expression profiles from three independent breast cancer clinical datasets.

MATERIALS AND METHODS

Cell culture and flow experiments

HMECs were obtained from Lonza (Walkersville MD, USA) and cultured as recommended. Cell monolayers were cultured on glass plates pre-coated with Rat Tail collagen I (Life Technologies Inc., ON, Canada) and grown to confluency. A bioreactor system consisting a parallel-plate flow chamber was used to expose triplicates of HMECs to an average shear stress of 1 Pa for 20 hours[6],

simulating exposure of cells to blood flow breast ducts and lobules. Flow was provided by a Masterflex peristaltic pump and tubing (Cole Parmer, Montreal, QC, Canada). Some plates were grown as static controls and media was replaced at the same time as flow was set up.

RNA extraction and gene expression analysis

Total RNA from both flow exposed cells and cells grown as static controls was isolated using the EZNA Total RNA Kit (Omega Bio-tek, Inc., Norcross, GA. USA). RNA was assayed using the Quant-iT™ Ribogreen RNA Assay Kit (Life Technologies Inc., Burlington ON, Canada) and standard curves generated using a microplate reader. Differentially expressed genes were quantified using Affymetrix Primeview microarrays, and analyzed with Partek Genomics Suite Version 6.6 (Partek Incorporated, Missouri, USA). Differentially expressed genes were those with fold changes greater than or equal to 2.0 and p-values less than 0.01. Microarray data was validated by real time quantitative polymerase chain reaction (qPCR), with beta-2-microglobulin used as the reference gene. PCR was carried out on a ViiA 7 Real Time PCR System (Life Technologies, Foster City CA, USA), and relative expression calculated using the comparative cycle threshold method[7].

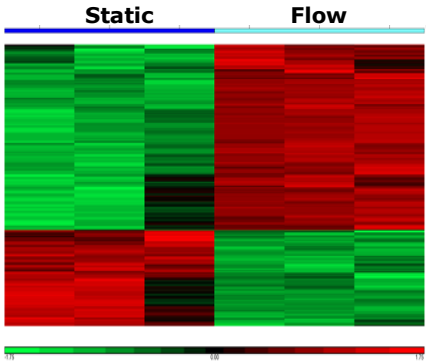

Fig. 1: Heat map of differentially expressed genes between static and flow-exposed HMECs. Fold change ≥ 2.0 and $p \leq 0.01$.

Gene patterns from microarray data were used to extract pronounced gene features by network analysis on the Cytoscape software platform[8,9]. Differentially expressed genes between flow-exposed and statically grown cells were mapped onto a human interactome obtained from integrated complex traits networks (iCTNet)[9].

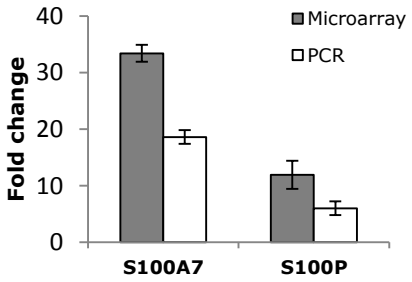

Fig. 2: S100A7 and S100P were among the most upregulated genes upon flow exposure both in microarrays and quantitative PCR. $p \leq 0.01$.

Clinical patient data analysis

Gene expression profiles from 5 healthy breast tissues and 9 ductal carcinoma in situ (DCIS) patients were analyzed to evaluate expression of S100 genes during early stages of breast cancer[16]. Prognostic value of S100P and S100A7 were evaluated via a breast tumor microarray data set from 1,809 patients, using Kaplan – Meier survival curves[9]. Expression data from The Cancer Genome Atlas (TCGA) RNA – Seq database[10] was also analyzed for differential expression of S100 genes.

RESULTS

Fluid flow affects gene expression in human mammary epithelial cells

Microarray data showed unique clusters of differentially expressed genes in flow-stimulated and unstimulated HMECs (Fig. 1), with more than 1200 genes being differentially expressed upon flow exposure. To identify key molecular functions and biological processes that were significantly affected by flow exposure, we performed Gene Ontology enrichment analysis using microarray data. Gene subsets involved in cellular processes involved in breast cancer development and progression, such as cellular response to TGF-β stimulus, regulation of EMT, cell adhesion, proliferation and motility were profoundly enriched upon flow exposure (data not shown).

S100 genes are upregulated in flow-stimulated HMECs and during early stages of breast cancer

S100 genes were among the most upregulated genes with flow stimulation; S100P and S100A7 were overexpressed more than 5 fold (Fig. 2). Overexpression of S100 genes was

also observed in DCIS patient tissue compared to healthy breast (Fig. 3), suggesting potential roles during early stages of breast cancer.

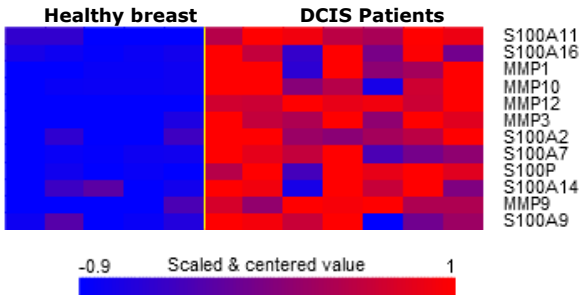

Fig. 3: S100 genes are involved during early stages of breast cancer development. Several S100 genes were upregulated in ductal carcinoma in situ (DCIS) patient tissue compared to healthy breast. $p \leq 0.01$.

Network analysis revealed interactions between S100 genes, including an interaction between S100A7 and S100P (data not shown). Interestingly, S100P was upregulated in several breast cancer cell lines upon flow stimulation, suggesting a potential role of fluid flow during breast cancer progression (Fig. 4).

Expression profiles of S100P and S100A7 in clinical datasets

To investigate the relevance of our findings to progression of breast cancer, we analyzed gene expression data from 517 patients obtained from TCGA database. We observed overexpression of S100P in patients with all subtypes of breast cancer and overexpression of S100A7 in patients with luminal B, HER2 positive and basal breast cancer (Fig. 5).

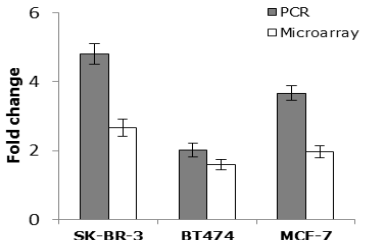

Fig. 4: S100P is also overexpressed when breast cancer cells are exposed to fluid flow in our bioreactor system. $p \leq 0.01$.

Finally, the prognostic value of S100P and S100A7 was evaluated in a microarray data set of breast tumors from 1,809 patients[11]. Kaplan – Meier curves of relapse-free survival times of breast cancer patients with lymph node positive status (n=936), stratified by S100P and S100A7 expression levels, demonstrate that elevated expression correlates with reduced survival times (Fig. 6).

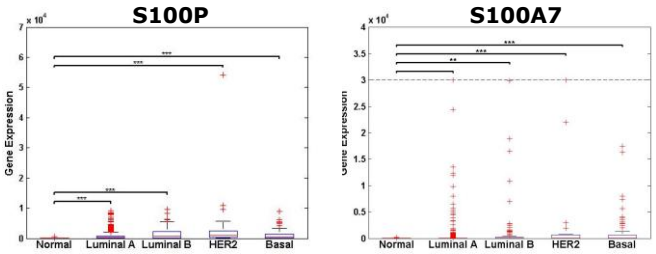

Fig. 5: Relative expression of S100P and S100A7 between healthy volunteers and patients with different stratifications of breast cancer. Expression data was obtained from TCGA and consisted of 104 healthy volunteers, 317 luminal A, 93 luminal B, 26 HER2 and 81 basal patients. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

DISCUSSION AND CONCLUSION

Breast cancer is caused by genetic mutations that bestow cells lining breast ducts or lobules with the ability to grow abnormally. These cancerous cells can eventually spread to nearby lymph nodes or other parts of the body in a process called metastasis[10,12]. Development and progression of this complex disease involves mechanical interactions between epithelial cells lining ducts or lobules and associated microenvironments, including exposure to fluid flow from either blood supply or lymphatic drainage[13,14,15]. The potential implications of this interaction in the context of breast cancer development have not been previously investigated. In this study, we used a bioreactor system to model exposure of HMECs to fluid flow and quantified changes in gene expression. Our findings suggest fluid flow has a role in breast cancer development by upregulating genes in the S100 family.

Two S100 genes, S100P and S100A7 were among the most upregulated genes in flow-stimulated cells. S100P and S100A7 have previously been implicated in tumor development due to their roles in cell adhesion, motility and proliferation[3,4]. Overexpression of S100A7 in pre-invasive early stage cancer has also been reported[1]. Compared to expression in healthy breast, tissue from patients presenting with early stage breast cancer showed elevated expression of S100 genes, supporting our position for these genes to be involved during

breast cancer development. Interestingly, S100P was also upregulated in three breast cancer cell lines upon flow stimulation, implying potential roles of fluid flow exposure during disease progression. Further evidence for this stance was obtained through overexpression of S100P and S100A7 in patients with most subtypes of breast cancer. Survival plot analysis revealed reduced survival times for patients with elevated expression levels of S100P and S100A7.

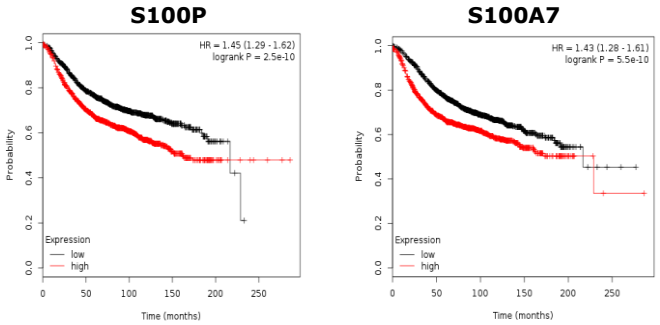

Fig. 6: Relapse-free survival analysis of breast cancer patients with lymph node positive status (n=936), stratified by S100P and S100A7 expression respectively. Data was obtained from http//kmplot.com/analysis. Statistical significance was determined by the log-rank test.

Network analysis revealed key interactions through which overexpressed S100 genes potentially contribute to tumorigenesis. This suggests that flow-induced upregulation of S100 genes in HMECs is possibly a fundamental adaptation pathway in normal tissue that is also active in breast cancer. However, the activated signaling cascades and underlying mechanisms through which fluid flow exposure potentially enhances breast cancer progression remain elusive. The knowledge gained from such investigations could lead to new diagnostic and therapeutic approaches for early stage breast cancer.

This study shows that exposing HMECs to fluid flow upregulates genes clinically identified to be overexpressed in breast cancer patient profiles, including S100P and S100A7. This suggests that our bioreactor platform is a useful tool for identifying how mammary epithelial cell exposure to blood flow affects early stages of breast cancer progression.

ACKNOWLEDGEMENTS

We acknowledge the Arnie Charbonneau Cancer Institute Microarray Facility for performing microarray experiments and Zymetrix (University of Calgary) for the ViiA 7 PCR System. This work was supported by grants from Alberta Innovation and Advanced Education, Canada Foundation for Innovation, Natural Sciences and Engineering Research Council of Canada (to KR) and Alberta Innovates Technology Futures (to KF).

REFERENCES

1. Bresnick R., Weber J. & Zimmer, D. B. S100 proteins in cancer. *Nat. Rev. Cancer* **15,** 96–109 (2015).
2. Gibadulinova A., Tothova V., Pastorek J. & Pastorekova, S. Transcriptional regulation and functional implication of S100P in cancer. *Amino Acids* **41,** 885–892 (2011).
3. Jiang, H. *et al.* Calcium-binding protein S100P and cancer: Mechanisms and clinical relevance. *J. Cancer Res. Clin. Oncol.* **138,** 1–9 (2012).
4. Arumugam T. & Logsdon D. S100P: A novel therapeutic target for cancer. *Amino Acids* **41,** 893–899 (2011).
5. Zhu, L. *et al.* Upregulation of S100P, receptor for advanced glycation end products and ezrin in malignant melanoma. *J. Dermatol.* **40,** 973–979 (2013).
6. Shepherd, R. D., Kos, S. M. & Rinker, K. D. Flow-dependent Smad2 phosphorylation and TGIF nuclear localization in human aortic endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **301,** H98–H107 (2011).
7. Livak J. & Schmittgen D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔ C(T)) Method. *Methods* **25,** 402–408 (2001).
8. Lopes T. *et al.* Cytoscape Web: an interactive web-based network browser. *Bioinformatics* **26,** 2347–2348 (2010).
9. Wang L., Khankhanian P., Baranzini E. & Mousavi P. iCTNet : A Cytoscape plugin to produce and analyze integrative complex traits networks. *BMC Bioinformatics* **12,** 380 – 386 (2011).
10. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* **490,** 61–70 (2012).
11. Győrffy B. *et al.* An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. *Breast Cancer Res. Treat.* **123,** 725–731 (2010).
12. Carey K A. and Lisa A. C. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. *Clin Breast Cancer.* **9,** 73–81 (2010).
13. Koumoutsakos, P., Pivkin, I. & Milde, F. The Fluid Mechanics of Cancer and Its Therapy. *Annu. Rev. Fluid Mech.* **45,** 325–355 (2012).
14. Mitchell, M. J. & King, M. R. Computational and experimental models of cancer cell response to fluid shear stress. *Front. Oncol.* **3(44),** 1–11 (2013).
15. Swartz M. & Lund W. Lymphatic and interstitial flow in the tumour microenvironment: linking mechanobiology with immunity. *Nat. Rev. Cancer* **12,** 210–219 (2012).
16. Seth, A., Kitching R., Landberg G., Xu J., Zubovits J., Burger A. Gene expression profiling of ductal carcinomas in situ and invasive breast tumors. *Anticancer Res.* **23(3A),** 2043 – 2051. (2003).