The Breast 20 (2011) 413–418

Contents lists available at ScienceDirect

The Breast

journal homepage: www.elsevier.com/brst

Original article

Calpastatin is associated with lymphovascular invasion in breast cancer

Sarah J. Storr [a], Rabab A.A. Mohammed [a,1], Caroline M. Woolston [a], Andrew R. Green [b], Tim Parr [c], Inmaculada Spiteri [d], Carlos Caldas [d], Graham R. Ball [e], Ian O. Ellis [b], Stewart G. Martin [a,*]

[a] Academic Oncology, University of Nottingham, School of Molecular Medical Sciences, Nottingham University Hospitals NHS Trust, City Hospital Campus, Nottingham NG5 1PB, UK
[b] Histopathology, University of Nottingham, School of Molecular Medical Sciences, Nottingham University Hospitals NHS Trust, City Hospital Campus, Nottingham NG5 1PB, UK
[c] University of Nottingham, Division of Nutritional Sciences, Sutton Bonnington Campus, Leicestershire LE12 5RD, UK
[d] CR-UK Cambridge Research Institute, Li Ka Shing Centre, Robinson Way, Cambridge CB2 0RE, UK
[e] John van Geest Cancer Research Centre, School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, UK

ARTICLE INFO

Article history:
Received 6 January 2011
Received in revised form
22 March 2011
Accepted 7 April 2011

Keywords:
Breast cancer
Lymphovascular invasion
Metastasis
Calpastatin
Calpain

ABSTRACT

Metastasis of breast cancer is a major contributor to mortality. Histological assessment of vascular invasion (VI) provides important prognostic information and demonstrates that VI occurs predominantly via lymphatics in breast cancer. We sought to examine genes and proteins involved in lymphovascular invasion (LVI) to understand the mechanisms of this key disease process. A gene expression array of 91 breast cancer patients was analysed by an Artificial Neural Network (ANN) approach using LVI to supervise the analysis. 89 transcripts were significantly associated ($p < 0.001$) with the presence of LVI. Calpastatin, a specific calpain inhibitor, had the second lowest selection error and was investigated in breast cancer specimens using real-time PCR ($n = 56$) and immunohistochemistry ($n = 53$). Both calpastatin mRNA and protein levels were significantly associated with the presence of LVI ($p = 0.014$ and $p = 0.025$ respectively). The data supports the hypothesis that calpastatin may play a role in regulating the initial metastatic dissemination of breast cancer.

Introduction

The major contributor to mortality in cancer is the ability of the tumour to metastasise, with vascular invasion (VI) being an important initial step. In breast cancer, recent evidence has shown that VI occurs primarily via lymphatic vessels with 96% being lymphovascular invasion (LVI) and only 4% blood vessel invasion (BVI).[1] The presence of LVI is an independent prognostic indicator of recurrence and death from the disease.[1] With such clinical relevance there is a need to understand the mechanisms of LVI, in the hope of identifying both biomarkers and therapeutic opportunities.

It is unclear what causes preferential LVI in breast cancer; however it has been postulated that the lack of continuous basement membrane and pericytes which surround blood capillaries or more sparse cellular junctions in lymphatic vessels may make entry easier.[2] However, the expression of lymphatic specific adhesion molecules or up-regulation of adhesion molecules has been shown to influence LVI suggesting that there may be mediators of LVI in addition to the influence of vessel structure.[3] Furthermore, tumour cells can migrate specifically towards lymphatics by both Vascular Endothelial Growth Factor-C (VEGF-C) autocrine and C–C chemokine receptor 7 dependent paracrine mechanisms.[4] Both lymphatic and blood endothelial cells have been subjected to gene expression profiling with results demonstrating that tumour endothelium are distinct at the molecular level from their respective normal counterparts.[5,6] In gastric cancer there are a number of genes potentially associated with LVI, including oligophrenin I and ribophorin II.[7] In breast cancer certain proteins have been shown, via IHC, to correlate with LVI, including VEGF-C, COX-2, and Bcl-2.[8,9] Attempts to use gene expression patterns to predict the presence of LVI have met with limited success, principally due to substantially overlapping clusters.[10]

To understand the microenvironment in breast cancer we compared microarray expression data from patients with and without LVI. We investigated the transcript identified from the microarray profile with the second lowest selection error, calpastatin, with a combination of real-time PCR (QPCR) and immunohistochemistry (IHC).

* Corresponding author. Tel.: +44 0 115 823 1846; fax: +44 0 115 823 1849.
E-mail address: stewart.martin@nottingham.ac.uk (S.G. Martin).
[1] Present address: Department of Pathology, Faculty of Medicine, Assiut University, Assiut, Egypt.

0960-9776/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.breast.2011.04.002

Materials and methods

Clinical samples

All clinical specimens were retrieved from the Department of Histopathology, Nottingham City Hospital. This project was approved by Nottingham Ethics Committee 2 with 20 year clinical follow up information available for analysis. Nottingham Local Ethics Committee waived the need for written informed consent. Formalin fixed paraffin embedded tissue was used for IHC. Fresh-frozen breast tissue was used for RNA extraction for both microarray analysis and QPCR. The mean age (±standard deviation) at diagnosis was 56.2 ± 9.0 for gene expression profiling, 52.7 ± 10.2 for QPCR and 52.9 ± 9.2 for IHC. Additional clinical characteristics for the three sample cohorts are shown in Table 1. QPCR was conducted on lymph node (LN) negative patients where LVI has been used to stratify patients into prognostic groups. The groups for the microarray analysis and QPCR studies had one overlapping sample and can be considered as independent groups for verification. The microarray analysis and IHC studies had 21 overlapping samples. LVI status of patient samples was assessed using IHC techniques as described previously.[1] Data on a wide range of biomarkers of known clinical relevance were available including oestrogen receptor (ER), progesterone receptor (PR) and basal phenotype status which was categorised as the expression of cytokeratin 5/6 and/or cytokeratin 14.[11,12] This study is reported according to REMARK criteria.[13]

Table 1
Clinicopathologic criteria of patients from the three sample cohorts.

Variables	Gene Expression ($n = 91$)	Real-time PCR ($n = 56$)	IHC ($n = 53$)
Age (mean ± SD), years	56.2 ± 9.0	52.7 ± 10.2	52.9 ± 9.2
Age			
≤40 years	5 (5.5%)	6 (10.7%)	8 (15.1%)
>40 years	86 (94.5%)	50 (89.3%)	45 (84.9%)
Tumour Size			
≤2 cm	66 (72.5%)	33 (58.9%)	34 (64.2%)
>2 cm	25 (27.5%)	23 (41.1%)	19 (35.8%)
Stage Classification			
I	60 (65.9%)	55 (98.2%)	41 (77.4%)
II	23 (25.3%)	1 (1.8%)	7 (13.2%)
III	8 (8.8%)	0 (0%)	5 (9.4%)
Grade Classification			
I	25 (27.5%)	7 (12.5%)	12 (22.6%)
II	40 (44.0%)	19 (33.9%)	14 (26.4%)
III	26 (28.6%)	30 (53.6%)	27 (50.9%)
LN Status			
Negative	61 (67.0%)	56 (100%)	39 (73.6%)
Positive	30 (33.0%)	0 (0%)	12 (22.6%)
Unknown	0 (0%)	0 (0%)	2 (3.8%)
Nottingham Prognostic Index			
Good <3.4	37 (40.7%)	17 (30.4%)	18 (34.0%)
Intermediate 3.4–5.4	50 (54.9%)	39 (69.6%)	31 (58.5%)
Poor >5.4	3 (3.3%)	0 (0%)	4 (7.5%)
Lymphovascular Invasion			
Absent	58 (63.7%)	28 (50%)	26 (49.1%)
Present	33 (36.3%)	28 (50%)	27 (50.9%)
Unknown	0 (0%)	0 (0%)	0 (0%)
ER Status			
Negative	35 (38.5%)	24 (42.9%)	18 (34.0%)
Positive	53 (58.2%)	29 (51.8%)	33 (62.3%)
Unknown	3 (3.3%)	3(5.4%)	2 (3.8%)
PR Status			
Negative	30 (33.0%)	30 (53.6%)	22 (41.5%)
Positive	54 (59.3%)	22 (39.3%)	26 (49.1%)
Unknown	7 (7.7%)	4 (7.1%)	5 (9.4%)

Microarray expression analysis

Gene expression was conducted on a cohort of 91 breast carcinoma patients as described previously.[14–16] Briefly RNA was isolated from tissue and RNA integrity was assessed using an Agilent 2100 Bioanalyzer and labelled with biotin using the Illumina TotalPrep RNA Amplification kit (Ambion). 1.5 μg biotin labelled cRNA was used for each hybridisation on Sentrix Human-6 BeadChips (Illumina). Illumina gene expression data were processed and summarised using Illumina BeadStudio Software. Analyses of the probe level data were performed using the beadarray Bioconductor package. The data are deposited in ArrayExpress at the EBI with the accession number E-TABM-576 (http://www.ebi.ac.uk/miamexpress/).

Identification of genes associated with LVI

Microarray data was analysed using a supervised ANN, using LVI as the supervising variable. ANNs were used to develop optimised predictive models using subsets of genes that could predict LVI status for a blind cohort. The ANN was conducted using a supervised approach with multi-layer perceptron architecture and sigmoidal transfer function, where weights were updated by a back propagation algorithm.[17,18] A random sample cross-validation approach was used where data was split into three subsets, 60% for training, 20% for testing and 20% for validation to allow confidence intervals to be generated. The final model contains transcripts that significantly associate with LVI.

Real-time reverse transcription PCR (QPCR)

QPCR was used to verify gene expression data in a cohort of 56 breast patients; 28 LVI negative and 28 LVI positive. RNA was isolated from 8 μm tissue sections using TRIZOL reagent (Invitrogen). RNA was quantified by absorbance at 260/280 nM using a NanoDrop and immediately reverse transcribed. 1 μg of total RNA was reverse transcribed using oligo(dT)$_{15}$ priming and the Reverse Transcription System (Promega) according to the manufacturers protocols. Following reverse transcription cDNA was stored at −80 °C prior to PCR. QPCR was conducted using SYBR premix Ex Taq II containing Ex Taq HS, dNTPs, Mg^{2+} and SYBR green I (Takara Bio) and a Stratagene MX3000p real-time cycler (Stratagene). A standard curve was included with each run and all samples were run in duplicate. Reaction conditions were 95 °C for 2 min, prior to 40 cycles of 95 °C for 5 s, 59 °C for 30 s and 72 °C for 15 s. Finally the reaction was held at 95 °C for 1 min followed by 55 °C for 30 s and 95 °C for 30 s. Calpastatin splice variants containing exon 3 and endogenous control gene HPRT specific primers were designed: calpastatin exon 3 sense primer 5′-AACCAGCAAGTCTTCCAGTATGAA-3′ and calpastatin exon 3 anti-sense primer 5′-GTCCTTCCATCTGTTGGCTGA-3′ (71 base pair product), HPRT sense primer 5′-AAATTCTTTGCTGACCTGCTG-3′ and HPRT anti-sense primer 5′-TCCCCTGTTGACTGGTCATT-3′ (122 base pair product). The calpastatin primers spanned an exon junction with the sense primer being on exon 2 (common to the predominantly expressed calpastatin mRNAs) and anti-sense primer on exon 3. A critical threshold cycle (Ct) value was determined for each reaction which reflects the cycle number at which DNA was first detected. Relative transcript expression ratios were calculated where $E = 2^{-\Delta\Delta Ct}$, where E is the expression ratio and ΔΔCt is the difference in crossing points between HPRT and calpastatin exon 3-containing mRNA for each sample relative to the mean expression of the total sample cohort.