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Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer

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Summary

Purpose. Inflammatory breast cancer (IBC) is the most aggressive form of locally advanced breast cancer (LABC). The IBC phenotype is characterized by an infiltrative growth pattern, increased (lymph)angiogenesis and the propensity to invade dermal lymphatics. In pancreatic cancer, interactions between caveolin-1 and RhoC GTPase, a key molecule in causing the IBC phenotype, regulate tumour cell motility and invasion. In this study we sought to investigate the role of caveolin-1 and -2 in IBC cell lines and in human IBC samples.

Experimental design. Differential methylation techniques identified the methylation status of the caveolin-1 and -2 promoters in human mammary epithelial cells (HMECs) and the SUM149 cell line. In cell line experiments, caveolin-1 and -2 mRNA and protein expression were compared in HMECs, MCF10A, the SUM102 non-IBC cell lines and 2 IBC cell lines (SUM149 and SUM190). Furthermore, caveolin-1 and -2 mRNA and protein expression were compared in human IBC and non-IBC samples using cDNA microarray, real-time qRT-PCR and immunohistochemistry. Results were correlated with RhoC protein expression data.

Results. In the SUM149 cell line, the caveolin-1 and -2 promoter sites were hypomethylated. A significantly increased expression of caveolin-1 and -2, both at the mRNA and protein level was found in IBC cell lines and in human samples of IBC: caveolin-1 and -2 mRNA were respectively 1.7 ($p = 0.02$) and 2.2 ($p = 0.03$) fold more expressed in IBC compared to non IBC and at the protein level, 41.4% of IBC specimens expressed either caveolin-1 or -2, compared to 15.6% of non-IBC specimens ($p = 0.03$). Furthermore a correlation was found between RhoC protein expression and caveolin-1 ($p = 0.1$) or caveolin-2 ($p = 0.09$) or either caveolin-1 or -2 protein expression ($p = 0.04$).

Conclusions. Although considered a tumour suppressor in breast cancer, we demonstrated overexpression of caveolin-1 and -2 in IBC cell lines and in human samples of IBC, most likely due to hypomethylation of their respective promoters. These results confirm the distinct molecular signature of IBC. Our data further suggest interaction between RhoC GTPase and the caveolins in IBC.

Introduction

Inflammatory breast cancer (IBC) is a distinct and aggressive subtype of locally advanced breast cancer (LABC). At time of diagnosis virtually all patients have lymph node involvement and 1/3 of the patients have distant metastases, resulting in a very poor prognosis. Even when receiving combined modality treatment including neo-adjuvant chemotherapy, less than 50% of IBC patients have an overall survival of more than 5 years [1]. The aggressive IBC phenotype is marked by a diffusely infiltrative rather than nodule-forming growth pattern, by increased angiogenesis and lymphangiogenesis [2, 3] and by the propensity of IBC to invade the dermal lymphatics and metastasize early in the development of the disease. It is the extraordinary invasiveness of the disease that suggests an important role for factors involved in cell motility and migration in the pathophysiology of IBC. Better understanding of the molecular mechanisms involved in the IBC phenotype can therefore lead to better understanding of the biology of breast cancer and metastasis 'an sich'.

Caveolin-1 is the major protein component of caveolae: specialized lipid rafts that are recognized in electron micrographs as 50–100 nm invaginations of the plasma membrane [4]. Although similar in distribution

and tissue expression to caveolin-1, caveolin-2 is less extensively studied. Many receptors and signal transduction molecules are concentrated within caveolae; therefore caveolae are proposed to be able to integrate and regulate cellular signalling pathways including GTPases (i.e. Ras and RhoA). Caveolin-1 has been shown to directly interact with small GTP binding proteins such as RhoA, a homologue of RhoC (95% identical) and with the epidermal growth factor receptor (EGFR) [5–6]. Abnormal EGFR expression and activation, as is often found in IBC, can lead to caveolin-1 phosphorylation and subsequent cell motility [7–9].

Although other Rho-family members are detected in human cancers, RhoC expression is associated with particularly aggressive human cancers [10–25]. RhoC has also been intimately linked to the unique IBC phenotype. RhoC is overexpressed and active in the majority of IBC and is involved in the motile, invasive, angiogenic and metastatic phenotype through mitogen activated protein kinase (MAPK) signaling. Overexpression of RhoC in HMECs recapitulated the IBC phenotype with regards to invasion, motility and angiogenesis [26–28]. Furthermore, in tumours smaller than 1 cm, RhoC expression is a good marker to identify breast cancer patients with increased risk of developing metastases [29].

Therefore, the aim of this study was to investigate the role of caveolin-1 and -2 in IBC. We studied the methylation status of the caveolin-1 and -2 promoters in IBC cell lines. Subsequently, the expression of caveolin-1 and -2 was compared, at the mRNA and protein level, in IBC and non-IBC cell lines and in human samples of IBC and non-IBC patients. Results were confronted with cDNA microarray experiments comparing IBC and non-IBC patient samples [30] and were correlated to RhoC protein expression levels [31].

Materials and methods

Cell line experiments

Culture conditions

The SUM190, SUM149 and SUM102 cell lines were maintained under defined culture conditions as previously described [32–35]. E6/E7 immortalized human mammary epithelial cells (HMECs: [36]) were grown in 5% FBS (Life Technologies, Gaithersburg, MD)-supplemented Ham's F12 medium (Life Technologies) containing insulin, hydrocortisone, epidermal growth factor and cholera toxin (Sigma Chemical Co., St. Louis, MO). MCF10A cells were maintained in serum free mammary epithelial growth medium (Clonetics, Rockland, ME) supplemented with cholera toxin. All cell lines were kept at 37 °C in a 90% air/10% CO_2 atmosphere.

Differential methylation analysis

Genomic DNA (gDNA) was isolated from the SUM149, HMECs and normal lymphocytes (isolated from the patient whose tumour the SUM149 cell line was isolated from) using Trizol (Invitrogen, Carlsbad, CA). Two µg aliquots of gDNA were digested with 20 units of each *Rsa*I, *Rsa*I + *Msp*I (a methylation insensitive restriction enzyme), or *Rsa*I + *Hpa*I (a methylation sensitive restriction enzyme) for 16 h at 37 °C. Restriction enzymes were heat inactivated for 20 min at 65 °C. The digested gDNAs were amplified by arbitrarily primed PCR (AP-PCR) according to the original published protocol [37] using a single-primer (AACCCTCACCCTAACCCCGG) or two primers (AACCCTCACCCTAACCGCGC + AACCCTCACAA CCCGCG). PCR reactions were performed in a final volume of 25 µl with the following components: 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM dNTPs, 2 µCi γ^{32}P-dATP (MP Biomedicals, Irvine, CA) , 25 pmol primer and 0.53 U Taq polymerase (Roche) at 94 °C for 30 s, 40 °C for 60 s and 72 °C for 90 s for 5 cycles followed by 94 °C for 15 s, 55 °C for 15 s and 72 °C for 60 s for 30 cycles. PCR products were separated on an 8 M urea 6% polyacrylamide gel, dried to filter paper and exposed to Kodak X-Omat film for 72 h at RT. Differentially methylated products were excised, eluted in TE buffer, reamplified by PCR, cloned into the pGEM-T Easy vector system (Promega, Madison, WI) and sequenced by the University of Michigan DNA sequencing core. Sequences were submitted to BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/).

Semiquantitative RT-PCR

Total RNA was harvested from actively growing cells at 60% confluence using Trizol Reagent (Invitrogen) and cDNA was made using the AMV-reverse transcriptase kit (Promega). Caveolin-1 and -2 transcripts were PCR amplified using 1:100 dilutions of caveolin specific primers; caveolin-1 forward 5′-CGTAGACTCGGAGGGA CATC-3′, reverse 5′-GATGCGGACATTGCTGAATA-3′, caveolin-2 forward 5′-ATGACGCCTACAGCCACCA CAG-3′, reverse 5′-GCAAACAGGATACCCGCAATG-3′. GAPDH was amplified as a loading control. PCR products were separated on a 1.2% TAE agarose gel and visualized by ethidium bromide staining (Sigma Chemical Co.). The intensity of ethidium bromide stained PCR products was measured using ImageJ (NIH shareware program) on a negative image. Caveolin message levels were calculated relative to GAPDH levels.

Western blot analysis

Cells were grown in 100 mm dishes until reaching 70% confluence. Protein was harvested with 500 µl RIPA buffer with the addition of 1 mM sodium orthovanadate, 0.3 mg/ml aprotinin, and 0.1 mg/ml phenylmethylsulfonyl fluoride. Aliquots of 50 µg were mixed with Laemmli buffer, heat denatured, separated by 12.5% SDS-PAGE and transferred to nitrocellulose. Nonspecific binding was blocked by 1 h incubation with 5% powdered milk in Tris-buffered saline with 0.05% Tween 20 (Sigma Chemical Co.). Immobilized proteins were probed using antibodies for caveolin-1 (clone 2297, Transduction Laboratories, BD Biosciences, San Diego,

CA) and caveolin-2 (clone 65, Transduction Laboratories). Levels of actin were determined as loading controls (actin antibody (C2), Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by ECL and exposed to hyperfilm (Amersham-Pharmacia Biotech, Piscataway, NJ). Densitometry was performed using ImageJ (NIH shareware program). Protein levels were calculated relative to actin levels.

Statistical analysis
Statistics were performed using Prism4 (Graphpad Software, San Diego, CA) graphing and statistics program. Mean relative caveolin-1 and -2 mRNA and protein expression were compared between cell lines, using One-Way ANOVA statistics and Bonferroni's *post hoc* testing.

Human tissue sample experiments

Tissue samples
All tumour specimens included in this study were obtained pre-treatment (before any neo-adjuvant chemo- and/or hormonal therapy) and after written informed consent. All protocols were reviewed and approved by the ethical committee of the General Hospital Sint-Augustinus. For the cDNA microarray and quantitative RT-PCR experiments, tumour specimens of 17 consecutive IBC and 20 control non-stage matched patients with breast cancer were collected and snap-frozen in liquid nitrogen. IBC cases were diagnosed by strictly respecting the criteria mentioned by the American Joint Committee on Cancer as T4d [38]. All IBC patients presented with a recently developed diffuse enlargement of the breast with redness and edema of more than one third of the skin of the breast. The presence of tumour emboli in dermal lymphatics was, as an isolated pathological finding, not sufficient for the diagnosis of IBC. The average age of the IBC patients was 59.9 years (range: 40.5–80.1 years). The non-IBC control population consisted of 6 T1, 4 T2, 7 T3 and 3 T4 breast tumours. The average age of the non-IBC patients was 61.6 years (range: 42.1–77.8 years). All but one IBC and all non-IBC resection specimens included for the qRT-PCR and cDNA microarray analysis were also included for the IHC analysis. Both groups were enlarged with 12 patients from whom no fresh frozen tissue was available. Clinico-pathological characteristics are listed in Table 1. Tumour size, lymph node status and estrogen receptor and progesterone receptor status were recorded by review of pathology files. Tumours were subtyped as ductal, lobular or special type (medullary, mucinous, tubular,...) carcinoma and histologically graded as I-III according to the Nottingham modification of the Bloom and Richardson histological grading scheme.

cDNA microarrays and Real-Time quantitative Reverse Transcriptase-PCR
Total RNA was extracted with the RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA). The Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to assess the quality of the RNA. The cDNA microarray experiments were performed as described before [30]. Briefly, one microgram of total RNA of each sample was reverse transcribed, amplified and labelled with Cy5 using the Amino Allyl MessageAmp aRNA Amplification Kit (Ambion, Woodward, TX). Reference RNA (Universal Human Reference RNA, Stratagene, La Jolla, CA) was processed accordingly and was labelled with Cy3. Sample and reference were competitively hybridized onto cDNA microarray slides obtained from the Sanger Center (Hixton, Cambridge, UK) as part of the Ludwig Institute for Cancer Research/Cancer Research United Kingdom (LICR/CRUK) Microarray Consortium. These slides were made by linking PCR products of cDNA clones on 3D-linked activated glass slides. Each array contained 10750 spots, representing 9932 sequence-validated cDNA elements of 6000 known and named human genes/ESTs. Slides were incubated 16 h at 47 °C, washed and scanned using ScanArray (Perkin–Elmer Life and Analytical Sciences, Boston, MA) software. Data were normalized, analysed as described before [30].

For the RT-PCR experiments, one microgram of total RNA was reverse transcribed into cDNA with random primers with High-Capacity cDNA Archive kit

Table 1. Clinico-pathological data of IBC and non-IBC patients

	Non-inflammatory breast carcinoma	**Inflammatory breast carcinoma**
N	32	30
Age, years – mean (range)	57.5 (33.0–80.5)	59.4 (33.0–80.1)
Lymph node status		
negative/positive	9/23	0/30
N0	9	0
N1	7	7
N2	4	14
N3	12	9
Tumour Size		
Small (< 2 cm)	10	*
Large (> 2 cm)	21	
Histology		
Ductal	24	27
Lobular	4	3
Special type	4	0
Grade		
I	8	1
II	15	11
III	9	18
ER		
Positive (> 10%)	21	11
Negative (< 10%)	11	19
PR		
Positive (> 10%)	15	9
Negative (< 10%)	17	21

* In IBC, exact tumour size cannot be assessed neither clinically, nor pathologically due to the diffuse enlargement of the breast and the diffusely infiltrative growth pattern of the tumour (ER, estrogen receptor; PR, progesterone receptor).

(Applied Biosystems, Foster City, CA). PCR primers and TaqMan probes targeting caveolin-1 and -2, 18S rRNA and β-actin were purchased as Assays-on-demand Products for Gene Expression (Applied Biosystems). Real-time quantitative reverse transcriptase-PCR was performed with the ABI Prism 7700 Sequence Detector (Applied Biosystems). 18S rRNA and β-actin were selected as internal controls for RNA input and reverse transcription efficiency with the TaqMan Human Endogenous Control Plate (Applied Biosystems). All PCR reactions were done in duplicate for both target genes and internal controls. Relative gene expressions were presented with the $2^{-\Delta\Delta Ct}$ method [39].

Immunohistochemistry

A representative formalin fixed paraffin embedded tissue block was selected, five-micrometer sections were cut, deparaffinised and rehydrated through graded alcohols. Antigen retrieval was performed by heating the slides in citrate buffer for 30 min using a warm water bath at 98 °C. Endogenous peroxidase was quenched for 10 min with peroxidase blocking reagent (DakoCytomation, Glöstrup, Denmark). Primary antibodies , anti-caveolin-1 (1:100; clone 2297; BD Biosciences Pharmingen, San Diego, CA) and anti-caveolin-2 (1:100; clone 65; BD Biosciences Pharmingen, San Diego, CA) were incubated for 60 min at room temperature. Antibody staining was visualized using the ChemMate Envision detection system (DakoCytomation). Sections were counterstained with hematoxylin and mounted for light microscopy. Caveolin-1 and -2 status of the tumour cells was evaluated by light microscopy as either positive (any tumour cell with immunohistochemical staining) or negative. IHC interpretation was done by 2 independent observers (GVdE and PV). Interobserver reproducibility was excellent (κ-value caveolin-1 and -2 : 0.91). For a few discrepant cases slides were reviewed and a consensus was reached.

From 46 of 62 tumour specimens, 15 IBC and 31 non-IBC, RhoC protein expression was determined using a tissue microarray (TMA) we previously validated [31]. Briefly, a TMA containing 34 IBC and 41 non-stage matched non-IBC tumours was constructed. After antigen retrieval, IHC staining for RhoC (antibody kindly provided by Prof. Dr. C.G. Kleer, 1/750, 1 h) was performed using the ChemMate Envision detection system (DakoCytomation). Interpretation of RhoC expression was done as described before [29] and the results were dichotomised: IHC score 0–1 = 0 and score 2–3 = 1.

Statistics

Statistical analysis was done using SPSS 12.0 software (SPSS, Chicago, IL). Median caveolin-1 and caveolin-2 mRNA expression in IBC and non-IBC tumour samples were compared using a Mann–Whitney U non-parametric test. Caveolin-1, caveolin-2 and RhoC protein expression were compared using Chi-Square statistics (or Fisher's Exact Test, if necessary). The correlation between caveolin-1 and -2 expression at the mRNA and protein level was analysed using a Spearman's correlation coefficient and Chi-Square statistics, respectively. The correlation between RhoC and caveolin-1 and -2 protein expression was analysed using χ^2 statistics.

Results

Cell line experiments

Using a differential methylation technique utilizing arbitrarily primed PCR [37], we previously identified 15 distinct hyper- and hypomethylated transcripts in the SUM149 IBC cell line compared with HMECs [40]. Genomic sequencing [41] was used to identify differentially methylated sequences. BLASTN analysis of isolated sequences revealed that two 249 bp and 248 bp hypomethylated transcripts, matched BAC clone CTB-11K1 (AC006159), proximal to chromosomal marker D7S522, corresponding to the *caveolin-1* and *caveolin-2* promoters, respectively.

Since hypomethylation of CpG islands in gene promoter regions typically lead to increased gene expression, RT-PCR and Western blot analysis were used to measure caveolin-1 and -2 levels in the SUM149 and SUM190 IBC cell lines relative to HMECs, MCF10As and the SUM102 non-IBC cell lines. Figure 1 shows the results of a typical RT-PCR and Western blot experiment (Figure 1). Relative caveolin-1 and -2 mRNA levels were measured three separate times and found to differ significantly in the cell lines (One-way ANOVA $p < 0.001$ and $p < 0.001$, respectively). In the SUM149 and SUM190 cell lines, mean relative caveolin-1 mRNA expression was significantly higher then in HMEC's (Bonferroni's *post hoc* test $p < 0.001$ and $p = 0.001$, respectively), MCF10A (Bonferroni's *post hoc* test $p < 0.001$ and $p < 0.001$, respectively) and SUM102 (Bonferroni's *post hoc* test $p < 0.001$ and $p < 0.001$, respectively) cell lines. For caveolin-2, significantly increased mean relative mRNA expression in IBC cell lines compared to HMECs and non-IBC cell lines was also found, although the difference between the SUM149 and MCF10A did not reach statistical significance (Bonferroni's *post hoc* test $p = 0.125$).

Relative caveolin-1 and -2 protein expression were also measured three separate times. Mean relative protein expression significantly differed between the cell lines (One Way ANOVA $p = 0.004$ and $p = 0.002$, respectively). Mean relative caveolin-1 protein expression was significantly higher in SUM149 and SUM190 IBC cell lines compared to HMECs (Bonferroni's *post hoc* test $p = 0.1$ and $p = 0.03$, respectively) and the MCF10A (Bonferroni's *post hoc* test $p = 0.03$ and $p = 0.01$, respectively) cell line. No statistical significant difference was found between both IBC cell lines and the SUM102 non-IBC cell line (Bonferroni's *post hoc* test $p = 0.33$ and 0.12, respectively). For mean relative caveolin-2 expression, comparable results were found.

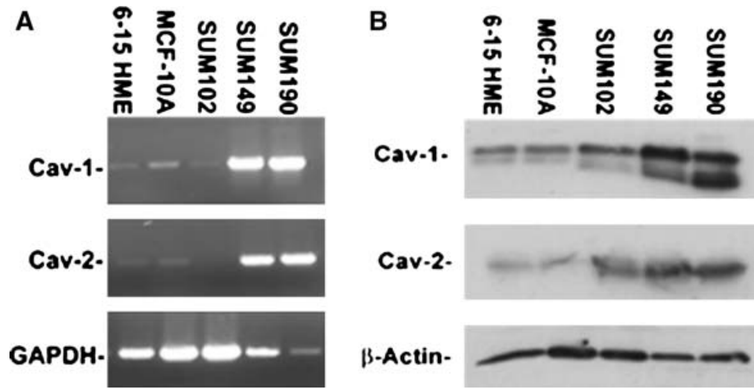

Figure 1. RT-PCR and Western blot of caveolin-1 and -2 expression in HMECs and IBC and non-IBC cell lines. (a) Gel electrophoretic image of RT-PCR amplified caveolin-1 and -2 mRNA in HMECs, MCF10A and SUM102 non-IBC and SUM149 and SUM190 IBC cell lines. GAPDH was used as a loading control. Increased expression of caveolin-1 and-2 mRNA in SUM149 and SUM190 compared to HMECs, MCF10A and SUM102 was observed. (b) Western blot image of caveolin-1 and -2 in the same cell lines. β-actin was used as a loading control. Protein levels of caveolin-1 and-2 mirrored mRNA expression. (HMEC: human mammary epithelial cell; IBC: inflammatory breast cancer).

Table 2 shows the mean relative caveolin-1 and -2 mRNA and protein expression levels in all cell lines investigated (Table 2).

Human sample experiments

Relative gene expression, measured by cDNA microarray analysis, for both caveolin-1 and -2 mRNA was found to be significantly higher in the human IBC samples: 1.7 fold for caveolin-1 ($p = 0.05$) and 1.8 fold for caveolin-2 ($p = 0.04$). This was confirmed by real-time quantitative RT-PCR analysis on the same samples: median caveolin-1 expression was 1.7 fold higher ($p = 0.02$) and median caveolin-2 expression was 2.2 fold higher ($p = 0.03$) in IBC compared to non-IBC (Figure 2). Furthermore, a correlation between relative gene expression for caveolin-1 and -2 in the same sample was found ($\rho = 0.55$, $p < 0.001$).

Table 3 shows the IHC results for caveolin-1 and caveolin-2 protein expression in IBC and non-IBC. When the expression of both molecules was considered separately, no difference was found between IBC and non-IBC for caveolin-1 ($p = 0.24$) expression. For caveolin-2 a statistical trend to overexpression in IBC ($p = 0.09$) was found. But 12 out of 29 (41.4%) IBC specimens did express either caveolin-1 or -2 compared to only 5 out of 32 (15.6%) non-IBC specimens ($p = 0.03$) (Table 3). Again, caveolin-1 and -2 expression were correlated ($p = 0.008$). Figure 3 represents the same zone of an IBC specimen showing IHC staining for caveolin-1 and caveolin-2 (Figure 3).

RhoC protein expression was significantly different between IBC and non-IBC: 66.7 % of IBC and 35.5% of non-IBC tumour specimens expressed RhoC ($p = 0.04$). When IBC and non-IBC samples were taken together, a trend to a correlation between caveolin-1 ($p = 0.1$) or caveolin-2 ($p = 0.09$) and RhoC protein expression was found. A significant correlation was found between RhoC expression and the combined caveolin-1 or caveolin-2 protein expression ($p = 0.04$) (Table 4).

Discussion

We used a differential methylation technique utilizing arbitrarily primed PCR [37] to demonstrate hypomethylation of the caveolin-1 and -2 promoter in the SUM149 IBC cell line. The sequences corresponding to the caveolin-1 and -2 promoters are 4 of the 10 distinct hypomethylated transcripts that we previously identified [40] in the SUM149 IBC cell line compared with HMECs. Engelman et al. demonstrated that hypermethylation of CpG islands in the caveolin-1 promoter was responsible for decreased caveolin-1 expression in the

Table 2. Mean relative caveolin-1 and -2 mRNA and protein expression levels (±SD) in HMECs, MCF10, SUM102, SUM149 and SUM190 cell lines

		HMECs	Non-IBC		IBC	
			MCF10A	SUM102	SUM149	SUM190
RT-PCR	Caveolin-1	45.47 (±13.16)	67.17 (±7.35)	36.63 (±5.07)	144.70 (±19.45)	204.60 (±15.98)
	Caveolin-2	32.93 (±8.69)	60.73 (±16.35)	17.57 (±5.30)	95.20 (±15.32)	134.40 (±18.98)
Western Blot	Caveolin-1	67.80 (±19.52)	53.43 (±18.24)	83.63 (±32.23)	141.53 (±31.93)	155.97 (±36.75)
	Caveolin-2	66.93 (±18.01)	49.00 (±7.50)	102.77 (±17.81)	122.33 (±14.63)	115.24 (±24.66)

Relative caveolin-1 and-2 mRNA and protein levels are higher in IBC compared to non-IBC cell lines. (HMEC: human mammary epithelial cell; IBC: inflammatory breast cancer)

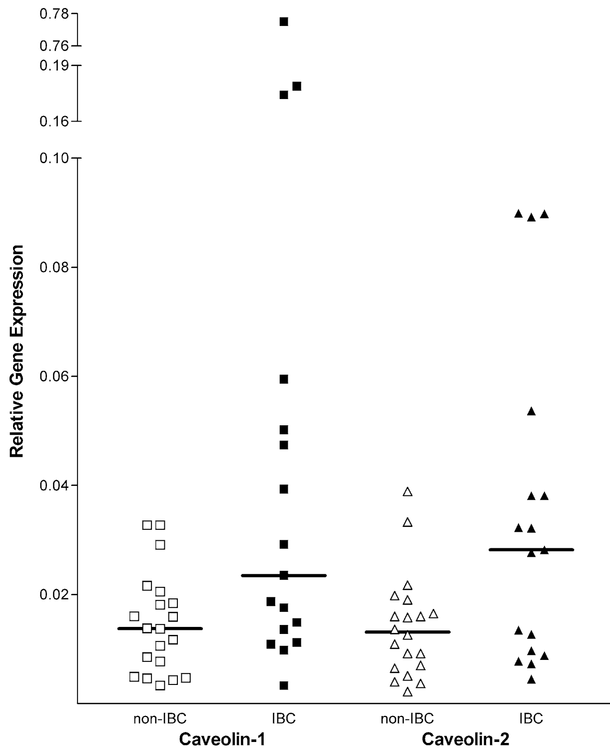

Figure 2. Relative gene expression of caveolin-1 and caveolin-2 mRNA measured by quantitative real-time RT-PCR in IBC and non-IBC human tissue samples. A 1.7 fold increase in caveolin-1 expression ($p = 0.02$) and a 2.2 fold increase in caveolin-2 expression ($p = 0.03$) was seen in IBC (IBC: inflammatory breast cancer).

MCF-7 and T47-D non-IBC breast cancer cell lines [42]. Since hypomethylation of CpG islands in gene promoter regions typically lead to increased gene expression [43,44], caveolin-1 and -2 gene expressions were measured in IBC cell lines and human tissue samples of IBC patients using cDNA microarray, RT-PCR and Western blot analysis. In IBC cell lines a higher mRNA and protein expression of both caveolin-1 and -2 was demonstrated compared with HMECs, MCF10A and SUM102 cell lines. The differences in expression between the two IBC cell lines and the HMECs, MCF10A and SUM102 cell lines did not always reach statistical significance, but were obvious and consistent as shown in Table 2. These results strongly suggest that hypomethylation of the caveolin-1 and -2 promoter leads to increased expression of caveolin-1 and -2 in IBC cell lines. Increased expression of caveolin-1 and -2 in IBC cell lines was then confirmed in human samples of IBC by cDNA microarray and real-time qRT-PCR experiments for mRNA expression and IHC for protein expression.

IBC is the most aggressive clinical subtype of breast cancer. The correlation of caveolin-1 expression with tumour progression varies with the tumour histiotype. In breast cancer, caveolin-1 is thought to act as a suppressor of tumour growth and metastasis. In breast cancer cell lines, caveolin-1 levels were inversely correlated to breast cancer progression *in vitro* and the overexpression of caveolin-1 resulted in substantial growth inhibition of breast tumour cells, which normally had no endogenous caveolin expression [45,46]. Using different animal model systems, Sloan et al. and Williams et al. demonstrated that caveolin-1 is a suppressor of mammary tumour growth and metastasis *in vivo* [47,48]. In human breast cancer tissues caveolin-1 and –2 expression, both at the mRNA and protein level, were significantly downregulated compared to corresponding normal tissues [49]. Caveolin-1 suppression correlated closely with that of caveolin-2 in breast cancer and caveolin-1 level was inversely correlated with tumour size. Overexpression of caveolin-1 and -2 in IBC, therefore seems contradictory. Nevertheless, evidence is increasing that IBC has to be considered as a biologically separate breast cancer entity. As previously described, the small GTP binding protein RhoC is overexpressed in IBC [32]. RhoC plays a key role in the increased motility, invasion and angiogenesis of the IBC phenotype [27,28]. Decreased expression of another gene, Wnt Induced Secreted Protein-3 (or Lost in Inflammatory Breast Cancer), was also found to be contributing to the IBC phenotype [50]. In trying to elucidate signal transduction pathways involved in IBC, we recently conducted comparative gene expression profile analysis of human IBC and non-IBC specimens [30]. When stringent clinical criteria for the diagnosis of IBC (AJCC) were taken into account, unsupervised hierarchical cluster analysis separated IBC and non-IBC specimens, again suggesting that IBC is a separate breast cancer entity with a distinct molecular signature. The present results suggest that increased expression of caveolin-1 and -2 has to be considered as part of this particular IBC molecular signature. This IBC signature contains a strikingly high number of NF-κB target genes

Table 3. Immunohistochemical staining results for caveolin-1 and caveolin-2 protein expression in human tissue samples of IBC and non-IBC

	IHC caveolin-1		IHC caveolin-2		IHC caveolin-1 or-2	
	neg	pos	neg	pos	neg	pos
Non-IBC	30	2	27	5	27	5
IBC	24	5	19	10	17	12
	p = 0.24		p = 0.09		p = 0.03	

Twelve out of 29 (41.4 %) IBC samples were either caveolin-1 or caveolin-2 positive, compared to 5/32 (15.6 %) non-IBC samples ($p = 0.03$). (IHC: immunohistochemistry; IBC: inflammatory breast cancer).

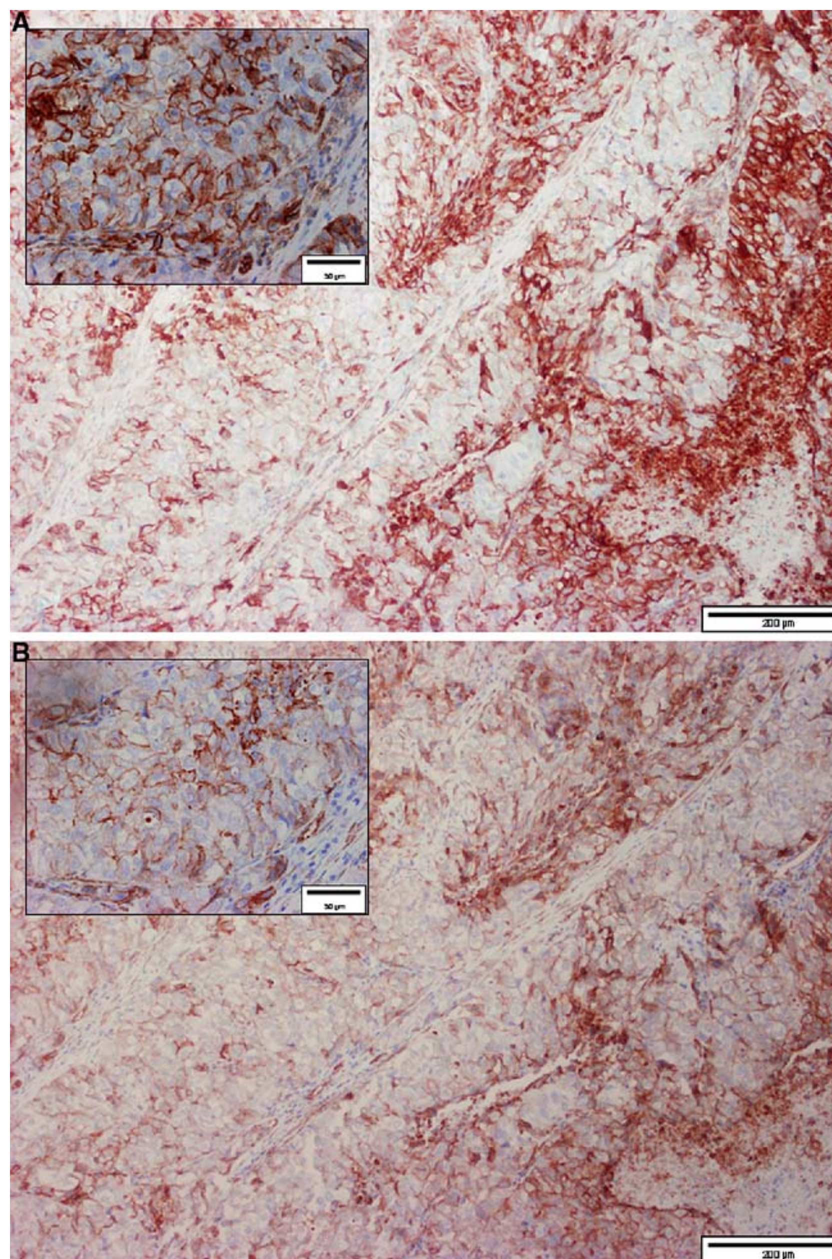

Figure 3. Immunohistochemical staining of caveolin-1 (a) and caveolin-2 (b) in the same tumour region of an IBC resection specimen. Insets show membranous staining of IBC tumour cells for caveolin-1 and -2 (IBC: inflammatory breast cancer).

that are overexpressed compared to non-IBC. In breast cancer [51], but also in multiple myeloma [52], NF-κB activation is responsible for invasiveness due to increased cell migration and motility. In the breast cancer model, this was due to induction of an epithelial-mesenchymal transition by TGFbeta that depended critically on NF-κB signalling, while in multiple myeloma, the NF-κB target gene caveolin-1 triggered tumour cell migration. Overexpression of caveolin-1 might contribute to the unique IBC phenotype, possibly by interaction or regulation of RhoC GTPase activity. As before, we demonstrated an increased protein expression of RhoC in IBC compared to non-IBC [31]. Furthermore, a correlation between caveolin-1 or caveolin-2 and RhoC protein expression was found. In other cell types, caveolin-1 has been shown to directly interact and regulate molecules such as RhoA, a homologue of RhoC (95% identical) [5,6]. Furthermore, we demonstrated that pancreatic cancer cells motility is controlled through interaction of RhoC and caveolin-1 [53]. In the previous cDNA microarray experiments IBC specimens were classified as belonging to the basal like subtype according to Perou et al. [54]. Since caveolin-2 belongs to the gene set that defines the basal-like subtype, overexpression of caveolin-2 confirms this classification of IBC to this subtype, indicative of a worse prognosis.

Although in breast cancer mainly considered to be a tumour growth and metastasis suppressor, in prostate [55–60] , bladder [61] and esophageal [62] cancer, caveolin-1 may function as a tumour promoter. Furthermore, Yang et al. demonstrated an increased expression of caveolin-1 in prostate cancer and primary

Table 4. Correlation between caveolin-1 or -2 and RhoC protein expression in human tissue samples of IBC and non-IBC

		IHC caveolin-1		IHC caveolin-2		IHC caveolin-1 or-2	
		neg	pos	neg	pos	neg	pos
IHC RhoC	neg	24	1	21	4	21	4
	pos	17	4	13	8	12	9
		$p = 0.1$		$p = 0.09$		$p = 0.04$	

A trend to a correlation was found between caveolin-1 ($p = 0.1$) or -2 ($p = 0.09$) and RhoC protein expression. A significant correlation was found between caveolin-1 or 2 protein expression and RhoC protein expression ($p = 0.04$) (IHC: immunohistochemistry).

and metastastic breast cancer specimens [63]. Caveolin-1 has also been shown to be essential for VEGF-triggered multiple myeloma migration. Caveolin-1 depletion by antisense methodology and administration of Bortezomib, a proteasome inhibitor, abrogates VEGF-triggered multiple myeloma migration [52]. The infiltrative growth pattern of IBC with tumour cell nests infiltrating between pre-existing tissue components instead of forming a well circumscribed tumour nodule and the multiple tumour cell emboli suggest an important role in the IBC phenotype for molecules involved in cell motility and migration. In order to reconcile contradictory data on the role of caveolin-1 in tumour progression, Carver et al. propose a biphasic model of caveolin-1 in tumour-cell growth and progression *in vivo* [4]. At the beginning of oncogenic transformation decreased caveolin-1 expression could lead to a growth advantage but in the later stages caveolin-1 re-induction could confer a survival benefit by suppressing apoptosis and allowing acquisition of multidrug resistance. Williams et al. suggest that caveolin-1 phosphorylation and mutations may override or inactivate the growth inhibitory activity of the caveolin-scaffolding domain [64].

Increased angiogenesis and lymphangiogenesis are probably necessary for the metastatic potential of IBC. In IBC increased expression of most angiogenic and lymphangiogenic mediators and increased blood vessel and lymph vessel endothelial cell proliferation were demonstrated [2,3]. Caveolae and caveolin-1 could coordinate and regulate angiogenic signalling pathways and modulate vascular tubulus formation. Targetted downregulation of caveolin-1 expression prevents vessel maturation *in vivo* and capillary-tube-like formation in cell culture, whereas overexpression of caveolin-1 seems to promote tubule formation *in vitro* [65,66].

In conclusion, we demonstrated hypomethylation of the caveolin-1 and -2 promoters leading to increased expression of caveolin-1 and -2 both at the mRNA and protein level in IBC, both in cell lines and in human breast cancer samples. In these samples, a correlation was found between caveolin-1 or -2 and RhoC protein expression. These results suggest a role for caveolin-1 and -2 in the aggressive IBC phenotype. Furthermore, the data confirm our cDNA microarray results classifying IBC as a separate breast cancer entity [30]. Our results encourage further exploration of the role of caveolin-1 and caveolin-2 in IBC and its interaction with RhoC GTPase. Caveolin-1 and-2 might be considered as new therapeutic targets in IBC. Recently Bortezomib (Velcade), the first proteasome inhibitor to have shown anti-cancer activity and reached clinical trials [67], has also been shown to decrease caveolin-1 expression, thereby probably targeting both the tumour stroma and the cancer cells [52].

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