ARTICLE
Journal of Cellular Biochemistry 112:3176–3184 (2011)

Journal of Cellular Biochemistry

Identification of Specific Nuclear Structural Protein Alterations in Human Breast Cancer

Manuel Debald,[1] Sebastian Franken,[2] Lukas Carl Heukamp,[3] Andrea Linke,[4] Matthias Wolfgarten,[1] Klaus-Jürgen Walgenbach,[1] Michael Braun,[1] Christian Rudlowski,[1] Volkmar Gieselmann,[2] Walther Kuhn,[1] Gunther Hartmann,[4] and Gisela Walgenbach-Brünagel[4]*

[1]*Department of Obstetrics and Gynecology, Centre for Integrated Oncology, University of Bonn, Bonn, Germany*
[2]*Institute for Biochemistry and Molecular Biology, University of Bonn, Bonn, Germany*
[3]*Institute of Pathology, Centre for Integrated Oncology, University of Bonn, Bonn, Germany*
[4]*Institute for Clinical Chemistry and Clinical Pharmacology, Centre for Integrated Oncology, University of Bonn, Bonn, Germany*

ABSTRACT

Breast cancer is the most commonly diagnosed type of cancer and a major cause of death in women. Reliable biomarkers are urgently needed to improve early detection or to provide evidence of the prognosis for each individual patient through expression levels in tumor tissue or body fluids. This proteomic analysis focused on the nuclear structure of human breast cancer tissue, which has been shown to be a promising tool for cancer biomarker development. The nuclear matrix composition of human breast cancer ($n = 14$), benign controls ($n = 2$), and healthy controls ($n = 2$) was analyzed by high-resolution two-dimensional gel electrophoresis and mass spectrometry. Validation studies were performed in an individual sample set consisting of additional breast cancer tissues ($n = 3$) and additional healthy control tissues ($n = 2$) by one-dimensional immunoblot. In this setting, we identified five proteins that were upregulated in human breast cancer tissue, but absent in the healthy and benign controls ($P < 0.001$). These spots were also present in the investigated human breast cancer cell lines but absent in the MCF10a cell line, which represents normal human epithelial breast cells. Two of the breast cancer-specific proteins have been confirmed to be calponin h2 and calmodulin-like protein 5 by one-dimensional immunoblot. This is the first study demonstrating the expression of both proteins in human breast cancer tissue. Further studies are required to investigate the potential role of these proteins as biomarkers for early diagnosis or prognosis in human breast cancer. J. Cell. Biochem. 112: 3176–3184, 2011.

KEY WORDS: BREAST CANCER; BIOMARKER; CALPONIN H2; CALMODULIN-LIKE PROTEIN 5; NUCLEAR MATRIX

Breast cancer is the most common cancer in women. Every year more than 1.3 million women worldwide are diagnosed with breast cancer, while nearly 500,000 patients die due to this disease [Jemal et al., 2010].

The early diagnosis of breast cancer in a potentially curable stage improves the prognosis and consecutively reduces mortality of breast cancer patients. Clinical breast examination, imaging by mammography as well as tumor biopsy are the only recommended methods for breast cancer screening in the non-high risk population [Smith et al., 2010]. The established screening by clinical breast examination and mammography is able to detect breast cancer in early stages and has been shown to reduce mortality [Tabar et al., 2003; Berry et al., 2005].

Nevertheless, mammography screening, especially in patients under the age of 50 remains controversial, especially due to significant rates of false negative as well as false positive results leading to overdiagnosis and overtherapy [Jorgensen and Gotzsche, 2009]. It is mainly young women (<50 years) with a high density of the breast who show low sensitivities in mammography [Buist et al., 2004]. The sensitivity of mammography in women aged 50 years

Grant sponsor: BONFOR, University of Bonn.
*Correspondence to: Gisela Walgenbach-Brünagel, Institute for Clinical Chemistry and Clinical Pharmacology, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany. E-mail: bruenagel@uni-bonn.de
Received 12 May 2011; Accepted 21 June 2011 • DOI 10.1002/jcb.23249 • © 2011 Wiley Periodicals, Inc.
Published online 5 July 2011 in Wiley Online Library (wileyonlinelibrary.com).

and older ranges from 68% to 90%, while in women aged 40–49 years, the sensitivity is lower, with estimates between 62% and 76% [IARC, 2002]. Likewise, a meta-analysis of randomized controlled trials showed a decreased mortality reduction of 15% in young women (39–49 years) undergoing regular screening by mammography, compared to 30% in women aged 49–59 years [Heron et al., 2009]. Therefore, established mammography screening programs are mainly addressed to older women (50–69 years) [Schopper and de, 2009]. However, you have to note that one of five breast cancers occurs in this non-screened younger subgroup, which suffers from more aggressive and rapidly growing forms of breast cancer [Brekelmans et al., 1996]. Moreover, breast cancer is the most common cause of cancer death among young females aged 20–59 years [Jemal et al., 2010].

Thus, it is of great clinical interest to use biomarkers for breast cancer detection in sublinical stages and/or to get prognostic or predictive information about the individual disease. During the last two decades, genomic and proteomic technologies have significantly increased the number of potential DNA, RNA, and protein biomarkers in breast cancer [Brennan et al., 2007; Goncalves and Bertucci, 2011]. However, no reliable non-invasive test has become available to date for clinical routine [Harris et al., 2007; Debald et al., 2010; Smith et al., 2010]. Regarding proteomic approaches, a major challenge is based on the complexity of the human proteome and its dynamic state. Even current mass spectrometry (MS)-based technologies are still failing to achieve comprehensive access to low abundance proteins in complex biological samples. However, low abundance proteins represent numerous potential tumor-specific biomarkers [Petricoin et al., 2006]. A promising strategy to overcome this problem would be to fractionate sophisticated samples or to examine specific sub-cellular proteins.

Our group investigates nuclear matrix proteins (NMP), which represent only 1% of the total cell proteome. In 1974, the nuclear matrix was first described as the structural framework scaffolding of the nucleus, consisting of the peripheral lamins, protein complexes, an internal ribonucleic protein network, and residual nucleoli [Berezney and Coffey, 1974]. Most of the NMP are common to all cell types, but numerous NMP are tissue and cell type specific [Getzenberg, 1994]. NMP have been demonstrated to participate in many vital cellular functions, such as steroid hormone binding, gene transcription, and gene translation [Leman and Getzenberg, 2008]. The main characteristics of cancer cells are alterations in the size and shape of the nucleus that reflect the analogous alteration of the nuclear matrix. Recently, alterations of several NMP have been shown to be cancer-specific biomarkers. These findings have been successfully developed into non-invasive, blood- and urine-based tests with high sensitivity and specificity for prostate, bladder as well as colon cancer, which have now to be validated in correctly designed large studies to demonstrate their clinical utility [Van Le et al., 2005; Leman et al., 2007a,b; Walgenbach-Brunagel et al., 2008]. The aim of this study was to investigate the NMP composition in human breast cancer, benign control, and healthy breast tissue by two-dimensional gel electrophoresis, silver staining, and mass analysis in an effort to identify unique cancer associated nuclear proteins.

PATIENTS AND METHODS

TISSUE SAMPLES AND CELL LINES

Human breast tissues were obtained from the Department of Obstetrics and Gynecology at the University of Bonn Medical Center and from the Department of Plastic and Aesthetic Surgery at the University of Bonn Medical Center in cooperation with the tissue bank of the Center for Integrated Oncology Cologne-Bonn. For all specimens, histopathological workup was performed by an experienced, board-certified breast pathologist. The characteristics of the cancer and the control patients for two-dimensional gel-electrophoresis as well as verification experiments by one-dimensional immunoblot are summarized in Table I. None of the patients received neo-adjuvant treatment prior to surgery. The investigations conformed to the principles outlined in the Declaration of Helsinki and were performed with permission by the responsible Ethics Committee of the School of Medicine, University of Bonn.

To verify the epithelial character of specific protein spots, we investigated the NMP composition of different breast cancer cell lines as well as the NMP composition of MCF10a, which represents normal human epithelial breast cells. Breast cancer cell lines (HCC1937, BT474, SkBr3, and MCF7) as well as the MCF10a cell line were obtained from the American Type Culture Collection and cultured under appropriate conditions.

NUCLEAR MATRIX PROTEIN PREPARATION

NMP were extracted according to techniques as previously described [Getzenberg et al., 1991]. Briefly, the frozen breast cancer samples were pulverized in a Micro-Dismembrator (B Braun Biotech International) and transferred to a buffer containing 0.5% Triton X-100 (Carl Roth, Germany) and 2 mM ribonucleoside vanadyl complexes (Sigma–Aldrich) to release lipids and soluble proteins. Next, the solution was filtered through a 350-μm nylon mesh and underwent treatment with DNase as well as RNase-A to remove the soluble chromatin and RNA. The remaining fraction, consisting of intermediate filaments and NMPs, was disassembled with 8 M urea and the insoluble components (mainly carbohydrates and extracellular matrix) were pelleted. After the urea was dialyzed out, the intermediate filaments were allowed to reassemble and were subsequently removed by centrifugation. In a final step, the NMPs were precipitated in ethanol and resolved in 2D sample buffer (9 M urea, 4% CHAPS, 82 μM TBP, 0.4% ampholyte) or phosphate-buffered saline (PBS) for one-dimensional immunoblot analysis. The described reactions, except for digestion with DNase and RNase, were performed on ice. All solutions contained 1 mM PMSF to inhibit serine proteases. To remove potentially interfering contaminants a sample cleanup was performed (ReadyPrep 2-D Cleanup; Bio-Rad). The protein concentration was quantified by a reducing agent compatible microplate BCA protein assay kit (Thermo Scientific) with bovine serum albumin as a standard. The final pellet containing NMPs represents $<1\%$ of the total cellular proteins.

HIGH-RESOLUTION TWO-DIMENSIONAL GEL ELECTROPHORESIS

Isoelectric focusing was carried out in a PROTEAN IEF Cell (Bio-Rad) according to the manufacturer's instructions. Samples containing

TABLE I. Characteristics of Cancer Patients as well as Healthy and Benign Controls

Sample	Age at diagnosis	Menopause	Histology	T-stage	Nodal-status	Metastasis	Grade
Cancer patients							
Tumor 1	50	Postmenopausal	Invasive-ductal	1	0	0	2
Tumor 2	72	Postmenopausal	Invasive-ductal	1	0	0	2
Tumor 3	74	Postmenopausal	Invasive-ductal	4	1	0	3
Tumor 4	63	Postmenopausal	Invasive-ductal	1	0	0	2
Tumor 5	58	Postmenopausal	Invasive-ductal	2	1	0	3
Tumor 6	65	Postmenopausal	Invasive-ductal	1	1	0	2
Tumor 7	66	Postmenopausal	Invasive-ductal	1	3	0	2
Tumor 8	64	postmenopausal	Invasive-ductal	3	3	0	3
Tumor 9	41	Premenopausal	Invasive-ductal	2	0	0	2
Tumor 10	48	Premenopausal	Invasive-ductal	2	1	0	3
Tumor 11	59	Postmenopausal	Invasive-ductal	1	0	0	3
Tumor 12	41	Premenopausal	Invasive-ductal	1	0	0	2
Tumor 13	56	Postmenopausal	Invasive-ductal	1	1	0	3
Tumor 14	70	Postmenopausal	Invasive-ductal	1	2	0	3
Tumor 15[a]	59	Postmenopausal	Invasive-ductal	1	1	0	2
Tumor 16[a]	63	Postmenopausal	Invasive-lobular	1	1	0	2
Tumor 17[a]	50	Premenopausal	Invasive-mucinous	2	3	0	3

Sample	Age at diagnosis	Menopause	Histology
Benign and healthy controls			
Control 1	48	Postmenopausal	Healthy breast tissue (breast reduction)
Control 2	20	Premenopausal	Healthy breast tissue (breast reduction)
Control 3	19	Premenopausal	Fibroadenoma
Control 4	18	Premenopausal	Fibroadenoma
Control 5[a]	48	Postmenopausal	Healthy breast tissue (breast reduction)
Control 6[a]	53	Postmenopausal	Healthy breast tissue (breast reduction)

[a]Sample was used for protein validation by one-dimensional immunoblot.

100 μg of NMP were added to 24 cm immobilized pH gradient (IPG) strips (Bio-Rad) in the range of IP 3–10. After 16 h of passive rehydration two wet paper wicks were inserted between the IPG strip and the electrode. A gradient at 200 V for 2 h, 500 V for 2 h, 2,000 V for 3.5 h and 8,000 V for 2 h was applied to the IPG strips. The temperature throughout this process was maintained at 20°C. After isoelectric focusing the IPG strips were equilibrated in EQ-buffer I (6 M urea, 0.375 M Tris/HCl pH 8.8, 20% glycerol, 2% SDS, 2% DTT) and EQ-buffer II (6 M urea, 0.375 M Tris/HCl pH 8.8, 20% glycerol, 2% SDS, 2.5% iodoacetamide) for 15 min each. The equilibrated strips were loaded onto 10% polyacrylamide gels and overlaid with 1% agarose. The separation in the second dimension was performed in a Investigator 2-D Electrophoresis System (Genomic Solutions) until the bromphenol blue front reached the end of the gel. After SDS–PAGE the separated NMPs were visualized by mass-compatible silver staining (SILVERQUEST silver staining kit; Invitrogen). The gels were analyzed using the PDQuest 2D analyzing software (Bio-Rad). Only clear and reproducibly identical spots in all of the gels were included in the analysis.

MASS SPECTROMETRIC ANALYSIS

Spots of interest were automatically cut from silver stained gels and processed using a trypsin profile IGD kit (Sigma–Aldrich) following the manufacturer's instructions. For analysis eluted peptides were separated using an Ultimate 3000 LC system (Dionex-LC Packings, Germany). Samples were loaded onto a monolithic trapping column (PepSwift, 200 μm × 5 mm) by the loading pump of the system operating at 10 μl/min, and 0.1% Heptafluorobutyric acid in water was used as mobile phase. After 5 min, valve was switched and the sample was eluted onto the analytical separation column (PepSwift monolithic capillary column, 200 μm × 50 mm), using a flow rate of 500 nl/min. The mobile phases used were H_2O/0.1% formic acid (v/v) for buffer A and 100% ACN/0.1% formic acid (v/v) for buffer B. Peptides were resolved by gradient elution using a gradient of 5−50% buffer B over 20 min, followed by a gradient of 50−90% buffer B over 1 min. After 5 min at 90% buffer B, the gradient returned to 5% buffer B preparing for the next run. Column effluent was monitored using a 3 nl UV flow cell (214 nm).

Mass spectrometric analysis was done via online ESI-MS/MS using an HCTUltra ion trap mass spectrometer (Bruker Daltonics, Germany). All measurements were carried out in positive ion mode. MS-spectra were acquired in standard-enhanced mode between 300 and 2,000 m/z at a rate of 8,100 m/z/s. Fragmentation of peptides from MS-spectra using CID was done in Auto-MS2 mode, selecting precursor ions according to the following parameters: number of precursor ions = 5, minimal ion intensity = 10,000, ion excluded after two spectra, exclusion release after 1 min. MS2 data acquisition was done in ultrascan mode with a scan range of 50–3,000 m/z at a scan speed of 26,000 m/z/s.

Raw MS data for each LC run were processed using DataAnalysisTM version 4.0. The spectrum was screened for compounds using the software's AutoMS/MS search feature applying the following parameters: intensity threshold = 10,000; max number of compounds = 500; retention time = 0.4. Identified compounds were subsequently deconvoluted and exported for protein database comparison with BioToolsTM version 3.1. In BioToolsTM, the exported compounds were run against an in-house SwissProt v51.6 database using the Mascot 2.2.02 algorithm. The searches were carried out using the following parameters: enzyme = trypsin; missed cleavages = 1; taxonomy = all entries; variable modifica-

tions = oxidation (M) and carbamidomethylation (C); peptide tolerance = 300 ppm; MS/MS tolerance = 1.1 Da; significance threshold $P = 0.05$.

NUCLEAR AND CYTOPLASMIC PROTEIN EXTRACTION

To rule out a cytoplasmic expression of calponin h2 by one-dimensional immunoblot, a NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) was used for the preparation of nuclear and cytoplasmic extracts. The protein concentration was quantitated by a reducing agent compatible microplate BCA protein assay kit (Thermo Scientific) with bovine serum albumin as a standard.

ONE-DIMENSIONAL IMMUNOBLOT

To validate the calponin h2, which was identified by MS analysis, NMP-extracts of human breast cancer, healthy breast tissue, and human breast cancer cell lines were studied.

One-dimensional immunoblotting was performed on NMP-extracts of additional breast cancer patients and additional healthy controls, which had not been used for protein separation by two-dimensional gel electrophoresis. To outline the specificity of the identified proteins we also investigated different histological breast cancer types (ductal, lobular, and mucinous). Ten micrograms of NMP per sample were separated by 12% SDS–PAGE (Invitrogen), transferred onto nitrocellulose membranes and blocked overnight in BSA in TBS with 0.5% Tween-20. The following day, membranes were incubated with anti-Calponin h2 1:200 (No: sc-16608; Santa-Cruz) for 2 h at room temperature. Anti-Lamin A/C 1:200 (No: sc-56140; Santa-Cruz) was used as a nuclear and anti-α-Tubulin 1:200 (No: sc-58666; Santa-Cruz) as a cytoplasmic control. Species-appropriate fluorescently conjugated secondary antibodies were applied for 1 h at room temperature. Membranes were analyzed using an Odyssey Infrared Imaging System (LI-COR, Australia).

STATISTICS

Statistical analyses were performed using GraphPad InStat, version 3.06 (GraphPad Software Inc). P values were determined using Fisher's exact test. Two-sided P values lower than 0.05 were considered statistically significant.

RESULTS

We investigated human breast cancer tissues of 14 different patients with ductal-invasive breast cancer as well as four non-malignant controls (two fibroadenoma and two healthy controls). The NMP were extracted and separated by high-resolution two-dimensional gel electrophoresis. Figure 1 shows a representative two-dimensional gel (2D gel) of NMP in human breast cancer tissue after mass-compatible silver staining as well as the location of the cancer-specific protein spots (A, B, C, D, E). Representative 2D gels of human healthy breast tissue and human fibroadenoma are shown in Figure 2. Performing a computer-based comparison of all 2D gels by PDQuest 2D analyzing software five protein spots (A, B, C, D, E) were identified to be present in all human cancer tissues (14/14) but not in any control (0/4; $P < 0{,}001$). The expression levels of the five

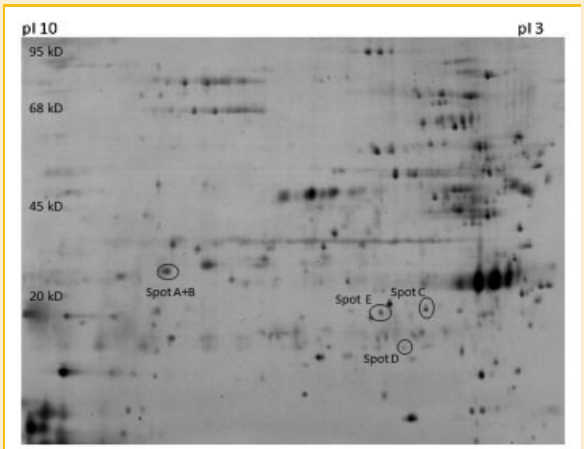

Fig. 1. Representative gel of nuclear matrix proteins (100 μg) in human breast cancer after high-resolution two-dimensional gel electrophoresis and silver staining. Spots A–E are present in the investigated breast cancer tissues, but absent in the healthy and benign controls.

protein spots (A, B, C, D, E) for each individual tumor sample are summarized in Table II. To rule out the possibility that the differences in NMP composition may be due to the detection of NMP from stromal, blood or other cell types, we also examined the NMP composition of human breast cancer cell lines as well as the NMP composition of MCF10a, which represents normal human epithelial breast cells. The cancer-specific spots A–E were all present in the investigated breast cancer cell lines, outlining the epithelial source of the identified protein spots (Fig. 3). To exclude a potential overlap of the benign or healthy epithelium in the non-malignant controls due to their stromal fraction, we analyzed the MCF10a cell line analogously. None of the breast cancer-specific spots was present in the MCF10a cell line, indicating that the missing spots A–E in the benign and healthy control tissues are not just artifacts due to stromal interference. In addition, one protein spot (F) was exclusively found in healthy breast tissue (2/2), but was absent in fibroadenoma (0/2) and human breast cancer (0/14; $P = 0.0065$).

To clarify the identity of each specific protein by MS analysis, up to four protein spots were automatically picked from silver-stained two-dimensional gels and pooled prior to trypsin digestion to enhance signal intensity and thus protein identification. Protein spots A, B, D were successfully identified by ESI-MS. The breast cancer-specific protein spots have been shown to be calponin h2 (CNN-2; Spot A), calmodulin-like protein 5 (CALML5; Spot B), and heat shock protein (hsp) beta 1 (hsp27; Spot D). The molecular weights and isoelectric points for each protein spot as well as the MOWSE (molecular weight search) scores for the identified proteins A, B, D are summarized in Table III. To date, MS analysis for spots C, E, and F has not resulted in protein identification.

One-dimensional immunoblotting with commercially available antibodies against CNN-2 was performed to validate the results of MS analysis. These validation experiments were performed in an individual sample set consisting of additional breast cancer tissues (n = 3) and additional healthy control tissues (n = 2), which have not been used for two-dimensional gel electrophoresis. As shown in

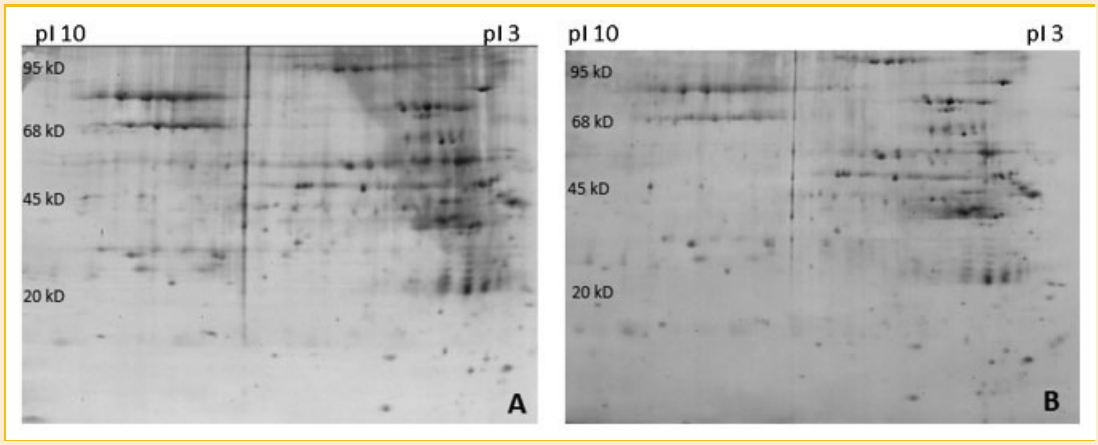

Fig. 2. Representative gels of nuclear matrix proteins (100 μg) in human healthy breast tissue (A) and human fibroadenoma (B) after high-resolution two-dimensional gel electrophoresis and silver staining.

Figure 4, the antibody against CNN-2 detected a protein band at 37 kDa in the NMP-fraction of histological different human breast cancer entities (ductal, lobular, and mucinous) but not in healthy human breast tissue. This band was also present in the NMP-fraction of the investigated breast cancer cell lines (Fig. 5). To rule out the possibility of a contamination of the NMP extraction with cytoplasmic proteins we performed a one-dimensional immunoblot analysis in the cytoplasmic and NMP fractions. A polyclonal α-tubulin antibody specific for cytoplasmic α-tubulin (approximately 51 kDa) was used as a control and identified α-tubulin exclusively in the cytoplasmic protein fraction. The NMP fractions did not contain a band recognized by the α-tubulin antibody ruling out a contamination of these fractions with cytoplasmic proteins. Additionally, performing one-dimensional immunoblot analysis in the cytoplasmic fraction of human breast cancer tissue the antibody against CNN-2 showed no positive reaction for calponin h2. These results demonstrate that a cytoplasmic expression of CNN-2 could also be excluded (Fig. 4).

TABLE II. Expression Levels of Breast Cancer-Specific Nuclear Matrix Proteins After Two-Dimensional Gel Electrophoresis and Silver Staining

Sample	Spot A	Spot B	Spot C	Spot D	Spot E
Tumor 1	3	1	2	2	3
Tumor 2	3	3	3	2	3
Tumor 3	1	1	2	1	2
Tumor 4	1	1	3	2	1
Tumor 5	2	3	1	1	2
Tumor 6	2	1	2	1	2
Tumor 7	2	2	2	2	1
Tumor 8	1	3	3	3	1
Tumor 9	1	2	3	3	3
Tumor 10	1	1	1	1	1
Tumor 11	2	1	2	2	3
Tumor 12	1	2	2	1	1
Tumor 13	1	2	1	1	1
Tumor 14	3	3	3	3	3

A scoring system of 1 = weak, 2 = moderate, and 3 = strong was used to evaluate differences in protein expression for each individual tumor sample.

DISCUSSION

In this study, we identified three proteins by two-dimensional gel electrophoresis that were upregulated in the nuclear matrix of human breast cancer tissue. These proteins were absent in healthy human breast tissue and in benign controls.

The first protein has been shown to be heat shock protein beta-1 (hsp27). Overexpression of hsp27 is observed in numerous cancer entities and, in particular, in human breast cancer [Ciocca and Calderwood, 2005]. Upregulated protein levels have been associated with estrogen receptor levels as well as better differentiation of tumor cells suggesting a good prognosis. These data are still controversially discussed as additional studies led to differing findings [Ciocca and Calderwood, 2005]. Moreover, further studies indicated a link between high levels of hsp27 and more aggressive tumors as well as drug resistance [Oesterreich et al., 1993; Hansen et al., 1999]. Likewise, hsp27 positive breast cancer from node-negative patients is correlated to lower overall survival and survival after first recurrence [Thanner et al., 2005]. The data showed that heat shock proteins are involved in several aspects of tumor biology, but their definite role in cancer diagnosis, prognosis and therapy needs to be clarified [Romanucci et al., 2008].

Posttranslational modifications, especially phosphorylation of hsp27 (Ser^{78} and Ser^{82}) have been shown to be associated with a nuclear translocation in transfection experiments [Geum et al., 2002]. Our data demonstrate an expression of hsp27 in the nucleus of human breast cancer tissue. A posttranslational modification of nuclear hsp27 might explain the nuclear localization in our study. These modifications could be specific for human breast cancer tissue. Further studies are in progress to evaluate this hypothesis.

The second breast cancer-specific protein was found to be calmodulin-like protein 5 (CALML5 = CLSP). Calmodulins are the major calcium-binding proteins in non-muscular tissue, which are known to interact with at least 50 different target enzymes and structural proteins, including protein kinases, phosphatases, phosphodiesterases, ATPases, and NO-synthases, mediating many biological processes [Crivici and Ikura, 1995; Tabernero et al., 1997].

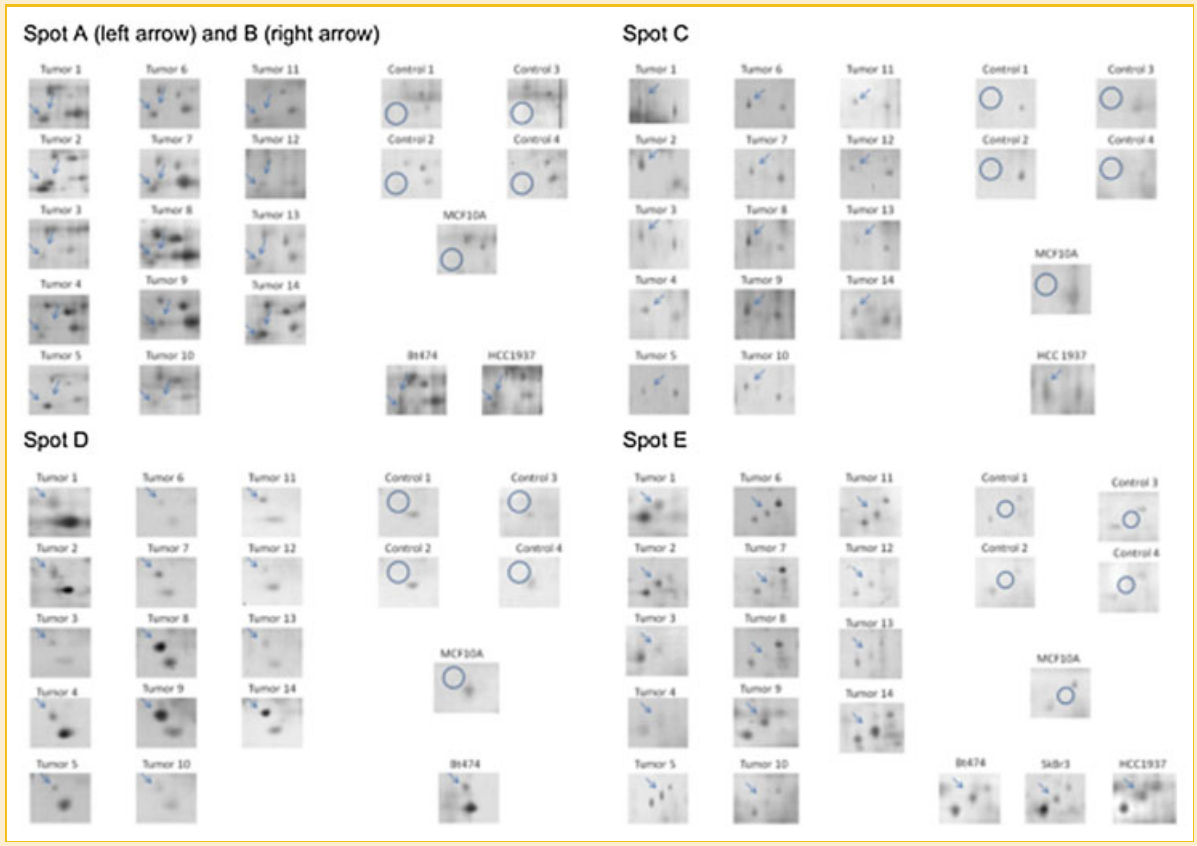

Fig. 3. Protein spots A–E are present in human breast cancer tissue, but absent in human benign and healthy controls. Comparing the expression of nuclear matrix proteins in human breast cancer tissue (n = 14), healthy control tissues (n = 2), and benign control tissues (n = 2) by high-resolution two-dimensional gel electrophoresis and silver staining, protein spots A–E were specific for normal human breast cancer tissue. These spots were also absent in the MCF10A cell line, which represents human normal epithelial breast cells. The epithelial source of these protein spots was confirmed by the expression in the human breast cancer cell lines SkBr3, Bt474, or HCC1937.

Calmodulin (CaM) is composed of 148 amino acid residues, two globular domains and a single peptide chain containing four calcium-binding sites. CaM is found in all eukaryotic cells and its amino acid sequence is highly conserved, in particular its primary amino acid sequence which is virtually identical in many different species.

Mehul et al. [2000] discovered and biochemically characterized a new skin-specific calcium-binding protein that was named calmodulin-like skin protein (CLSP) based on its homology (52%) with CaM and its tissue-specific expression in the epidermis. A real-time PCR analysis in human tissue, including heart, testis, skeletal muscle, pancreas, liver, small intestine, placenta, spleen, kidney, and epidermis, demonstrated the specificity of CLSP in the epidermis, while a faint expression was also found in lung tissue [Mehul et al., 2000]. CLSP and CALML5 are different names for the same protein, which can be used equivalent. It could be shown that CLSP is

TABLE III. Protein Identification by Mass Analysis

Protein spot	Breast cancer	Fibroadenoma	Healthy control	Identified protein	MW (Da)	pI	MOWSE score	Sequence coverage (%)
Specific nuclear matrix proteins in human breast cancer								
A	100% (14/14)	0% (0/2)	0% (0/2)	Calponin-2	33,675	6.95	140	12
B	100% (14/14)	0% (0/2)	0% (0/2)	Calmodulin-like protein 5	15,883	4.34	588	76
C	100% (14/14)	0% (0/2)	0% (0/2)	Unidentified	25,000[a]	4[a]	–	–
D	100% (14/14)	0% (0/2)	0% (0/2)	hsp beta1	22,768	5.98	184	20
E	100% (14/14)	0% (0/2)	0% (0/2)	Unidentified	20,000[a]	4.5[a]	–	–

Protein spot	Breast cancer	Fibroadenoma	Healthy control	Identified protein	MW (kDa)	pI	MOWSE score	Sequence coverage (%)
Nuclear matrix protein only found in healthy breast tissue								
F	0% (0/14)	0% (0/2)	100% (2/2)	Unidentified	20,000[a]	4[a]	–	–

[a]Approximate MW and pI in 2D-gel.

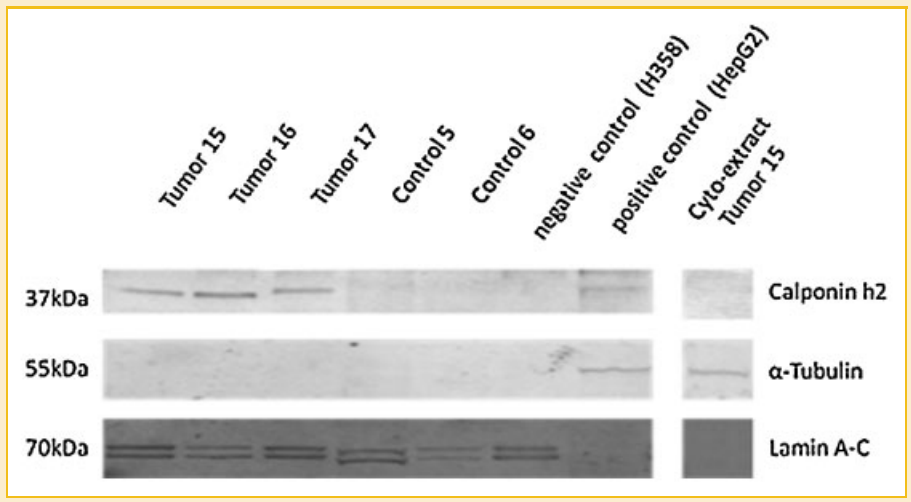

Fig. 4. Calponin h2 in the NMP-fraction of different human breast cancer subtypes. Nuclear matrix protein (NMP) extracts (10 μg/lane) of ductal-invasive (Tumor 15), lobular-invasive (Tumor 16), and mucinous-invasive (Tumor 17) human breast cancers as well as two healthy control tissues were fractionated on SDS–PAGE and subjected to immunoblotting with a specific antibody against human Calponin h2. A specific band at 37 kDa indicates the expression of Calponin h2 in different human breast cancer subtypes. This band is not found in both human healthy breast tissue controls, indicating the specific expression of Calponin h2 in human breast cancer. Total cell lysates of H358 cells (human lung cancer cell line) were used as a negative, cytoplasmic lysates of HepG2 cells (human hepatocellular carcinoma cell line) were used as a positive control. Lamin A–C (70 kDa) was used as a loading control for the NMP fraction. To confirm the nuclear expression of Calponin h2 we also investigated a cytoplasmic extract (10 μg/lane) of human breast cancer tissue (cyto-extract Tumor 15). The specific antibody against human Calponin h2 did not detect Calponin h2 in the cytoplasmic fraction. The absence of nuclear proteins was confirmed by a negative reaction for Lamin A–C (70 kDa). A cytoplasmic contamination of the investigated NMP extracts could also be excluded by a negative reaction for α-Tubulin (55 kDa).

involved in epidermal differentiation [Mehul et al., 2001]. Investigating gene expression patterns in human breast cancer tissue, Porter et al. [2003] found CLSP to be upregulated in invasive and metastatic breast cancer. To our knowledge, we are the first to describe the presence of CLSP in human breast cancer on a protein level.

The third protein has been shown to be calponin h2, a member of the calponin family. Calponins are a family of 34–37 kDa cytoplasmic Ca^{2+}-binding proteins, which bind in vitro to F-actin and tropomyosin [Takahashi et al., 1986]. There are three isoforms

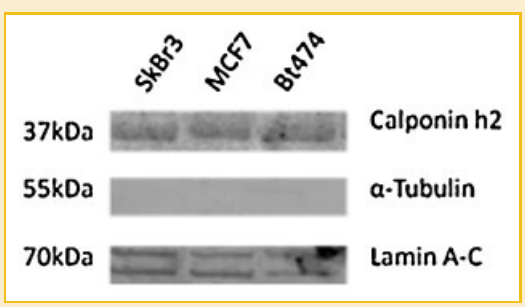

Fig. 5. Calponin h2 in the NMP-fraction of breast cancer cell lines. Nuclear matrix protein (NMP) extracts (10 μg/lane) of the breast cancer cell lines SkBr3, MCF7, and Bt474s were fractionated on SDS–PAGE and subjected to immunoblotting with a specific antibody against Calponin h2. A specific band at 37 kDa indicates the nuclear expression of Calponin h2 in the investigated breast cancer cell lines, confirming the epithelial source of Calponin h2. A cytoplasmic contamination of the investigated NMP extracts could be excluded by a negative reaction for α-Tubulin (55 kDa). The presence of a nuclear protein fraction was confirmed by a positive reaction for Lamin A–C (70 kDa).

(basic h1, neutral h2, and acidic h3) sharing high sequence identity within the first 273 amino acids, but differing significantly in their carboxyl-terminal sequences [Tang et al., 2006]. Calponin h1 was first characterized by Takahshi et al. as a smooth muscle-specific protein that inhibits the actin-activated ATPase activity of myosin in vitro [Takahashi et al., 1986].

Strasser et al. [1993] demonstrated the existence of calponin h2, which is encoded by a different gene. This neutral isoform is expressed in both, smooth muscle as well as non-muscle cells [Wu and Jin, 2008]. It is found in fibroblasts and epidermal keratinocytes with a role in stabilizing the actin filaments [Fukui et al., 1997]. Hossain et al. [2006] analyzed the expression of calponin h2 in smooth muscle and several non-muscle organs in the mouse and found high levels of expression in the spleen and in lung alveolar epithelials. The high amount of calponin h2 found in the spleen may reflect the expression described in endothelial cells [Sakihara et al., 1996; Tang et al., 2006]. A significant upregulation of calponin h2 is found in the growing smooth muscles, such as embryonic stomach and urinary bladder as well as the uterus during early pregnancy in mice [Hossain et al., 2003]. The high expression of calponin h2 decreases in adult smooth muscle cells, indicating its potential role during cell proliferation. It is known that calponins are involved in numerous functions in muscle and non-muscles cells. However, the expression of calponins, particularly of calponin h2, in cancer cells and their biological function has not been widely studied to date [Wu and Jin, 2008].

The upregulation of calponin h2 in human breast cancer and also its nuclear localization has not been described before. Validation experiments in an individual sample set of patients with ductal-invasive, lobular-invasive as well as mucinous-invasive breast

cancer could confirm the expression of calponin h2 in the NMP-fraction of all investigated breast cancer subtypes. The detection of calponin h2 in human breast cancer cell lines confirms the epithelial expression and excludes potential impurites by other cell types, such as smooth muscle, blood, or endothelial cells. A cytoplasmic contamination of the investigated NMP-fractions was excluded.

Calponin h2 is an actin-binding protein [Rozenblum and Gimona, 2008]. While the existence of actin filaments in the nucleus has been doubted for a long time, nuclear actins have been shown to play an important role in chromatin remodeling, transcriptional regulation, RNA processing, and nuclear export [de Lanerolle et al., 2005; Grummt, 2006]. Recently, a number of other actin- and actin-binding related proteins have been identified in the nucleus, but the question whether these proteins and how they participate in nuclear actin regulation processes remains largely unanswered [Dingova et al., 2009]. Further studies are required to evaluate these nuclear functions during carcinogenesis, particularly with regard to the demonstrated upregulation of calponin h2 in human breast cancer.

Apart from the nuclear localization of calponin h2, CLSP, and hsp27, our data show a specific expression in human breast cancer tissue compared to healthy and benign controls. Studies performed by Schenk et al. [2008] and Sheng et al. [2006] demonstrated the existence of calponin h2 as well as CLSP in the human plasma proteome. As these proteins can be found in human blood, a blood-based assay detecting the upregulation of calponin h2 as well as CLSP might be used to separate healthy controls from breast cancer patients. The challenge will be to perform correctly designed large retrospective studies including independent validation sets, followed by prospective validation studies to demonstrate the clinical benefits for patients [Pepe et al., 2001; Ludwig and Weinstein, 2005]. We are currently investigating the potential role of the identified proteins as diagnostic and prognostic biomarkers in human breast cancer that could potentially help to improve the management of this disease.

ACKNOWLEDGMENTS

Manuel Debald is funded by BONFOR, University of Bonn.

REFERENCES

Berezney R, Coffey DS. 1974. Identification of a nuclear protein matrix. Biochem Biophys Res Commun 60:1410–1417.

Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, Mandelblatt JS, Yakovlev AY, Habbema JD, Feuer EJ. 2005. Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med 353:1784–1792.

Brekelmans CT, van Gorp JM, Peeters PH, Collette HJ. 1996. Histopathology and growth rate of interval breast carcinoma. Characterization of different subgroups. Cancer 78:1220–1228.

Brennan DJ, Kelly C, Rexhepaj E, Dervan PA, Duffy MJ, Gallagher WM. 2007. Contribution of DNA and tissue microarray technology to the identification and validation of biomarkers and personalised medicine in breast cancer. Cancer Genomic Proteomics 4:121–134.

Buist DS, Porter PL, Lehman C, Taplin SH, White E. 2004. Factors contributing to mammography failure in women aged 40–49 years. J Natl Cancer Inst 96:1432–1440.

Ciocca DR, Calderwood SK. 2005. Heat shock proteins in cancer: Diagnostic, prognostic, predictive, and treatment implications. Cell Stress Chaperones 10:86–103.

Crivici A, Ikura M. 1995. Molecular and structural basis of target recognition by calmodulin. Annu Rev Biophys Biomol Struct 24:85–116.

de Lanerolle LP, Johnson T, Hofmann WA. 2005. Actin and myosin I in the nucleus: What next? Nat Struct Mol Biol 12:742–746.

Debald M, Wolfgarten M, Walgenbach-Brünagel G, Kuhn W, Braun M. 2010. Non-invasive proteomics-thinking about personalized breast cancer screening and treatment. EPMA J 1:413–420.

Dingova H, Fukalova J, Maninova M, Philimonenko VV, Hozak P. 2009. Ultrastructural localization of actin and actin-binding proteins in the nucleus. Histochem Cell Biol 131:425–434.

Fukui Y, Masuda H, Takagi M, Takahashi K, Kiyokane K. 1997. The presence of h2-calponin in human keratinocyte. J Dermatol Sci 14: 29–36.

Getzenberg RH. 1994. Nuclear matrix and the regulation of gene expression: Tissue specificity. J Cell Biochem 55:22–31.

Getzenberg RH, Pienta KJ, Huang EY, Coffey DS. 1991. Identification of nuclear matrix proteins in the cancer and normal rat prostate. Cancer Res 51:6514–6520.

Geum D, Son GH, Kim K. 2002. Phosphorylation-dependent cellular localization and thermoprotective role of heat shock protein 25 in hippocampal progenitor cells. J Biol Chem 277:19913–19921.

Goncalves A, Bertucci F. 2011. Clinical application of proteomics in breast cancer: State of the art and perspectives. Med Princ Pract 20: 4–18.

Grummt I. 2006. Actin and myosin as transcription factors. Curr Opin Genet Dev 16:191–196.

Hansen RK, Parra I, Lemieux P, Oesterreich S, Hilsenbeck SG, Fuqua SA. 1999. Hsp27 overexpression inhibits doxorubicin-induced apoptosis in human breast cancer cells. Breast Cancer Res Treat 56:187–196.

Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF, Bast RC, Jr. 2007. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. J Clin Oncol 25:5287–5312.

Heron MP, Hoyert DL, Murphy SL, Xu JQ, Kochanek KD, Tejada-Vera B. 2009. Death: Final data for 2006. National vital statistics reports. vol 57, no 14. Hyattsville, MD: National Center for Health Statistics.

Hossain MM, Hwang DY, Huang QQ, Sasaki Y, Jin JP. 2003. Developmentally regulated expression of calponin isoforms and the effect of h2-calponin on cell proliferation. Am J Physiol Cell Physiol 284:C156–C167.

Hossain MM, Smith PG, Wu K, Jin JP. 2006. Cytoskeletal tension regulates both expression and degradation of h2-calponin in lung alveolar cells. Biochemistry 45:15670–15683.

IARC. 2002. IARC Handbooks of cancer prevention: Breast cancer screening. Lyon, France: IARC Press.

Jemal A, Siegel R, Xu J, Ward E. 2010. Cancer statistics, 2010. CA Cancer J Clin 60:277–300.

Jorgensen KJ, Gotzsche PC. 2009. Overdiagnosis in publicly organised mammography screening programmes: Systematic review of incidence trends. BMJ 339:b2587.

Leman ES, Getzenberg RH. 2008. Nuclear structure as a source of cancer specific biomarkers. J Cell Biochem 104:1988–1993.

Leman ES, Cannon GW, Trock BJ, Sokoll LJ, Chan DW, Mangold L, Partin AW, Getzenberg RH. 2007a. EPCA-2: A highly specific serum marker for prostate cancer. Urology 69:714–720.

Leman ES, Schoen RE, Weissfeld JL, Cannon GW, Sokoll LJ, Chan DW, Getzenberg RH. 2007b. Initial analyses of colon cancer-specific antigen

(CCSA)-3 and CCSA-4 as colorectal cancer-associated serum markers. Cancer Res 67:5600–5605.

Ludwig JA, Weinstein JN. 2005. Biomarkers in cancer staging, prognosis and treatment selection. Nat Rev Cancer 5:845–856.

Mehul B, Bernard D, Simonetti L, Bernard MA, Schmidt R. 2000. Identification and cloning of a new calmodulin-like protein from human epidermis. J Biol Chem 275:12841–12847.

Mehul B, Bernard D, Schmidt R. 2001. Calmodulin-like skin protein: A new marker of keratinocyte differentiation. J Invest Dermatol 116:905–909.

Oesterreich S, Weng CN, Qiu M, Hilsenbeck SG, Osborne CK, Fuqua SA. 1993. The small heat shock protein hsp27 is correlated with growth and drug resistance in human breast cancer cell lines. Cancer Res 53:4443–4448.

Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. 2001. Phases of biomarker development for early detection of cancer. J Natl Cancer Inst 93:1054–1061.

Petricoin EF, Belluco C, Araujo RP, Liotta LA. 2006. The blood peptidome: A higher dimension of information content for cancer biomarker discovery. Nat Rev Cancer 6:961–967.

Porter D, Lahti-Domenici J, Keshaviah A, Bae YK, Argani P, Marks J, Richardson A, Cooper A, Strausberg R, Riggins GJ, Schnitt S, Gabrielson E, Gelman R, Polyak K. 2003. Molecular markers in ductal carcinoma in situ of the breast. Mol Cancer Res 1:362–375.

Romanucci M, Bastow T, Della SL. 2008. Heat shock proteins in animal neoplasms and human tumours—A comparison. Cell Stress Chaperones 13:253–262.

Rozenblum GT, Gimona M. 2008. Calponins: Adaptable modular regulators of the actin cytoskeleton. Int J Biochem Cell Biol 40:1990–1995.

Sakihara C, Nishimura J, Kobayashi S, Takahashi S, Kanaide H. 1996. Expression of calponin mRNA in porcine aortic endothelial cells. Biochem Biophys Res Commun 222:195–200.

Schenk S, Schoenhals GJ, de SG, Mann M. 2008. A high confidence, manually validated human blood plasma protein reference set. BMC Med Genomics 1:41.

Schopper D, de WC. 2009. How effective are breast cancer screening programmes by mammography? Review of the current evidence. Eur J Cancer 45:1916–1923.

Sheng S, Chen D, Van Eyk JE. 2006. Multidimensional liquid chromatography separation of intact proteins by chromatographic focusing and reversed phase of the human serum proteome: Optimization and protein database. Mol Cell Proteomics 5:26–34.

Smith RA, Cokkinides V, Brooks D, Saslow D, Brawley OW. 2010. Cancer screening in the United States, 2010: A review of current American Cancer Society guidelines and issues in cancer screening. CA Cancer J Clin 60:99–119.

Strasser P, Gimona M, Moessler H, Herzog M, Small JV. 1993. Mammalian calponin. Identification and expression of genetic variants. FEBS Lett 330:13–18.

Tabar L, Yen MF, Vitak B, Chen HH, Smith RA, Duffy SW. 2003. Mammography service screening and mortality in breast cancer patients: 20-year follow-up before and after introduction of screening. Lancet 361:1405–1410.

Tabernero L, Taylor DA, Chandross RJ, VanBerkum MF, Means AR, Quiocho FA, Sack JS. 1997. The structure of a calmodulin mutant with a deletion in the central helix: Implications for molecular recognition and protein binding. Structure 5:613–622.

Takahashi K, Hiwada K, Kokubu T. 1986. Isolation and characterization of a 34,000-dalton calmodulin- and F-actin-binding protein from chicken gizzard smooth muscle. Biochem Biophys Res Commun 141:20–26.

Tang J, Hu G, Hanai J, Yadlapalli G, Lin Y, Zhang B, Galloway J, Bahary N, Sinha S, Thisse B, Thisse C, Jin JP, Zon LI, Sukhatme VP. 2006. A critical role for calponin 2 in vascular development. J Biol Chem 281:6664–6672.

Thanner F, Sutterlin MW, Kapp M, Rieger L, Morr AK, Kristen P, Dietl J, Gassel AM, Muller T. 2005. Heat shock protein 27 is associated with decreased survival in node-negative breast cancer patients. Anticancer Res 25:1649–1653.

Van Le TS, Miller R, Barder T, Babjuk M, Potter DM, Getzenberg RH. 2005. Highly specific urine-based marker of bladder cancer. Urology 66:1256–1260.

Walgenbach-Brunagel G, Burger B, Leman ES, Walgenbach KJ, Tolba R, Heukamp L, Hirner A, Getzenberg RH. 2008. The use of a colon cancer associated nuclear antigen CCSA-2 for the blood based detection of colon cancer. J Cell Biochem 104:286–294.

Wu KC, Jin JP. 2008. Calponin in non-muscle cells. Cell Biochem Biophys 52:139–148.